Bacterial Sensor Systems for On-line Water Monitoring and Pollution Characterization

A Ph.D. Research Proposal

Submitted by:

Tal Elad

Under the Supervision of:

Prof. Shimshon Belkin

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Abstract

The necessity for monitoring the presence of toxic chemicals in water has led to an increased need for early warning devices. Current analytical detection techniques, though highly accurate and sensitive, are hard to integrate into simple field monitoring instrumentation. More suitable is the bioassay-based approach, which utilizes live organisms in order to monitor water quality. One manifestation of this approach is based on genetically engineered microorganisms, tailored to respond by a dose-dependent signal to the presence of pollutants. While numerous such constructs have been presented over the past decade, they have yet to be incorporated in actual sensing systems. I seek to establish means for the immobilization and long-term activity maintenance of reporter cells under continuous-flow conditions using a laboratory simulation system, and to incorporate reporter cells in an on-line water monitoring prototype. At the same time, I seek to develop methods for the characterization of the unknown toxic contents of a water sample, by applying well-established decision and clustering algorithms to the response pattern of small and large collections of reporter strains. Pursuing these two avenues, I aim to bring about progress from recombinant bacterial reporter strains towards real-world devices for water monitoring.
Background and Significance

Recent decades have seen an increase in environmental pollution levels dangerous to human and environmental health. At the same time, there is a growing worry of bio-terror and the use of chemical warfare. The subsequent necessity for monitoring the presence of accidentally or intentionally introduced toxicants has led to an increased need for early warning devices that can detect toxic chemicals in the environment in general and in water in particular.

The traditional approach for monitoring chemicals in aquatic environments is based on chemical or physical analysis and allows highly accurate and sensitive determination of the exact composition of any sample. However, such methodologies, while essential for regulatory purposes, require skilled personnel and sophisticated equipment. They also consume a substantial amount of time, especially when there is no preliminary knowledge about the sample’s contents. These drawbacks make it hard to use analytical techniques for early warning or to integrate them in field monitoring instrumentation. More suitable for these purposes is the bioassay-based approach, which utilizes live organisms in order to monitor water quality. The organisms, ranging from fish to invertebrates, are exposed to the tested sample, and changes in their physiology and behavior indicate the presence of toxic substances (Gerhardt et al. 1998; Shedd et al. 2001). Unicellular microorganisms, in particular bacteria, are advantageous for such use. Their large population size, high growth rate, low cost, easy maintenance, and rapid response make them an appealing option for pollution monitoring. An additional attractive characteristic of bacteria is that they can be genetically engineered to respond by a detectable dose-dependent signal to prespecified changes in their environmental conditions (Belkin 2003).

Indeed, numerous bacterial reporters have been constructed, most of which harbor a plasmid-borne fusion of a stress- or chemical-specific gene promoter to a reporter gene whose expression can be readily measured (Köhler et al. 2000; Hansen and Sørensen 2001; Gu et al. 2004). Common among reporter genes are the bacterial luxCDABE genes
and the \textit{gfp} gene. The bacterial \textit{luxCDABE} genes, isolated from luminous bacteria such as \textit{Photobacterium phosphoreum}, catalyze a biochemical reaction in the course of which 490 nm light is emitted. The \textit{gfp} gene encodes for the Green Fluorescent Protein (GFP), which exhibits bright green fluorescence when exposed to blue light and was first isolated from the jellyfish \textit{Aequorea victoria}. In both cases, when the designated environmental conditions are met, the transcription of the reporter gene(s) is promoted and an optical signal proportional to the stimuli is produced. Examples include the construction of bacterial reporters responsive to heat shock (van Dyk et al. 1994; Sagi et al. 2003), oxidative stress (Belkin et al. 1996) and genotoxic agents (Vollmer et al. 1997; van der Lelie et al. 1997; Kostrzynska et al. 2002; Hwang et al. 2008), as well as heavy metals (Corbisier et al. 1999), BTEXs (benzene, toluene, ethyl benzene and xylene; Applegate et al. 1998), PAHs (polycyclic aromatic hydrocarbons; Heitzer et al. 1992) and antibiotics (Eltsov et al. 2008) – a growing concern in recent years.

In order to progress from genetically engineered reporter cells to an actual biological early warning system, several obstacles need to be overcome. Prominent among them is the need to immobilize the cells in such a manner that will not only place the cells in proximity to the device's signal transducer, but will allow long-term maintenance of cell viability and activity (Biran et al. 2003; Elad et al. 2008; Elad et al. \textit{in press}). Agar – a polysaccharidic polymer used by microbiologists as a solid growth medium – was shown to be inert to the activity of reporter cells immobilized in it. Furthermore, reporter cells immobilized in agar maintained their activity for a number of weeks when kept in a refrigerator (Schreiter et al. 2001; Ripp et al. 2003; Lee et al. 2005; Park et al. 2005). The use of agar as an encapsulation matrix is especially appealing due to its ease of handling and virtually inherent biocompatibility. It is also superior to other proposed techniques. Bioreactor-based systems, for example, unlike agar, require constant and complex cell maintenance (Kim and Gu 2005; Lee and Gu 2005; Bjerketorp et al. 2006). Despite all the above, the long-term viability and activity of agar-immobilized reporter cells under continuous flow conditions has not been examined, nor has such constellation been incorporated in a functioning water monitoring prototype device.
Another important research avenue in the pursuit for the realization of cell-based sensing equipment focuses on signal analysis. Microbial sensor cells test the effect of the target chemical(s), but, contrary to analytical techniques, lack the ability to identify the chemical itself. While, as indicated above, many chemical-specific bioreporters have been described, their applicability is very limited when one is trying to identify unknown pollutants. Were sensor cells able to identify or at least classify, even to some extent, the chemical in question, it could be an important added-value to cell-based monitoring systems. In a partial response to this challenge, Belkin et al. (1997) have suggested and described the use of a panel of five specific stress-responsive luminous bacteria to detect water toxicity and indicate the type of biological stress involved. This approach was later utilized by Ben-Israel et al. (1998), who used discriminant analysis to identify toxic chemicals according to the response pattern of a panel of seven *Escherichia coli* reporter strains carrying *lux* genes fused to stress promoters. However, the algorithm was described in general terms, and a limited amount of repeats was used for training.

The number of reporter strains used for toxicant identification should not be confined to a mere few. A bacteria library, containing thousands of reporter strains, may prove more useful for this purpose than a panel composed of a small number of reporters for several reasons: (a) its identification range is expected to be broader; (b) it is likely to be more robust and less sensitive to artifacts; (c) it will better reflect the overall cellular response of the bacterium to the sample. The latter is particularly important because it may facilitate the detection of the pollutant's family – a more pragmatic approach than detecting its exact identity, since obtaining a data set of the response patterns to all possible toxic chemicals is a daunting undertaking.

Large-scale collections of bacteria and yeast have become of interest in recent years owing to developments in the fields of molecular biology and robotics (Winzeler et al. 1999; Zaslaver et al. 2006). They have been used for high-throughput investigations of gene function, genetic interaction networks and the mode-of-action of pharmacologically relevant compounds (Tong et al. 2001; Chang et al. 2002; Parsons et al. 2004; Parsons et
al. 2006; Typas et al. 2008), while the environmental applications of such collections remained unexplored. As suggested above, high-throughput screening of this sort for environmental pollutants may prove useful for the characterization of the unknown toxic contents of a water sample. Furthermore, when this notion is combined with whole-cell array technology (Elad et al. 2008; Elad et al. *in press*), one can envisage thousands of reporter strains arrayed at a high-density on a chip platform. A drop of a sample could then be dripped on the chip, and the induced fingerprint would reveal the sample’s contents.

**Objectives**

The overall objective of my research is to bring about progress from recombinant bacterial reporter strains towards real-world devices for water quality monitoring. The envisioned devices are designated for on-line continuous water monitoring as well as for the characterization of the toxic contents of a suspected water sample. In order to achieve this objective, the following specific goals were set:

1. Establish means for the immobilization and long-term activity maintenance of recombinant bacterial reporter strains under continuous-flow conditions.

2. Integrate polymer-entrapped reporter strains in a functioning easy-to-handle prototype for on-line water monitoring, and demonstrate the prototype’s performance when challenged with various toxic compounds and environmental samples.

3. Determine the feasibility of applying well-established recognition algorithms for the identification of toxicants according to the response pattern of a reporter strain panel.

4. Develop a method for the characterization of the unknown toxic contents of a water sample, which will be based on the response pattern of a large-scale reporter strain collection.
Proposed Research

Bacteria Immobilization and Long-term Maintenance

In order to establish means for the immobilization and long-term activity maintenance of bacterial reporters under continuous-flow conditions, a laboratory system simulating such conditions was built (Figure 1). The system enables to probe the responsiveness of agar-immobilized reporters, which are exposed to a constant flow of tap water. In practice, the system is set as described below.

Bacterial reporters are grown overnight in Luria-Bertani (LB) broth supplemented with the appropriate antibiotics to prevent plasmid loss. The overnight culture is diluted and re-grown to the mid-exponential growth phase. Then, the bacteria are washed with phosphate buffer solution (PBS) and mixed with melted 1.5% agar kept at 50°C. Aliquots of the mixture are pipetted onto agar beds laid out beforehand across four 8-well strips. After the aliquots solidify, they are covered with agar solution to minimize "escape" of bacteria.

The four strips in which the reporters are immobilized are placed in four identical flow-through chambers in a plastic frame. The flow-through chambers, designed to fit the well strips in size, have each its own inlet and outlet. Using two 4-channel head-equipped peristaltic pumps, each inlet is separately and continuously fed with tap water and LB broth.

Additional tubes are fused to the tap water feeding tubes upstream to the peristaltic pump. In order to simulate a sudden incidence of water contamination, a toxic agent dissolved in tap water is pumped into the tap water stream through these additional tubes. Altogether, this configuration enables to expose each of the four lanes in a separate fashion and at different time points to the analyte. Luminescence is measured in fixed intervals for three hours using a Victor^2 luminometer (Turku, Finland). After each measurement, the strips are put back in the chambers till the next reading.
Figure 1. The continuous-flow simulation system. The system consists of tap water and LB reservoirs, peristaltic pumps, plastic framed flow-through chambers, and inducers inlets, through which inducers can be pumped into the tap water stream in order to simulate a sudden incident of water contamination. The reporter strains are immobilized in agar in 8-well strips, depicted here inside the flow chambers.

On-line Water Monitoring Prototype

An early warning flow-through prototype was built according to our specifications by Prof. Yosi Shacham-Diamand’s group from the Faculty of Engineering at Tel-Aviv University (Figure 2). Briefly, the prototype consists of four polydimethylsiloxane (PDMS) modules and three aligned Single-Photon Avalanche Diodes (SPADs; Hamamatsu, Japan) connected to a single-axis linear stepper motor. Each of the PDMS modules is perforated with a 3x4 well matrix and special tubing and channeling systems allow for the separate feeding of each of the well matrices. This configuration allows one to immobilize three replicates of four reporter strains in a single module, and to use, for example, one of the modules for the tested water and another as a negative control. As the immobilized reporters are challenged with samples, the SPADs scan along the modules column-by-column, stopping above each well column to measure the signal. The data are transferred to a PC station for processing.
Bacterial immobilization takes place according to the following protocol: refreshed cultures of luminescent bacterial reporters are concentrated in order to enhance their light signal. The concentrated bacteria are mixed with melted agar and aliquots of the mixture are immediately pipetted into the PDMS wells and let to solidify.

Experiments will seek to develop and optimize an on-line toxicity test using the designed prototype. Specifically, the prototype’s function and the behavior of the immobilized reporters will be examined. Challenging the reporters with different concentrations of the same compound will establish dose-dependency and challenging them with different compounds of different types will establish selectivity. Finally, the system will be challenged with real environmental samples.
Toxicant Identification by Pattern Classification Algorithms

Several datasets will be used in order to determine the feasibility of applying pattern classification algorithms for the identification of toxicants according to the response pattern of a reporter strain panel: a dataset previously constructed in our lab (Benovici 2003), and others constructed through the course of the current research. All datasets consist of the response of a few reporter strains (each carrying a different gene promoter fused to the lux operon) to selected model toxicants in a sufficient number of repeats. Bacteria are harvested at the early exponential growth phase; exposure to model chemicals takes place in opaque white 96- or 384-well microtiter plates; and luminescence is measured using a Victor^2 luminometer.

The response patterns of the bacterial reporter panels to the selected compounds will be classified using two well-established schemes: Bayesian decision theory and the nearest-neighbor technique (Witten and Frank 2000; Duda et al. 2001). The feature vector represents the response of each of the panel members, and the possible states of nature are the model chemicals in addition to a non-toxic control. The classifiers’ performances will be evaluated by a repeated 10-fold cross-validation procedure as recommended by Witten and Frank (2000). All the above will be implemented using MATLAB software (version 7.4 R2007a, The MathWorks).

To test algorithm validity in liquids other than laboratory media, the reporters’ response to model chemicals will be examined in spiked tap water and wastewater samples. The wastewater samples will be filtered to dispose of suspended solids and bacteria. Disinfected wastewater and tap water will be amended by Na-thiosulfate to remove any residual chlorine. Bacteria will be prepared and assayed as before.

Toxicant Screening against a Large-scale Reporter Collection

A comprehensive library of fluorescent transcriptional E. coli reporters (Zaslaver et al. 2006) was kindly provided by Prof. Uri Alon from the Department of Molecular Cell Biology at the Weizmann Institute. The library consists of transcriptional fusions of gfp
to each of about 2,000 different promoters in *E. coli* K12, covering the great majority of the promoters in the organism.

After optimizing the assay parameters using selected reporters, the library will be exposed to potential environmental pollutants of various classes, e.g., heavy metals, carbamate pesticides and antibiotics (all are available at our lab). Sets of 96 reporter strains, pre-arranged in a 96-well microtiter plate, will be exposed to model chemicals and to a non-toxic control. Prior to exposure, the reporter strains will be incubated overnight and refreshed in deep 96-well plates. Then, each reporter strain will be exposed to each of the treatments separately. In this fashion, where portions of the same batch culture are exposed to different treatments in parallel to one another, observed differences between their responses are expected to be of higher significance. The data will be analyzed using methods such as hierarchical clustering, a widely used technique for DNA microarray expression data interpretation (Ehrenreich 2006; Fan and Ren 2006).

**Results to Date and Remaining Tasks**

**Bacteria Immobilization and Long-term Maintenance**

Feasibility of agar immobilization was verified. Three reporter strains were used: DPD2511, carrying a *katG::lux* fusion; DPD2540, carrying a *fabA::lux* fusion; and TVI1061, carrying a *grpE::lux* fusion. The three strains were immobilized in agar exposed to a double dilution series of hydrogen peroxide, phenol and ethanol – three well-known inducers of *katG*, *fabA* and *grpE*, respectively (Belkin et al. 1997). All immobilized reporters exhibited a clear dose-dependent response. The use of sol-gel silicate as an alternative entrapment matrix was also tested. Sol-gel silicate enjoys good optical properties and was proven to maintain bacteria viability and activity (Nassif et al. 2002; Premkumar et al. 2002; Bjerketorp et al. 2006; Mitchell and Gu 2006). Reporter immobilization in sol-gel silicate was carried out as described elsewhere (Premkumar et al. 2002; Sharabi 2006) and initial results looked promising. However, tetramethyl
orthosilicate (TMOS) — a key precursor in sol-gel silicate preparation — exhibited inconsistent behavior across different batch solutions, and the technique was thus abandoned.

Following the verification of agar immobilization feasibility, a reporter strain carrying a sulA::lux fusion was immobilized in agar in all wells of all flow-through chambers of the continuous-flow simulation system. After the system was set, tap water was made to flow continuously through the system for two weeks, during which five pulses of the genotoxic agent nalidixic acid (NA) were introduced into the tap water flow. Each of the first four pulses was introduced to a different flow-through chamber. The fifth and last pulse was introduced after two weeks to a chamber of previously-exposed bacteria. As depicted in Fig. 3, the reporter was responsive for the entire period. Moreover, cells that had been already exposed to the genotoxicant exhibited an ability to recover and to respond again when challenged with another NA pulse a few days later. Similar performance was exhibited by a katG::lux reporter; not only did the reporter maintain a constant level of sensitivity to pulses of hydrogen peroxide for at least two weeks, its response was not hindered by an exposure to NA a few days earlier. The ability of the reporter cells to recover from an exposure to a certain toxicant and to respond when challenged with either the same or a different toxicant a few days later is particularly interesting, since it might save one the need to replace the cells, if and when toxicity is detected.

These results demonstrate for the first time that agar-immobilized reporter strains preserve their activity under continuous-flow conditions for a period of at least two weeks. They also constitute the first demonstration of the cells’ ability to produce signals over consecutive exposures. Next, I plan to immobilize a few reporters simultaneously in the continuous-flow simulation system, and examine their response pattern to different toxicants. This will further establish the use of agar for immobilizing reporter cells and preserving their activity.
Figure 3. *sulA::lux* reporter strain was immobilized in all four chambers of the continuous-flow simulation system and was exposed to NA pulses at five time-points. Response Ratio denotes the ratio between the luminescence measured 2 h after the exposure and the luminescence measured right before it, averaged across all wells.

On-line Water Monitoring Prototype

Strain ST1, harboring a *recA::lux* fusion, was immobilized in agar in all the wells of all four PDMS modules. 20, 10 and 5 mg nalidixic acid/L dissolved in LB were made to flow through three of the modules. The fourth and last module was left as an LB-only control. A similar procedure was followed with three other strains: *katG::*, *micF::* and *zntA::lux* reporter strains were exposed to double dilution series of hydrogen peroxide, paraquat and cadmium chloride, respectively. As illustrated by Figure 4, which depicts the average luminescence values, a clear dose-dependent signal was picked up by the system in all four experiments. A more careful scrutiny of the luminescence values themselves rather than their averages confirmed the practically uniform behavior among all the wells of the same module. Since each module is designated to house triplicates of four different reporters, it is obvious why such confirmation is of extreme importance.
Figure 4. Dose-dependent responses recorded by the on-line water monitoring prototype. (A) recA:lux reporter exposed to NA. (B) katG:lux reporter exposed to hydrogen peroxide. (C) micF:lux reporter exposed to paraquat. (D) zntA:lux reporter exposed to cadmium chloride. All legends depict concentrations in mg/L.

Figure 5. recA, micF, katG and zntA reporters were immobilized in our prototype in triplicate in each of three modules. 50 mg/L paraquat were made to flow through the first module (the sample module), 10 mg/L NA through the second module (the positive control module), and toxicant-free LB through the third module (the negative control module). (A) The differences between the average values of the sample and the negative control. (B) The differences between the average values of the positive and negative controls.
In a following experiment, I set out to demonstrate the prototype's operation when a panel of four different reporter strains is used. Reporter strains recA, katG, micF and zntA were immobilized in triplicate in each of the first, second and third modules. Paraquat presence was detected by the micF reporter, which produced a strong signal in comparison to the negative control (Fig. 5A). The recA reporter’s response to NA was very apparent in comparison to its background luminescence level (Fig. 5B). Above all, these results demonstrate the advantages in the use of a reporter panel. Not only does it grant our prototype the ability to detect toxicity of different kinds (in this case both genotoxicity and oxidative stress), the identity of the responsive reporter can indicate the type of toxicity involved.

In order to complete the demonstration of the prototype’s performance, I plan to take several steps: (a) Challenge the prototype with additional environmentally-relevant compounds; (b) Challenge the prototype with environmental samples; (c) Challenge the prototype with toxic samples after operating for an extended period of time (till now exposure took place immediately after the system was set). By this I will demonstrate our water monitoring prototype’s ability to detect a wide range of toxic chemicals, screen real-world samples, and work in a continuous mode.

**Toxicant Identification by Pattern Classification Algorithms**

Bayesian classifiers were applied to a previously constructed dataset (Benovici 2003). The dataset consists of the response of five reporter strains (carrying grpE::, nhoA::, oraA::, lacZ:: and mipA::lux fusions) to five model toxicants (dichlorvos, ethyl parathion, potassium cyanide, nitrogen mustard and paraquat) and to a toxicant-free control in a 40-repetition format. The classifiers were applied to the luminescence data collected 30, 60 and 120 min after exposure, and their estimated error rates were obtained in a repeated 10-fold cross-validation procedure. All three classifiers exhibited an error rate estimate that did not exceed 3% at a 95% confidence level, with the 120-min classifier exhibiting the lowest error rate estimate, 1.04% ± 0.25%. Additionally, no false negatives were recorded at any time-point. That is, no toxic sample was misclassified as an LB-only
control. The use of Bayesian decision theory in employing the responses of two *E. coli* gene promoters (*oraA* and *lacZ*) to discriminate between two toxicants (parathion and paraquat) is exemplified in Figure 6A.

When the nearest-neighbor rule was applied to the same data, its error rate estimates decreased with exposure time; the 30-, 60-, and 120-min classifiers yielded error rate estimates of 9.79, 6.42, and 1.87%, respectively. Unlike the Bayesian classifiers, the nearest-neighbor classifiers were not free of false negatives. Yet, the average number of false negatives of each of the classifiers did not exceed 4, with the 120-min classifier exhibiting an average of only 1.9 false negatives out of 240 processed observations.

All differences between the error rate estimates of the Bayesian classifiers and their nearest-neighbor counterparts were found to be statistically significant in a 5% level one-sided paired t-test. These results suggest that the Bayesian scheme, which displayed the lower error rate estimates, is preferable over the nearest-neighbor one in the examined time frames. The differences between the error rate estimates of different classifiers within the same scheme were also tested for significance using the above hypothesis test. Similarly, all differences, except the difference between the 30-min and the 60-min Bayesian classifiers, were found to be statistically significant, suggesting that the best performance was demonstrated by the 120-min Bayesian classifier.

To test the validity of the pattern classification algorithm in environments different from laboratory media, the reporter panel's response to two model chemicals (potassium cyanide and paraquat) was examined in tap water and in wastewater samples; the latter included both untreated and treated domestic wastewater. By using the 60 min luminescence values of each of the reporter strains, the Bayesian classifier correctly recognized the contents of all spiked samples. This is demonstrated for both chemicals and for treated wastewater in Figure 6B. In this figure, the response patterns of the reporter panel to the spiked wastewater are superimposed on the two toxicants' fingerprints, displaying a distinct similarity. To facilitate the comparison of the data, the
responses are plotted in this case as the logarithm of the response ratio exhibited by each of the panel members.

In conclusion, I demonstrated the feasibility of applying well-established recognition algorithms for the identification of toxicants according to the response pattern of a reporter panel. The decision algorithms applied were Bayesian and nearest-neighbor and their performances were evaluated and compared. For more details see Elad et al. (2008).

Toxicant Screening against a Large-scale Reporter Collection

As satisfactorily as the performance of the above-mentioned algorithms may be, they have only been tested in a very small "universe" containing a short list of potential hazards and a small number of reporter strains. Of course, the real world contains a practically unlimited number of toxic compounds that a panel of a few reporters might fail to cover. A possible solution to this problem is to use a large collection of reporter strains, under the assumption that the more strains we use the more chances to get a signal. In terms of future application it is also more likely that for thousands of potential chemicals, classification only to a chemical family level would be offered. Such classification could be more accurate, given the overall cellular response rather than the response of few gene promoters.

In order to study the possibility of identifying a toxicant’s family according to the fingerprint it induces on a large collection of reporter strains, a comprehensive library of fluorescent transcriptional E. coli reporters was obtained and is now available at our lab. The library has been screened against several families of compounds, namely heavy metals, carbamate pesticides and antibiotics, but the data have not been fully analyzed yet. When hierarchical clustering was applied to another dataset constructed, the heavy metals cadmium and arsenite clustered together as well as the structurally-similar phenol and 2-chlorophenol. Response to the carbamate pesticides oxamyl and aldicarb was barely detected, stressing the need to broaden the panel of reporters (Fig. 7).
Figure 6. (A) Bayesian decision boundary for parathion and paraquat based upon the luminescence emitted 30 min after exposure by reporters harboring omA:: and lacZ::lux fusions. (B) The response patterns of a 5-reporter panel to treated wastewater spiked with potassium cyanide (20 mg/L, blue solid line) and paraquat (31.25 mg/L, red solid line) superimposed on potassium cyanide and paraquat fingerprints (blue and red dashed lines, respectively) obtained based upon a 40-repetition sample in laboratory media. Values denote the logarithm of the response ratio.

Figure 7. Shown are a heat map (middle) representing the response pattern of a panel consists of recA::, katG::, mfp::, zntA::, and cydA::lux reporter strains to nine tested chemicals (columns are reporter strains, rows are the tested chemicals), and a tree diagram (left) illustrating the arrangement of the clusters produced by hierarchical clustering. Values are the logarithms of the maximum response ratios during 120 min of exposure.
To summarize, the tasks remaining to be carried out are:

1. Immobilizing a panel of reporter strains in the continuous-flow simulation system, exposing them to sudden pulses of environmental pollutants during two weeks time, and studying their response pattern. Special attention will be given to the issues of cross-contamination and pattern classification.

2. Challenging the on-line water monitoring prototype with additional toxic compounds as well as with real environmental samples. Operating the prototype for an extended period of time and examining its ability to function properly for 24 hours or more.

3. Analyzing the data accumulated during the large-scale screen of the reporter collection. Searching for unique patterns which characterize different families of pollutants. Validating the results by repeating the experiments and by exposing the collection or a selected sub-group of reporters to other chemicals from the same families.
References


