Publishable Summary of MicroAQUA (N. 265409)

Executive summary

At the onset of the activities of the µAQUA project, the methods to monitor water quality in terms of presence of contaminating pathogenic microorganisms relied mainly on standard microscopic observations and on culture-based approaches. Because these approaches present a considerable number of drawbacks, the µAQUA consortium decided to resort to state-of-the-art molecular biology methods to enhance greatly the ability to identify with improved accuracy and sensitivity harmful species of microorganisms and toxins. The molecular methods developed by the µAQUA consortium, mainly through the rational use of specific molecular probes combined in a universal microarray chip, have the potential of advancing the technical capabilities of monitoring the quality and safety of European waters, offering a very sensitive and reliable tool for the detection of harmful organisms and substances contaminating the waters. Furthermore, the methods devised by the µAQUA consortium have the merit of substantially increasing the number of samples that can be examined. Thus, the large body of data obtainable through the use of these methods can also yield important information with respect to gene flow and species distribution in time and space. More specifically, the main results obtained by the consortium were: i) the development of a standardized sampling and handling program for all fresh waters to be tested; ii) the development of a validated microarray that makes use of appropriately designed and tested probes and is capable of detecting toxin-producing organisms, water-borne pathogens such as cyanobacteria, bacteria and protozoa as well as a select number of diatom species serving as bio-indicators of water quality. If it were not for the fact that the chip does not yet allow the detection of viruses (due to some technical difficulties the viral probes will be added with a few months delay) the microchip developed by the µAQUA consortium can be defined “universal”; iii) the development of sensitive methods for the detection of several types of harmful toxins in environmental samples.

Finally, it should be stressed that at least a reduced-scale version of the microchip developed and validated by the µAQUA consortium is ready for commercial use and could be possibly adopted by European water authorities and by the water provider industry.

Project Context

The µAQUA project was conceived and designed in light of the evidence that the availability of reliable and safe water supplies has become a serious, health- threatening emergency also in Europe. Both the WHO and the UNECE (United Nations Economic Commission for Europe) have expressed their concern for this alarming situation and issued a protocol asking governments to take appropriate actions to cope with the water emergency which is bound to deteriorate further if no direct and strong measures are taken.

Indeed, a large proportion of European drinking water is obtained from surface waters, even though pathogenic organisms occur in lakes and rivers used as drinking water reservoirs. It can be estimated that every year cases of water-related diseases are no less than 170,000. Moreover, global climate changes and massive migration fluxes from Africa and Asia are expected to perturb the existing ecological balance determining major changes in the type, abundance and distribution of pathogenic microbes and likely causing (re)-emergence of water-related pathogens.

One of the essential measures to be taken at the European level is the implementation of a reliable and sensitive system for the early detection of water-borne pathogens and toxins whose presence in European waters is of paramount importance for public health.

The µAQUA project was conceived to cope with this particular aspect of the "water emergency". Because the presence of water-borne pathogens (bacteria, viruses, protozoa and cyanobacteria) and pathogenic substances (toxins) is either not routinely checked, or checked by traditional methods that are laborious, technically demanding and time-consuming, the purpose of the µAQUA project was to develop easy-to-use, advanced molecular tools for the detection in European freshwaters of known and emerging water-borne pathogens and also for monitoring the presence of select species of diatoms which represent reliable bio-indicators to assess overall water quality. More specifically, the aim of µAQUA was to design and develop in parallel: i) a state of the art, extremely sensitive, quantitative method for toxin detection and ii) a universal microarray chip for the high-throughput detection of bacteria, cyanobacteria, potential toxin-producers, protozoa, viruses and biomarker diatoms. Another aim of the project was to use the above-mentioned methodologies, in parallel with traditional methods, to test the quality of environmental waters taken from different locations in six European countries in a two-year sampling campaign.

In addition to the development of these monitoring tools, the µAQUA project included the investigation on the possible use of lytic cyanophages isolated from samples of environmental waters for setting up mitigation strategies for the control of cyanobacterial blooms.

In conclusion, the main objectives of the µAQUA consortium were:

i) The development of a multiplex sensor-based systems for the early detection of target toxins produced by organisms in fresh water. Important steps towards this end were the identification and isolation of the target toxins, their delivery for assay development, the production and characterization of proteins binding the target toxins with high sensitivity and specificity (i.e. monoclonal antibodies and phage) and their delivery for the development of the assay, the identification and characterization of the toxin receptors, the construction of an immuno-affinity-based device to prepare the samples, the development and the evaluation of the first prototype and of the final prototype multiplex assay.

ii) the development of a microchip containing nucleic acid probes capable of pairing specifically with the nucleic acids (RNA or DNA) obtained, either directly or upon PCR amplification, from a select number of pathogenic (bacteria, cyanobacteria, parasitic protozoa and viruses) or biomarker (diatoms) microorganisms;

iii) an investigation of the possibility of using cyanophages to mitigate harmful cyanobacterial blooms (HCBs) on a pilot scale.

Description of the main S & T results/foregrounds

The following text makes reference to figures and tables that can be found in the "attached document" (Figures & Tables Final Report MicroAQUA).

During the time span of the project (Figure FR.1), the µAQUA partners succeeded in establishing rigorous criteria and a detailed protocols for sampling and processing environmental water and extracting nucleic acids. All partners involved in water sampling campaigns have employed this standardized protocol in their water sampling campaigns (first and second year sampling) that have been carried out at different sites of six European countries (Ireland, Italy, France, Germany, Bulgaria and Turkey) encompassing a wide range of water types (Figure FR.2).

According to the established protocol water is concentrated using a hollow-fiber tangential dialysis filter and fractionated by a series of micro-filtrations to collect microorganisms of different size (Figures FR.3 and FR.4). Through a demo-video and practical workshop all partners were trained in the use of a standardized, quantitative RNA extraction protocol ensuring homogeneous and reliable yields from the samples. This protocol was implemented by the entire consortium. However, it proved to be necessary to design two additional protocols, one for the extraction from viruses of both RNA and DNA and another for the extraction of DNA from diatom biomass.

For the detection of individual water-borne microorganisms a very large list of probes was initially compiled. The probes were chosen based on their complementarity to regions of ribosomal RNA displaying sequence divergence among different orders, genera and species of select microorganisms and possessing the desired physical-chemical characteristics. These probes were reassessed for their specificity *in silico* and subsequently *in vitro*, through a series of hybridizations on microchips (generations 1 and 2) until only those that had proven successful and specific were retained and spotted on generation 3 microchip. Likewise, probes targeting species-specific protein-encoding genes were designed, synthesized and validated. A total of 417 probes were considered valid and spotted on the same generation 3 microchip (Table FR-1). The microchip technology for monitoring water safety and quality was proven suitable for the highly sensitive and selective detection of water-borne pathogens such as pathogenic bacteria, cyanobacteria and parasitic protozoa (Figure FR.5). Performance of the microchip was further improved by the use of probes capable of detecting toxin-producing microorganisms. In fact, the chip was implemented with 70 validated probes designed to target and detect cyanobacterial toxin genes being expressed, even at low levels, through a new method involving direct PCR amplification on the microarray that allows their immediate detection (Figure FR.6).

The designed probes were spotted on microchips that were subjected to several rounds of hybridization experiments using fluorescently labeled nucleic acids obtained from pure cultures of the target microorganisms. These were either RNA directly extracted from the microorganisms or DNA amplicons obtained after amplification by polymerase chain reaction (PCR) in the case of DNA. The probes that proved to be non-specific in these initial hybridization experiments were discarded, whereas those proven to be valid

were retained and spotted on the so-called generation-3 microarray. This microarray was eventually used for the analysis of the RNA extracted from environmental samples collected during the year one and year two sampling campaigns.

Meanwhile work was carried out to identify and count, by the best available traditional methods the microorganisms present in the field samples. This was done in order to establish a quantitative correlation between cell counts (nature and abundance of the microorganisms) and intensity of microarray hybridization signals, present in the samples analyzed by the partner laboratories.

The hybridization experiments were carried out on generation-3 microchip using the samples that gave acceptable R^2 and curves equation (indicated in green in Table FR-2). The hybridizations with these field samples allowed the successful detection of water-borne pathogenic organisms (bacteria, cyanobacteria, parasitic protozoa) in all collected samples (Figures FR.7 FR.8 and FR.9). By and large, the results obtained were consistent with the analyses carried out with the traditional methods concerning nature and abundance of the microorganisms present in the same samples. Thus, it was claimed that a microchip for the detection of these pathogens is ready for commercialization.

No comparable analysis was possible for the viruses, not only because viruses do not contain ribosomal RNA and many of them contain DNA and not RNA in their genomes, but also because their low number requires an amplification step whose establishment proved difficult and time consuming. The extraction of nucleic acids from viral standards, which had been obtained from other laboratories and purchased commercially, did not yield nucleic acids which were suitable from the point of view of quality as well as quantity. The use of viral nucleic acids from serum or cell cultures always yielded insufficient quantities for microarray hybridization (for which quantities of more than 1µg would be necessary).

To be suitable for use in microarray experiments, the nucleic acids must be pure enough to give spectrophotometric 260/280 and 260/230 ratios which are >1.8. Although several different extraction methods have been used (see 18-36 and 37-45 month reports), the extracted nucleic acids, also from different PTRs, were always insufficient in quantity and purity. The viral nucleic acids obtained after use of different extraction protocols gave poor 260/280 and 260/230 ratios and yielded poorly labelled probes which gave poor hybridization results.

Thus, the design and validation of the viral probes was delayed with respect to the other organisms. Only at the very end of the project satisfactory results were obtained, mainly through the use of newly designed PCR primers. The latest results are very positive and promise the identification of nine different viruses on a dedicated “virus” microchip.

As to the biomarker diatoms, the results obtained in the hybridization experiments were technically sound, but for the reasons given below it seems unlikely that an rRNA-based microchip could be of any use for evaluating water quality. Another type of chip developed during the project is more likely to serve this purpose. In fact, already at the onset of the work with diatoms, it was realized that the degree of sequence divergence of the rRNAs does not allow a reliable species-specific identification of a sufficiently high number of these biomarkers. This conclusion was confirmed by the results obtained upon hybridization of the RNA extracted from environmental samples with generation-3 RNA microchip. The good quality of the hybridization signals obtained was paralleled by the

overall inconclusiveness of the results in judging water quality. Thus, an alternative approach was followed, consisting in the search and identification of species-specific sequences of protein-encoding genes (eEF-1a and SIT). This choice has the advantage of preventing the amplification of the DNA possibly extracted from bacteria inevitably present in the diatom cultures. In fact, both chosen proteins are typically eukaryotic and SIT (silica transporter protein) is a typical diatomic protein. The species-specific sequences (ca. 400 bp) must be flanked by conserved sequences that are essential for the construction of "universal" forward and reverse primers that allow the PCR amplification of the selected genomic sequence. After a large number of trials to optimize the primer sequences and amplification conditions, it was possible to select two pairs of primers (one for eEF-1a and one for SIT) that allow the tandem, simultaneous amplification of the two genes in all ten target biomarker diatoms.

The PCR amplification step prior to microchip hybridization represents an additional, yet necessary complication introduced in the protocol for microchip hybridization. Indeed, the diatoms are mainly benthic organisms and one cannot expect to find many planktonic cells in the sampled waters and for this reason a PCR amplification step appears to be necessary. Overall, the work carried out so far for the species-specific identification was very successful, although the delay in obtaining the necessary number of validated probes did not allow challenging the diatom probes with field samples but only with lab cultures of these organisms. However, before final implementation of this innovative method for diatom detection, it will be necessary to verify that the distribution of planktonic and benthic diatom species correspond with one another. In fact, the indices for water quality assessment are based on presence and distribution of benthic and not on planktonic diatoms. In conclusion, at the end of the µAQUA project it can be stated that the detection of 10 biomarker diatoms species, characteristic of five levels of water quality (Table FR-3), is feasible by hybridization on a diatom-devoted microchip spotted with "protein probes" the amplicons obtained through a duplex PCR reaction. Because the list of the diatom species considered indicators of water quality is different in different geographic regions, the further development and validation of equivalent "protein-probes" targeting other diatom species appears necessary before the successful commercialization of this methodology.

The work carried out within the µAQUA project resulted in the preparation of conjugated high-affinity antibodies and receptors capable of recognizing target toxins and in the rapid preparation of target toxin samples by solid phase extraction (SPE) technology. These advancements were instrumental in obtaining one of the most important achievements of the µAQUA project, namely the development, optimization validation and application of different physico-chemical methods such as the multi-toxin detection Luminex assay and the UPLC-MS/MS method to detect and quantify in environmental water samples harmful toxins such as saxitoxin, domoic acid, cylindrospermopsin, microcystin and anatoxin. The anatoxin analogues H2-ATXa and H2-homo-ATX-a were detected by the use of a new UPLC-MS/MS method.

Indeed, toxin detection and quantification methods were applied to the analysis of 194 samples from 6 European countries resulting from a two year water sampling campaign (Table FR-4).

The results obtained in these analyses indicate that Luminex and UPLC-MS/MS yielded mainly comparable results, without any major deviations of the results observed in freshwater samples. Thus, it was concluded that the multi-toxin detection assay developed in this project promises to be a useful tool for the simultaneous detection of multiple toxins in freshwaters in a single assay, unlike traditional analytical methods.

As to the specific results of the analyses carried out on the environmental samples of year one and year two, a detailed description can be found in the 37-45 months periodic report (WP5). The overall conclusions that can be drawn from these results (summarized in Table FR-4) is that no significant levels of the five toxins were detected in any of the samples. However, some permanent levels of ATX-a analogues were detected for the first time across European waters. In fact, two Anatoxin-a analogues, H2-ATX-a and H2-homo-ATX-a, were identified in most of the samples analyzed. In addition, levels of microcystin-LR (MC-LR) and MC-RR were detected in one sample from Germany and one from Turkey, respectively.

Finally, one of the goals of the project was to investigate the possibility and the conditions under which cyanophages could be used for mediation of harmful cyanobacterial blooms (HCBs). Because freshwater cyanophages are poorly characterized in comparison to their marine counterparts, understanding this diversity was considered important to reach conclusions as to the feasibility of this endeavor. Thus, a collection of freshwater cyanophage isolates was obtained and some of them were characterized by microscopy, genetic techniques and analysis of their infection kinetics. In particular, a cyanophage named ΦMHI42 (Figure FR.10), the first freshwater cyanophage ever characterized in-depth, revealed novel and particularly interesting properties. It proved able to infect hosts from a range of different cyanobacterial genera and to possess properties different from those of any previously described phage, in regard to its morphology, host range and infective behavior. Ultimately, the genome sequence of this phage was also elucidated. The nature of the interactions between phage ΦMHI42 and its hosts appeared complex and likely dynamic in response to environmental influence, such as temperature. Overall, the results obtained on the interactions between phages and their hosts suggested the existence of a number of co-antagonistic relationships. Due to the existing evolutionary arms race between phages and their host, and the complex nature of host-phage relationships in the environment, application of phages isolated from previous seasonal blooms, or from temporally or spatially distinct areas related to contemporary samples, are likely to have very little impact on bloom structure. This also relates to the genetic structures of cyanobacterial communities, which are dynamic and may be composed of more than one genus and many hundreds of genotypes. Broad-range phages may lyse a range of species, or even genera, of cyanobacteria but their efficiency for doing so, and their affinity for a particular genus or species may vary dramatically according to their surroundings. In addition, any of the species of cyanobacteria used in this study would also release toxins characterized as part of the µAQUA project into the environment.

Taken together the results obtained indicated that it may not be possible to control naturally occurring cyanobacterial blooms in freshwaters with the use of previously isolated cyanophages.

Description of the potential impact and the main dissemination activities and the exploitation of results

A large proportion of Europeans do not have access to safe drinking water while the health of the entire European population is at risk because of the possible presence of pathogenic organisms and toxins in lakes, rivers and reservoirs used as drinking water supply or for recreational purposes. Indeed, in addition to the microorganisms capable of infecting directly humans and/or animals, many species of microorganisms, especially algae, produce toxins that can cause a variety of poisonings ranging from gastro-intestinal disturbances to death. Most pathogens are small and tend to occur in low numbers, making them very difficult to measure directly.

Global climate changes and massive migration fluxes from Africa and Asia are expected to worsen this situation by perturbing the existing ecological balance determining major changes in the type, abundance and distribution of pathogenic microbes, likely causing (re)-emergence of water-related pathogens. Among the essential measures that should be taken at the European level to cope with this alarming situation is to implement a reliable system for the sensitive early detection of water-borne pathogens and toxins. This was precisely the purpose of the present µAQUA project, conceived with the aim of developing a knowledge-based technology for monitoring, through an efficient, sensitive, robust, rapid and inexpensive test, the presence of toxic algae, pathogenic bacteria, parasites and viruses, and to detect the presence of toxins and of potential toxin-producers in European freshwaters.

The results of the µAQUA project are expected to have a strong impact on the way water safety and quality will be assessed in the future. In fact, whereas the traditional methods employed to this end are laborious, technically demanding and time-consuming, the µAQUA project developed easy to use advanced molecular tools (physical chemical methods and a microarray chip) for the high-throughput detection of toxins and of numerous water-borne pathogens (bacteria, protozoa and cyanobacteria) and of select bio-indicators (diatoms) to assess overall water quality. Through the use of the innovative molecular tools developed by the µAQUA project it should be possible to monitor in real time water quality routinely and semi-continuously. Furthermore, it should also be possible to monitor the dynamics of the populations of pathogens and to make reliable predictions that should allow rapid management response to new situations brought about by environmental changes and to improve the performance of water treatment in terms of efficacy and cost efficiency.

Consequently, the production and commercialization of a microchip for the detection of several target organisms (bacteria, cyanobacteria, toxin-producing organisms and parasites) appears imminent. Furthermore, in a foreseeable future the same or another chip will also be spotted with probes capable of detecting several water-borne viruses whose design and validation has suffered some delay, due to objective technical difficulties.

A method based on a microchip spotted with probes targeting species-specific genomic sequences encoding portions of two proteins hybridized with amplicons (PCR products) obtained by use of just two pairs of primers (one pair for each gene) has also been developed and should allow the detection of diatom species representing biomarkers

of water quality. Because the list of diatom species characteristic of different levels of water quality varies depending upon the geographic region where the water bodies are located, the success obtained within the µAQUA project with the development of this methodology can be taken as proof of principle and the experience so far gained exploited for the design of probes targeting the same genes of other diatoms belonging to other geographic areas. Once completed, a microchip suitable for use in large geographic regions will be prepared and will be ready to be commercialized, together with a kit enabling the user to obtain the necessary amplicons in a single PCR reaction. It is the intention of some µAQUA partners to make commercial use of the microarray designed and developed to detect water-borne pathogens, mainly bacteria, cyanobacteria and toxin producers.

For the commercial success of the µAQUA products, a vigorous dissemination activity is important. Indeed, several dissemination actions are planned for the best use and exploitation of the results of this project. During the course of the project, many opportunities for the dissemination of the µAQUA approach, goals and results have been offered by publications, conferences, videos produced by the µAQUA partners that have been mentioned elsewhere in this as well as in the two previous periodic reports. Of particular relevance from this point of view were the International Symposium "*Small Solutions for Big Water-Related Problems Innovative microarrays and small sensors to cope with water quality and food security*" held in Rome at the end of October 2014 and the Euronews reportage produced by the coordinator (PTR1) with the collaboration of PTR11, PTR9 and PTR12 and seen by millions of European television viewers (https://www.youtube.com/watch?v=i7vvq7dcynU).

For the future, several dissemination activities are foreseen. First of all, numerous articles, contributed by essentially all the µAQUA partners, will appear in the special issue that the on line open access International Journal of Environmental Research and Public Health has devoted to the aforementioned International Symposium. Furthermore, several µAQUA PTRs are scheduled to present talks in various scientific meetings and events. For instance, the coordinator PTR1 will present two talks on his µAQUA results in two International Congresses; in addition, he will take advantage of his imminent four-months stay in China, where he has been invited to present his accomplishments on the development of new antibiotics, to present also the results obtained by his group on the species-specific detection of biomarker diatoms. China represents a big market and its government is placing increasing emphasis on environmental issues; it is not unreasonable to imagine that PTR1 might be able to attract investments to support the pursuit of his research on diatoms detection by microchip, possibly by the same investors who are interested in investing in the development of antibiotics.

Overall, these dissemination activities, presented in several European and extra-European countries should help to bypass/remove potential biases and to increase the awareness of the nature of the Microarray, and of its advantages over the current analytical methods.

Finally, for the future at least some µAQUA partners plan to organize a practical demonstration of the molecular tools developed by the µAQUA project to a select public, mainly constituted by potential users of these tools. Although it is difficult to establish where and when this demonstration may take place, the most likely location will be in the

laboratory of PTR12 (Scienion), who has a strong interest in the production and commercialization of the microchips. It is hoped that this event could take place before the summer of 2016.