AQUAVALENS

Protecting the health of Europeans by improving methods for the detection of pathogens in drinking water and water used in food preparation

Key Findings of the FP7 Aquavalens Project

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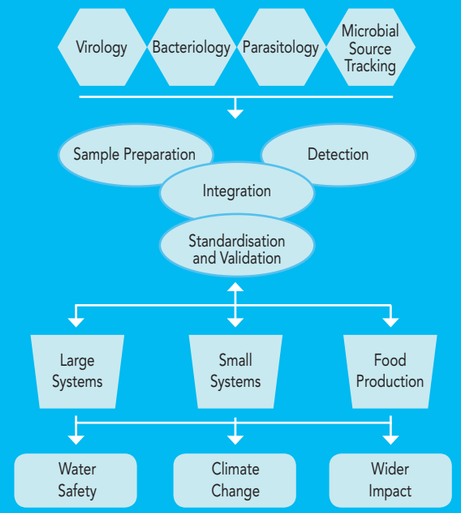
Contents

The €8.9 million Aquavalens research project aims to improve the safety of European drinking water through the development of technologies for more rapid detection of viruses, bacteria and parasites in water.

The Aquavalens project, led by the University of East Anglia, has developed new molecular techniques to allow routine detection of waterborne pathogens (microbes that cause disease) and improve the provision of safe, hygienic water for drinking and food production throughout Europe. The Aquavalens project is funded by the European Union Framework Programme 7 project and has brought together small-medium enterprises (SMEs), industries, universities and research institutes with the mission of protecting the health of European citizens from contaminated drinking water, including water used in food processing. We have achieved this by developing sustainable technologies to enable water system managers whether in large or small water systems or within food growers/ manufacturers to better control the safety of their water supplies. Aquavalens is centred on the concept of developing suitable platforms that harness the advances in new molecular techniques to permit the routine detection by non-scientists of waterborne pathogens and improve the provision of hygienically safe water.

Whilst in recent years there have been considerable developments, especially in molecular technology, very few systems are available that meet the current needs of water providers. These new techniques result in detailed insight into the pathogenic load, hygienic quality and the specific microbial strains (viruses, bacteria, protozoa) responsible for outbreaks of waterborne infections. Although Europe has some of the safest drinking water in the world, outbreaks of waterborne disease still occur each year. Currently it takes two or more days for the detection of infectious risks in drinking water, Aquavalens has developed easy to use techniques that would allow this to be done in far less time with greater insight into the type, strain, pathogenic load, and importantly, source of the contamination.



Overview of the Aquavalens project
Water is essential to life, clean water is essential to health. The quality of the water we use every day is taken for granted every day.

Professor Paul Hunter
Coordinator

Robust molecular systems for detection and typing of health-significant enteric viruses

The European Legislation for drinking water microbiological analysis requires the detection and enumeration of bacteria to determine water safety. But is the detection of bacteria enough to determine the safety and good quality of drinking water?

Several outbreaks have been reported in the European Union caused by non-bacterial organisms, such as viruses and protozoa in drinking water samples that were considered safe according to the EU legislation based only on bacterial parameters. In 2016, a large outbreak that resulted in more than 4,000 people becoming ill with norovirus (sometimes referred to as the winter vomiting bug) in northeastern Spain. The cause was later found to be contaminated bottled spring water. Currently, any drinking water (for instance, tap or bottled water) must be analyzed only for bacteria to ensure it is safe for human consumption. However as shown by this outbreak, the absence of bacteria does not necessarily mean the absence of other organisms such as viruses or protozoa. This is due to the fact that bacteria, viruses and protozoa are completely different organisms with very distinct resistance to disinfection treatments such as chlorine and UV, as well as differential survival rates in environmental waters such as rivers or lakes.

Why does the drinking water legislation only stipulate the detection of bacteria?

Standard methods for the detection of bacteria are well established. These methods are easy and cheap to perform, and do not require specialized personnel. Detection of viruses is trickier, since they are tiny. These infectious agents (about 1,000,000 times smaller than a centimeter) can only grow inside the cells of living organisms, including human cells, which makes them difficult to grow in the laboratory. Unlike bacteria, viruses require tissue culture (growth of cells outside of the body) for their replication but there are currently no suitable cell lines (cell types that would allow this), therefore, molecular detection of viral genetic material using polymerase chain reaction (PCR) is now extensively used. PCR is a process that copies a targeted sequence of DNA. A short artificially produced length of DNA called a primer (that has a sequence complimentary to the target sequence) binds to its target and an enzyme called DNA polymerase copies the DNA. This process is repeated many times until a high concentration of the target DNA is produced.

PCR detection is fast (providing results in about 2h), extremely sensitive and specific for the virus of interest and is less expensive than the use of cell lines, although it is still expensive for diagnostic laboratories.

What approaches have been used within the Aquavalens project to address the issues related to viral detection?

A PCR technique that combines the detection of multiple targeted viruses was developed. This allowed the simultaneous detection of norovirus and Hepatitis A virus in a single procedure, decreasing the associated costs and hands-on time, without compromising the high levels of sensitivity obtained when analyzing one virus at a time.

Viruses are usually more resistant to disinfection treatments than bacteria. PCR is capable of detecting what is infectious and what is not. Noroviruses were subjected to disinfection by one of the most commonly used disinfectants, free chlorine. The goal was to increase the current knowledge of the effects of the disinfectant on the various viral components and to expand the use of PCR to distinguish between infectious and non-infectious viruses. The new approach allowed the differentiation between resistant and sensitive virus components. Other disinfectants have been tested with varying levels of success.

In case of an outbreak, how can clinical samples isolated from infected patients be related to drinking water?

Outbreak investigation, an important aspect of public health, can help pinpoint the source of ongoing infections and prevent additional cases. To determine the source of infection, laboratory tests comparing the bug infecting the patients and the bugs that exist in suspected sources (water, food, etc…) are required. Within the Aquavalens project, several tools have been developed and applied successfully to outbreak cases, including the Spanish outbreak aforementioned. The developed tools were also used in an outbreak related to bathing waters in Finland, where around 1,400 persons fell ill. The Aquavalens tools determined that norovirus was the main causative agent and a linkage to small lakes and beach users was made. From these outbreaks, guidelines have been published for a tighter control because analysis of bacteria was not sufficient to ascertain the hygienic quality of water.

Are the methods developed within Aquavalens robust enough to allow for the inclusion of viruses in future Directives?

By requiring only the analysis of bacteria, the current EU Potable Water Directive would miss the presence of other bugs that could present high risks to human health. Therefore, as proven by the recent outbreaks, the addition of other microorganisms to the European Directive is paramount to protect human health. Developments achieved within the Aquavalens consortium provide better, more robust, cheaper, and faster methodologies for the detection of infectious viruses, which can be used for routine laboratory analysis and thereafter could assist the inclusion of viral detection in future water Directives.

The Aquavalens tools determined that norovirus was the main causative agent and a linkage to small lakes and beach users was made.

Advancement of quantitative and sensitive detection of pathogenic bacterial species and genotypes in drinking water using molecular techniques

Major human health threats from waterborne bacteria and why we need molecular detection tools

In the 20th century, people of Europe suffered large outbreaks of waterborne bacterial pathogens, such as ***Vibrio cholerae***. The last outbreak of cholera in Italy in 1973 infected almost 1000 individuals. Many of the waterborne pathogens, such as *Vibrio, Salmonella* and *Escherichia*, are difficult to detect because they do not grow in the laboratory but are still infective, i. e. they are in a **viable but non-culturable (VBNC) state**. Therefore, advanced molecular, DNA-based detection technologies are needed to respond quickly to outbreaks, to provide guidance for patient treatment and limit disease transmission.

Such DNA-based technologies were developed and applied to investigate how the abundance of *Vibrio* populations had fluctuated during the last 50 years in the North Atlantic and North Sea using preserved plankton samples collected by the Continuous Plankton Recorder (CPR) survey. Presence and relative abundance of *Vibrio* bacteria were measured in CPR samples showing that *Vibrio* populations, including the human pathogen *Vibrio cholerae*, increased significantly as the sea water warmed up. This increase was associated with an unprecedented occurrence of environmentally acquired *Vibrio* infections in the human population of Northern Europe, especially along the Irish Sea, the English Channel and the North Sea, in recent years. This study suggests a rise in people contracting *Vibrio* infections after swimming in the sea or eating raw seafood, like oysters, could be related to ocean warming linked to climate change. Most bacteria present in our oceans are not harmful to humans, but a better understanding of the occurrence of vibrios whose pathogens are directly associated with gastroenteritis, outbreaks of pandemic diseases and infections in open wounds exposed to seawater causing septicemia can be seen as a relevant contribution to **climate change preparedness**.

Next Generation Sequencing (NGS) has revolutionized the analysis of natural and man-made microbial communities by massive parallel sequencing of taxonomic marker genes obtained after polymerase chain reaction (PCR) (technique that copies target DNA). Nowadays, NGS is frequently applied for the assessment of microbial water quality by determination of the whole bacterial microbiome, i.e. the sum of all microorganisms and their genes from a specific site in the ***Drinking water supply system (DWSS).*** Thus, high-throughput sequencing of PCR products has the potential to provide valuable information regarding not only the environmental distribution and diversity of bacterial species but also their temporal and spatial behavior.

This developed NGS approach provides a new molecular surveillance tool to monitor all major species of targeted bacteria.

To this end, we pursued a new NGS approach comparing a specific bacterial genus with the whole bacterial microbiome based on the amplification of 16S rRNA genes from environmental DNA extracted from respective water samples. Genome standards of reference strains of the targeted individual species were used to calibrate the molecular assay and determine its accuracy and precision. Validation of the developed approach was done by using a set of representative freshwater samples from different drinking and raw water, and included comparison with real-time PCR measurements (the standard method allowing quantification of copied DNA) to assess the accuracy of quantification by the NGS-based approach (see **Figure** overleaf).

We need molecular standards for absolute quantification of bacterial pathogens

For determination of the absolute abundance of the targeted bacterial pathogens by NGS and real-time PCR, molecular standards are needed. These standards should be based on genomic DNA from the reference strains of bacteria. They should contain a defined number of genome copies to allow determination of **genome units (GU)** per assay using a calibration curve. Such **genomic standards**, called **GenoStand®**, were developed by the Aquavalens partner Genetic PCR Solutions (GPS). These calibrated genomic standards are accurately quantified and purified **genomic DNA** of certified reference strain species. These genomic standards are crucial for the calibration of molecular assays and are extensively used as positive controls or to make calibration curves to determine the absolute abundance of target pathogens in terms of GUs.

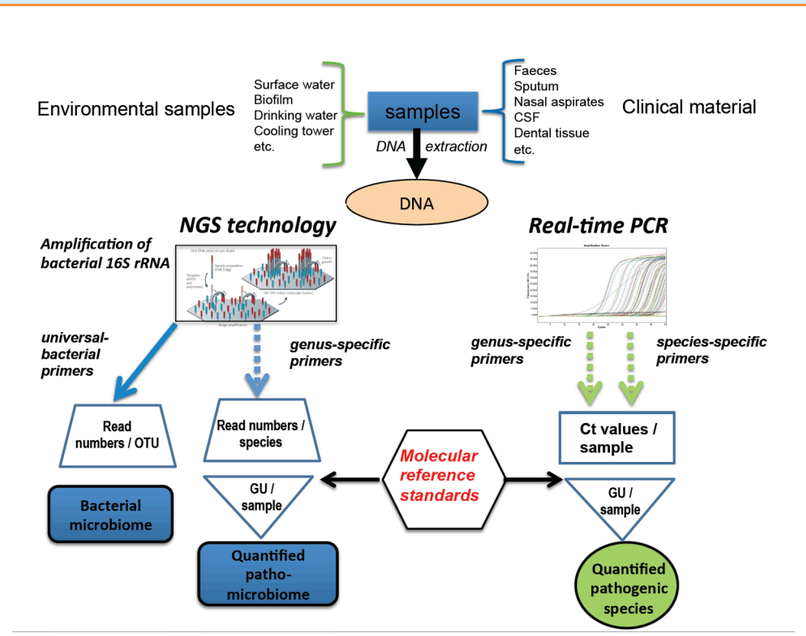
Application of genus-specific NGS approach to various genera

The NGS approach was validated for the genera ***Pseudomonas*** and ***Legionella*** targeting the 16S rRNA gene and applying genome standards for *P. aeruginosa* and *L. pneumophila*. Our results revealed that the generated NGS libraries presented a low average raw error rate per DNA base (<0.5%) due to the use of high-fidelity enzymes during PCR. This approach also showed high specificity (>95%) and very good repeatability. NGS read counts did not reveal considerable biases and showed a sensitive as well as precise quantification along a dilution range using the certified genome standards. The sensitivity of our genus-specific approach was at least one order of magnitude higher compared to the universal NGS approach, i. e. using universal primers targeting the 16S rRNA gene of all bacteria. Furthermore, the taxonomic resolution of the genus-specific NGS approach could be increased by using another target gene than the 16S rRNA, i. e. gyrase B gene. The in-situ applicability of this NGS approach was demonstrated for *Escherichia coli* and *Salmonella enterica* with high specificity and accuracy in contaminated river water. Overall, the genus-specific NGS approaches could reach comparable detection limits to real-time PCR (10 GU per assay).

Whole Genome Enrichment for high resolution analysis of waterborne bacterial species and their virulence genes

The detection and typing (classification) of waterborne pathogens in aquatic environments encounter major methodological challenges related to the fact that bacteria are often present in very low concentrations and cannot be easily grown in the laboratory. Therefore, NGS was combined with **whole-genome enrichment (WGE)** for direct genotyping and metagenomic (genetic material recovered directly from environmental samples) analysis of low abundance bacteria (<50 genome units/L). To this end, a WGE protocol was developed for the enrichment of target bacterial DNA in water samples using ***V. cholerae*** as a model. WGE was capable of enriching single-copy nuclear loci corresponding to >99.5% of the capture target region. Based on this assumption, the detection limit of the assay downstream of water filtration and DNA extraction is theoretically estimated to be close to 1GU per reaction and allowed the detailed sequence analysis of specific **virulence genes** (genes that aid infection).

The developed NGS approaches provide **new molecular surveillance tools** to monitor major water-related bacterial pathogens, such as ***Vibrio, Escherichia, Salmonella, Legionella*** **and** ***Pseudomonas***, in qualitative and in quantitative terms at the species level if appropriate genome standard are used to calibrate the assys. Overall, the genus-specific NGS approach opens up a new avenue to substantial diagnostics in a quantitative, specific and sensitive way. To this end, genus-specific NGS could replace or supplement real-time PCR for the quantitative determination of waterborne pathogens. NGS could furthermore be combined with WGE to improve the taxonomic (classification) resolution and enable direct genome analysis of low abundance waterborne pathogens, like individual genotypes of *V. cholera,* including their virulence genes.



Comparison of quantitative molecular detection of bacterial pathogens by NGS technologies with real-time PCR. Dotted arrows = detection of specific pathogens (genus/species), blue arrows = 16S rRNA gene based, OTU = operational taxonomic unit, GU = genome units.

Molecular markers for the detection and genotyping of waterborne protozoan parasites

Toxoplasma gondii: Optimisation of water sample processing and validation of a detection method using field samples.

The protozoan parasite *Toxoplasma gondii* is one of the commonest parasites to infect warm blooded animals worldwide, with cats being the only definitive host. Studies have confirmed that environmental contamination with *T. gondii* is widespread. Indeed the significance of toxoplasmosis has increased globally and it is now considered to cause the highest burden of all food-borne pathogens. The waterborne transmission of this parasite is also likely to be more important than previously thought as evidenced by large scale outbreaks of toxoplasmosis caused by contamination of drinking water. These outbreaks have been responsible for serious public health issues; abortions and birth defects through congenital infection in pregnant women, severe symptoms in people with weakened immune systems and more recently, eye disease in healthy people due to the emergence of uncommon strains of the parasite in countries such as Brazil. Eating raw or undercooked meat, and cross infection from cat faeces, are recognised ways of becoming infected with *T. gondii*. Another route is through contamination of drinking water. Very little is known about water transmission because no approved methods for water testing are currently available.

In this project, we optimised and validated methods for the processing of water samples and developed a sensitive and specific test for the detection of *T. gondii* in water. We then validated these methods using 1427 water samples from raw and finished waters, collected from 147 public water supplies throughout Scotland, where 8.79% of the samples were positive. One third of the water plants tested yielded at least one positive

sample and these were widespread geographically. This is the first study reporting the detection of *T. gondii* DNA in water supplies in Scotland, where environmental contamination with the parasite is known to be widespread. These findings have been discussed with Scottish Water, the Drinking Water Quality Regulator for Scotland and Health Protection Scotland. As a result, funding has been obtained to determine whether the *T. gondii* DNA originated from oocysts (infective stages in the parasite life cycle) and if so, investigate if they are viable (infective) or not. This research project will be completed by June 2018.

Cryptosporidium: There was a lack of genetic markers for assessing the relationship between Cryptosporidium samples during epidemics. We sequenced the entire genome so we could find new markers for a genotyping scheme.

Cryptosporidium is another common parasitic organism and is well known for causing waterborne outbreaks of diarrhoeal illness when water treatment is inadequate. Standard methods for detection have been in place for many years, including an International Standards Organisation method (ISO 15553:2006). There is already a lot of information about *Cryptosporidium* occurrence in source and drinking water, so we needed to address a different problem from *T. gondii*. When *Cryptosporidium* epidemics occur, it is important to find out whether patients have been infected from the same source; this could be livestock, wildlife or other people. Looking at the genetic code of the parasites isolated from patients and suspected sources such as drinking water can help establish how related they are and if they might be transmitted between hosts. To do this cost-effectively, genetic markers that can be investigated quickly and cheaply in an epidemic situation need to be identified. To find suitable markers, we developed a way to examine the entire genetic code (genome) for *Cryptosporidium parvum* and *Cryptosporidium hominis* because these are the species that cause most human disease. By examining the entire genomes, we found markers that met our criteria for inclusion in a scheme to compare the relatedness of these parasites. We linked in with another EU funded project **(http://www.euro-fbp.org/)** to establish the process for new markers to be incorporated into an internationally-accepted scheme.

We optimised and validated methods for the processing of water samples and developed a sensitive and specific test for the detection of *T. gondii* in water.

Cryptosporidium: Implementation of management solutions based on the results of a Cryptosporidium study in a catchment with a history of public water supply contamination and impact on water quality and cattle health.

Cryptosporidium parvum, an important zoonotic parasite (spread from animals to people), represents a threat to livestock health, water quality and public health. The main reservoirs of *C. parvum* are known to be farm livestock but the contribution of wildlife in water catchments is unclear. The aim of this study was to establish *Cryptosporidium* prevalence, species and genotypes present in livestock, wild deer and water samples from a water catchment area with a history of *Cryptosporidium* contamination in the public water supply. We recorded the impact of catchment management improvements on the resulting water quality. Results indicated a very high prevalence of *Cryptosporidium* with a predominance of *C. parvum* in livestock, deer and water samples tested. The predominance of *C. parvum* in livestock and deer suggested that they represent a significant risk to water quality and public health. Genotyping results suggested that all animal species had a role to play in contamination of water sources. Management solutions, such as farmer meetings, livestock fencing and provision of water troughs to reduce *Cryptosporidium* on farms and in the public water supply, have been implemented and shown to improve both animal health and water quality. The impact of water management solutions driven by this research is illustrated by data from Scottish Water: In the six months before the improvements, there were 16 final water positive samples and 21 raw water positives, whereas in the two years following water supply improvements, there was one final water *Cryptosporidium* positive and two raw water positives.

Cryptosporidium genotyping results suggested that all animal species had a role to play in contamination of water sources.

What is polluting my water? Microbial Source Tracking to determine the source of faecal pollution in water

What is polluting my water? Microbial Source Tracking to determine the source of faecal pollution in water

The availability of drinking water for the global human population is one of the great challenges facing humanity in this century. Water resources are limited and there is an increased water demand mainly due to the increasing human population and its concentration in large urban areas. Concurrently, the water cycle is impacted by pollution caused by different water uses (agriculture, animal husbandry, industries, food processing, urban sewage, etc.). However, organic compounds (mainly faecal matter) are the main pollution source. This faecal contamination is conducive to the transmission of waterborne pathogens, which use water as a transmission vehicle to spread the disease to new hosts and as such is associated with significant human risk. The identification of faecal pollution sources is very desirable and this area of research, is called Microbial Source Tracking (MST). The name reflects the most important parameter, which is the detection of microbes associated with the host producing the faecal contamination. The MST technique allows us to pinpoint water pollution sources for enabling legal action and the avoidance of further pollution. So, why do we need to determine the faecal source of pollution in water? There are many reasons to do this including: assessment of microbial risk, mapping of catchments and search for solutions to conflicts between land users. Consequently, determining the source of faecal contamination is increasingly important in the management of water resources to update control measures, reduce or eliminate contamination, or identify environmental responsibilities.

Defining parameters for the prediction of contamination sources

Within the Aquavalens project, an approach for the prediction of the origin of faecal contamination with high accuracy has been developed. This procedure is based on the use of inductive learning machine methods (computer based predictions) to select a small number of analytical parameters that act as indicators of the origin of the faecal contamination. Many of these parameters are determined based on analyses of certain microorganisms (bacteria or viruses) that are associated with the faecal matter of the host (human or animal). We have also considered some other indicators such as chemical compounds associated with human activities and some markers of host cells (mitochondria).

Initially, Aquavalens partners evaluated the use of more than 40 MST parameters identified in previous research studies. All these parameters were measured at the source in waters with faecal contamination from a unique known origin (human, cattle, pigs, poultry, etc.), in different European regions from Portugal to Finland. These samples allowed "training of the computers" based on real situations in order to define the best prediction models using the lowest number of parameters. Once the computational system was trained, the computer was programed to simulate thousands of situations where pollution is diluted in water ways and/or its concentration decays by the natural degradation process over time. Dilutions of up to 10,000 times were considered, bringing wastewater quality to those levels requested for bathing water. Moreover, the natural decay of the contamination (aging) was estimated to occur up to 300 hours after the introduction of the contaminant into the water resource. Therefore the trained computer system developed thousands of situations such as those we could find in the environment.

Subsequently, faecally contaminated water environmental samples, with different fold-dilutions and aging, were analysed to validate the developed models. The faecal source, dilutions and aging of this set of samples was not previously indicated to the computer with a goal of testing its accuracy. This set of samples was used to assess the capability of the developed prediction models to give answers to different situations such as distinguishing human from animal faecal contamination or identifying the main source of faecal pollution (human, bovine, swine, or poultry). It is also possible to asses the accuracy of predictions regardless of whether the models are based on any subset of the studied parameters (cultivable or molecular) or exclusively based on molecular parameters which could potentially be incorporated into automated platforms or commercial kits.

The predictive model can accurately identify sources of faecal contamination

The predictive models succeeded in determining the source of faecal contamination with high accuracy. It was possible to differentiate whether the source of pollution was human or animal with an accuracy of 98% in cases using only four parameters. It was also possible to determine which was the major faecal source, among the four analysed, with an accuracy of 96% using only five parameters. If users of the prediction software want to use molecular parameters only with the aim of subsequent automation, the accuracy was slightly lower (92% for differentiating human versus animal contamination, and 80% for identifying the main source within the four studied).

The predictive models succeeded in determining the source of faecal contamination with high accuracy.

Aquavalens has identified subsets of MST indicators, which are most appropriate for the further development of automated platforms or easy to use commercial kits for resolving MST cases. The developed numerical approach allowed the predictive models to characterise real situations occurring in environmental systems. These predictions could be improved if the selected MST indicators are highly sensitive and specific to the host producing the faecal pollution. Finally, any additional data provided by users to the trained computational platform and used for further calculations would further improve the accuracy of the predictions.

Advanced qPCR innovations for microbial testing in water: Knowledge transfer through an small-medium enterprise (SME)

One of the strengths of AQUAVALENS has been to incorporate several SMEs into the consortium. One such SME is Genetic PCR solutions™, who have been developing and validating quantitative PCR solutions for the consortium. This SME has been working with Aquavalens partners to produce all-in-one kits for the accurate and robust detection and quantification of targeted pathogens. These developed kits included the following bacterial targets selected for water testing in the frame of the **Aquavalens** project: *Salmonella enterica, Campylobacter coli, Campylobacter jejuni, Vibrio cholera, Escherichia coli species-specific, Escherichia coli* O157:H7, *Pseudomonas aeruginosa, Legionella pneumophila, Listeria monocytogenes,* and *Arcobacter*. These kits are pictured below.

GPS™ has developed genomic standards (GenoStand®) for all Aquavalens targeted pathogens.

From research to applications of better solutions to meet new requirements. GPS™ Innovations during Aquavalens project

Although the qPCR technology was available many years ago, some simple drawbacks have contributed to delay its implementation as a standard detection technique in regards to water testing. We have dedicated some of **GPS™** research resouces in the **Aquavalens** project with the aim of solving these problems and providing solutions that facilitate the transfer of this technology to a ready-to-use product.

For instance, a component of qPCR kits has to be maintained at low temperature (4°C) which makes transport very expensive and is a risk for quality assurance. We resolved this issue and produced qPCR "F100 format" kits used by **Aquavalens** partners, importantly the components of these kits are stable at room temperature. Kits may now be transported in small envelops and quality control is certified by single batch calibration which reduces cost, an important aspect if this technology is to be used widely within the field of water testing.

More than 300 units of F100 kits (>30,000 tests) for bacterial and protozoan pathogens were used by the Aquavalens research partners, as a reference test during process/ technique development and ring-test validation (comparative/validation testing within/between laboratories).

Another issue is mistrust generated by the lack of validations that comply with international standards, which could lead to loss of objectivity. In response, qPCR kits were subjected to **Internal Validation** following guidelines of international norm **UNE/EN ISO/ IEC 17025:2005** and French Standard NF T90-471. The terms of validation defined by these standards relied on severe criteria for acceptance, namely all experiments had to be repeated ten times.

Often, the need for accurate **Molecular Reference Standards** has been a great obstacle, particularly to ascertain absolute abundance of bacterial pathogen strains in water samples. This is not only a demand of the market, but also a request from Aquavalens partners to conduct research.

To solve this matter, GenoStand® was developed for all Aquavalens selected pathogens. This product contains a known number (calibrated) of genomic DNA copies of certified reference strains and species from public culture collections This was widely used by the Aquavalens project as a reference material for several tasks, such as quantitative and sensitive detection of pathogenic bacterial species and genotypes (collection of genes) in drinking water by Next Generation Sequencing (NGS), as well as a reference material in two ring-tests organized within Aquavalens.

Implementation: learning by listening to the existing market needs.

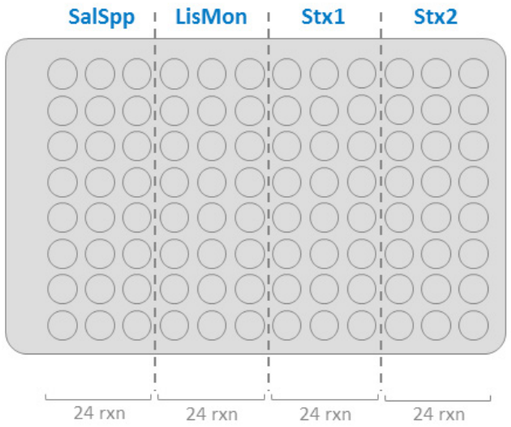
Many problems intrinsic to classical PCR techniques derives from the tedious handling needed to set up the assay for testing a high number of samples. There is also a risk of cross-contamination, damage induced by freezing/thawing, deterioration by UV light, and is altogether a considerably time-consuming process. MONODOSE dtec-qPCR developed by GPS™ minimises these issues. The kit comprises all dehydrated qPCR reagents for each specific pathogen detection in single PCR ready-to-use tubes and only requires the addition of samples to perform the qPCR. This decreases the

time it takes to carry out water testing which is an essential factor as water is usually consumed rapidly after treatment, for this reason rapid water testing is very important for the Aquavalens project.

Another great advance was the design of a unique PCR protocol, allowing all different pathogens to be tested at the same time, within the same assay. This gave rise to the **qPCR Panel Concept**. Although PCR is highly sensitive (it may detect a single cell, at least theoretically), the main problem of the methodology resides in the sampling and extraction/purification steps. Currently, the best sampling and extraction techniques are often poor at concentrating the targets but, ironically, are efficient concentrators of compounds that may inhibit the PCR reaction making it more difficult to detect pathogens in collected water samples.

Given this problem, we developed two solutions: first, a series of GPSpin® kits, for the fast and easy extraction/purification of DNA/RNA from bacteria and viruses; and second, FastCleaner® which is a buffer that removes inhibitors from DNA/RNA extracts in 5 minutes.

Finally, reagents and conditions were optimized to reduce PCR timing to a minimum, with the qPCR FastCycling Protocol, being the fastest reported reaction of just 17 minutes!



Sample processing to maximize recovery rates of pathogens in large volume water samples

To improve water safety, it would be very helpful if we could quickly and easily prepare a usable water sample to analyse for pathogens (microbes that cause disease) on-site, or off-site where facilities are lacking. Therefore, efficient methods to concentrate pathogens in water samples are needed to control water quality and ensure the safety of water supplies.

Why sample large volumes?

Microorganisms, such as viruses, bacteria and parasites, that may cause illness, can be present in water in such low numbers that they are difficult to detect and yet still represent a major threat to human health. To maximise the chance of detection, a large volume (10-1000 litres) of water needs to be sampled and concentrated to less than a few millilitre or microlitres that can then be analyzed.

What are the requirements?

A good concentration procedure should meet several criteria:

- technically simple
- fast
- affordable
- suitable for a wide variety of agents (viruses, bacteria and protozoa) and types of water (drinking water, irrigation water, source water)
- provide high pathogen recoveries without concomitantly concentrating inhibitory compounds that may interfere with detection

What is the best filtration technology for sample processing?

Previous investigations have evaluated filter efficiencies but it is difficult to combine and compare their findings because the studied factors varied greatly including: pathogen(s) tested, pathogen concentration, water types and detection methods. Considering the requirements outlined above, the Aquavalens research focussing on sample preparation, tested and evaluated three different filtering techniques, in particular their efficiencies to concentrate viruses, bacteria and/or protozoa from various volumes and types of water. The filters tested were:

- the low-cost Rexeed 25AX ultrafiltration (DEUF)
- the very easy monolithic affinity filtration (MAF)
- the very cheap glass wool filtration (GWF).

In principle, the methodology consists of the following five steps:

- Concentration of microorganisms from the entire water sample by filtration
- Elution of the microroganisms from the filter
- Further concentration of the microorganisms from the eluted suspension
- Purification of the the genetic material (nucleic acids) of the microorganisms
- Detection of target nucleic acids from the microorganism of interest using molecular methods

Some methological improvements such as reagents used, either during primary and secondary concentration, for microorganism elution from filters and/or during nucleic acid extraction, have been introduced by the Aquavalens partners. This was undertaken to optimise concentration efficiencies to allow for detection by polymerase chain reaction (PCR) (technique for the amplification of target DNA/RNA to allow easier detection), using commercially available detection kits provided by Genetic PCR Solutions and CeeramTools.

The concentration techniques have been tested to evaluate performances using qPCR (a quantitative form of PCR to measure relative concentrations) detection of spiked microbes in different types of water. They have also been applied in outbreak investigations of virus presence in drinking water as well as to survey source water quality. In addition, MAF and GWF filters were used to concentrate virus from sewage samples in the context of metagenomics (sequencing from environmental samples).

Overall, the results obtained showed that all three concentration methods could recover bacteria, viruses and parasites from 60-100 L of spiked water samples. The GWF was optimised to recover enveloped viruses, porcine transmissible gastroenteritis virus and a β-coronavirus (model for the Middle East Respiratory Syndrome coronavirus). The recovery efficiencies and inhibition varied with the type of microorganism and water quality. A summary of overall method characteristics for the three concentration techniques tested are schematically presented in Table 1.

All three trialled concentration methods could recover bacteria, viruses and parasites.

Investigation of drinking waterborne outbreaks of gastroenteritis occuring in Sweden, Denmark and Spain during the project period, using Aquavalens techniques, allowed the detection of norovirus in contaminated water samples concentrated using all three filtering techniques.

Are there alternatives to filtration?

We have developed microfluidic devices, containing flow channels with dimensions of the order of a single human hair, targetting specifically waterborne parasites to allow their concentration. A high-throughput method has been developed, in which spiral channel geometries and appropriate flow rate selection control the behavior of parasites directing them into a narrow collection channel and removing them from the rest of the sample (see Figure 1). High recovery rates of parasites (95% for *Cryptosporidium* and 86% for *Giardia*) have been achieved, significantly exceeding traditional approaches. This system has also been tested successfully using tap water. Additionally, by stacking together several of these systems (e.g. a 10 layer stack), 50mL of sample was concentrated in approximately 10 mins, with a performance comparable to the single layer system. This demonstrates that microfluidics could offer the potential to replace centrifugation since sufficient volumes can be processed in a reasonable time frame.

Another component of the microfluidics work was targeting species as well as viability based separation using microfluidic devices, with active forces incorporated. In this case, microfluidic systems were manufactured with electrodes included to generate electric fields inside the flow channel. By appropriate choice of electric field, species and viability based separations can be successfully performed (e.g. 95% of viable oocysts extracted to one channel outlet), thus enabling improved characterisation of identified pathogens.

Nevertheless, some challenges remain including creating easy-to-use automated systems for use by relatively inexperienced (in terms of microfluidics) end-users such as water utilities. Further performance validation with a wider range of pathogens and a variety of water sources would also strengthen the evidence for wider adoption of this type of system.

Is automated and online concentration and detection possible?

None of the current concentration techniques can be used on-line (plugged into a pipe) and a fully automated method does not seem realistic. This is because the secondary concentration in all three methods requires equipment (e.g. a centrifuge) not normally present at water treatment plants, see table 1. Therefore, concentrates need to be processed at a laboratory, along with the qPCR detection.

However, the Rexeed 25AX-filter has been incorporated in a filtration device developed by Aquavalens, enabling on-line automated filtration in either a dead-end or tangential flow manner. Filtration can also be performed on-site by connecting filters to a tap. For MAF and GWF, it may be possible to develop disposable concentration units, which for GWF is presently discussed within Aquavalens. Utilising a microfluidics set-up could avoid the need for centrifugation and thus enables a greater degree of processing and detection on-site. However, further method optimisation is needed before prototypes could be tested in the field.

Where can the methods be applied?

The concentration methods developed within the Aquavalens project could have a great value for stakeholders through contribution to improved public health. They could be used in field studies in European drinking water systems such as large or small water supplies or for testing water used for food production.

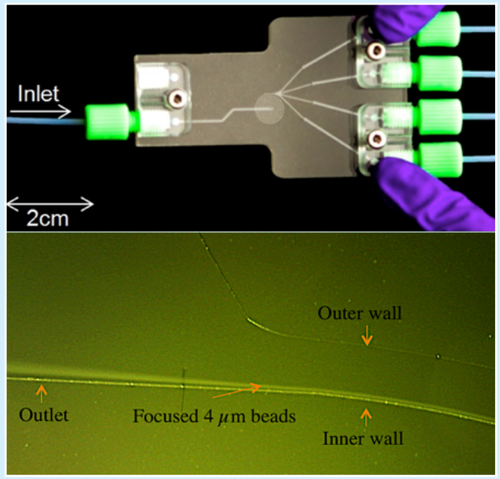


Figure 1 Top panel – image of the types of system developed showing the spiral channel and four different outlets. Parasites will be collected in one of these. Systems could be redesigned with more outlets for a greater concentration factor. Bottom panel – image of the system performance with beads allowing us to visualise the concentration and lining up of particles into a single stream that can be directed to a chosen outlet.

Steps in procedure	Method characteristics		Filtration techniques		
			Hollow fiber	Monolithic affinity	Glass wool
Water type			Drinking water Surface water	Drinking water, surface water, sewage water	Surface water Drinking water, sewage water
Volume filtered	Liter		50-200	10-60	5 – 50
Microroganisms	Virus*		NoV, MNV, HAdV, MC_0, MS2	NoV, MNV, HAdV, MC_0	NoV, MNV, HAdV, HAV, TBGV, MC_0
	Bacteria		*C. jejuni, L. mono S. enteritidis, E. coli O157*	*C. jejuni, L. mono, S. typhimurium*	*C. jejuni, L. mono, S. typhimurium*
	Protozoa		*Cryptosporidium, Giardia*	Cryptosporidium	Cryptosporidium
1° Concen-tration	Mechanism		Size exclusion	Adsorption by ionic interaction	Adsorption by ionic interaction
	Requirement of preconditioning of filter		Yes	No	Yes
	On-site filtration		Yes (tap or pump)	Yes (tap or pump)	Yes (tap or pump)
	On-line filtration		No	No	No
	Elution from filter		Back flush (pump, tubing)	Rinse (pump, tubing)	Rinse (pump, tubing)
	Water type sensitive	Turbidity	Yes (turbid eluate)	Yes (dirt decrease the sample volume)	No
		pH	No	No	No
2° concen-tration	Virus, bacteria and protozoa		Polyethylene glycol/NaCl or Centrifugal filter		
	If not all in one extraction		Bacteria and protozoa: centrifugation Protozoa: Imuno magnetic separation		

Table 1. Summary of overall method characteristics for the three applied filter concentration techniques

*NoV: norovirus, MNV: murine norovirus (model for human norovirus), HAV: hepatitis A virus, HAdV: adenovirus, MC0: mengovirus (process control), MS2: Bacteriophage (process control), TBGV: Porcine transmissible gastroenteritis virus (model for the Middle East Respiratory Syndrome coronavirus)

Detection of pathogens in water with DNA techniques – a pragmatic approach

Freeze dried soup may not be a culinary experience to remember. However, it is cheap, easy to distribute and anyone can make it resulting in almost the same taste. The idea behind our technology developed for the detection of pathogens in water is basically the same, but instead of adding hot water to a cup, you add your sample to a test tube.

How likely is it that a dish made from the same recipe by two different chefs would taste the same? Due to small differences in, for example raw products, kitchen models, cooking times and measuring cups, they would most likely taste different. This is also the case when different laboratories analyze samples taken from the same water source at the same time. Despite following a strict protocol (*e.g.* recipe), differences in results could be seen from testing standardized samples across, and even within, laboratories. Therefore we wanted a method to be so simple that anyone could perform the testing without prior learning, resulting in comparable and reliable results.

In Aquavalens research, we have developed and refined different methods for the rapid detection of pathogens. We selected and tested a series of methods according to the following criteria: (1) limit of detection, (2) species definition, (3) robustness of detection, (4) manufacturing and operational costs, (5) potential for simultaneous analysis of multiple pathogens, (6) market demand, (7) fit with existing water management systems, (8) system reliability, (9) size and portability of the system and (10) potential for automation. Whereas some of the selected platforms performed well over a large range of these criteria, others excelled in a specific subset, dealing with specific classes of target organisms and for specific market applications. Here, we present the most widely used technology in Aquavalens field studies: GPS™ MONODOSE dtec-qPCR kit, which is a real-time PCR (technique that copies and measures target DNA), specifically developed to match a maximum number of criteria listed above. Most importantly, the format has been shown to be suitable for simultaneous detection of all prioritized pathogens within the project.

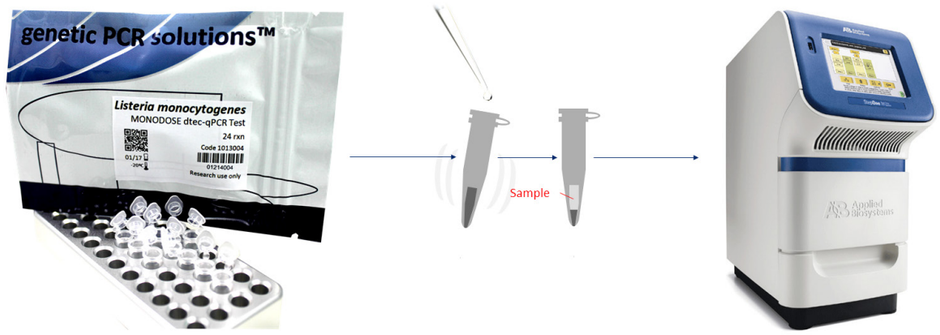


Figure 1 GPS™ MONODOSE qPCR kit for the detection of Listeria monocytogenes. Each bag (left) contains 24 single PCR ready-to-use tubes with dehydrated qPCR reagents, all that is needed is your sample and a thermocycler (right) essential for the PCR reaction.

Using our tests it was possible to run several single-plex PCR reactions targeting different pathogens simultaneously.

Real time PCR is a highly sensitive method for the detection of specific parts of the DNA that could be used as fingerprints providing information on which organisms are present in the sample and how many of them. In order to find the fingerprints of the pathogens, millions of copies are made so it can be easily visualized. This technique has been available for more than three decades but is little used in the area of waterborne pathogen detection; GPS™ R&D resources were exploited fully to develop this technique for this new setting. GPS™ has developed and produced this sophisticated technology to provide an easy, ready-to-use product, which is the key for wider adoption and commercial success. Aquavalens research yielded data that were exploited in order to find suitable "fingerprints" for prioritized pathogens. We successfully developed a method suitable for the detection of all pathogens at the same time. However, there were some obstacles to overcome in order for this method to be useful for water monitoring, *e.g.* robustness of detection, detection of multiple targets at the same time and automation potential.

Identification of multiple pathogens simultaneously

There are more than one hundred different diseases that could be transmitted via drinking water. Within the Aquavalens project, we have prioritized the most important ones. Having one analytical technique for all pathogens would save valuable time and money. Different dishes work better with specific seasoning and cooking times, similarly, analyses of different microorganisms have their optimal prerequisites. Luckily, many of those have been dealt with in the sample processing steps. Preparing different dishes in the same cooker is not possible because the different flavors will mix. For the same reason, the results from several PCR reactions for different pathogens in the same tube, so-called multiplex, is unpredictable. Therefore our approach was to prepare different recipes with the same cooking times and intensity of fire. Just as you can prepare several different kinds of freeze-dried soup, adding water from the same kettle in different cups, we have made it possible to run several PCR reactions for many different pathogens simultaneously, but in separated tubes. The advantages are amazing and easy to see: ask a chef how easy life would be if he/she could have the chance to prepare a full menu of several starters, main course, and a choice of desserts, all cooked at the same time, each having its own taste.

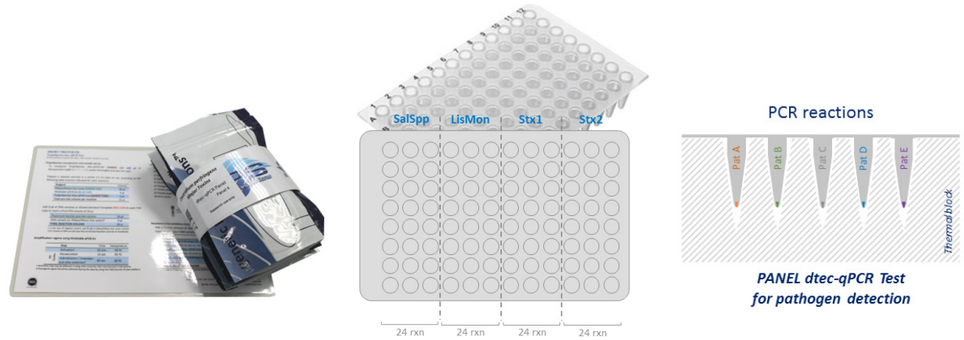


Figure 2 GPS™ MONODOSE dtec-qPCR kits for the detection of different pathogen targets could be run simultaneously with the same temperature and cycle times. This makes it possible for a PANEL of tests created in plates or strips to be used simultaneously to test the water sample for several bacterial pathogens.

Automation of Pathogen Detection

In the eighteenth century, Adam Smith foresaw the industrialisation in his work "The wealth of Nations", in which the division of labour to the most simple tasks would lead to an increased productivity. In the long run, simple tasks could be automated. The developed detection system has been simplified to the extent that you only need to add your sample, which can be done by anyone in the laboratory. With many samples to process, a machine will likely do it better and more efficiently. Another advantage with ready-to-use kits is that variation in performance is minimized. This makes results more robust and comparable between measurements and laboratories. Going back to the example of the freeze dried soup: if we all add the same volume of water (the sample), using the same time and intensity of cooking, the result will be, almost, the same. The soup may not be fancy, probably not very tasty, but it is cheap, easy to distribute and anyone can make it. For our purpose, these were the most important criteria. An added value is that automation is simplified since the only thing needed is to add a specific amount of your sample to the test tube. If genetic detection of pathogens in water will be a future regulatory standard, this will push the market demand for automated detection systems and Aquavalens companies are on track to provide the best solutions.

Automated sampling, filtration and detection systems for water

The development of automated filtration, sampling and/or detection platforms to improve water testing, reducing analysis cost, human handling time and the potential for contamination is highly desirable. We summarise here the main achievements of the Aquavalens project related to this field of research. On the sampling and filtration side, the detection of low amounts of microbial pathogens in water requires the analysis of large volumes (40-1,000L) of water using various filtration techniques to reduce water volume while retaining the microorganisms. The potential to automate the filtration process exists and can include megasonic-assisted agitation for the sonication (ultrasound disruption technique) and elution (collection of sample) of membranes and filters whilst guaranteeing that a single filter could accommodate the three kingdoms of viruses, bacteria and protozoa. On the detection side, an automated optical imaging system for the quantification of fluorescent bacterial cells was improved allowing the rapid quantification and classification of cells. Additionally, an automated platform was designed, manufactured and tested for the rapid detection of living bacteria by measuring ATP (an energy source for organisms).

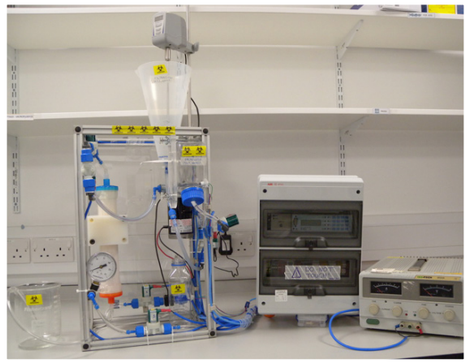


Figure 1 The automated sampling and filtration system for the detection of parasites

Automated filtration and sampling system based on megasonic enhanced elution of filters and membranes

Large amounts of water, up to 1,000L, are necessary for the detection of low concentration of highly virulent protozoan parasites such as *Cryptosporidium parvum*. Filtering large quantities of water requires the use of special filtering systems that use filters and membranes to capture such parasites. Moreover, a system that could also capture bacteria and viruses would be extremely advantageous to ensure the water quality. Within Aquavalens, we tested a particular filter that does this and uses megasonic energy to enhance the agitation of membranes and filters in order to recover as many pathogens as possible for further molecular detection (Figure 1). Preliminary tests using this filter with a megasonic transducer showed very promising recovery rates, which increased by 18% for the tangential flow filtration system and 12% for the dead end filtration elution comparing to traditional filtration systems.

An automated platform was designed, manufactured and tested for the rapid detection of living bacteria.

Automated platform for the rapid detection of living bacteria by measuring Adenosine Tri-Phosphate (ATP)

The rapid detection of living bacteria by measuring ATP, one of the chemicals indicative of living organisms, is based on measuring the optical signal from the chemiluminescent reaction (chemical reaction that produces light) between ATP and another biochemical compound. This principle was used for the development of a robust and stand-alone detection test.

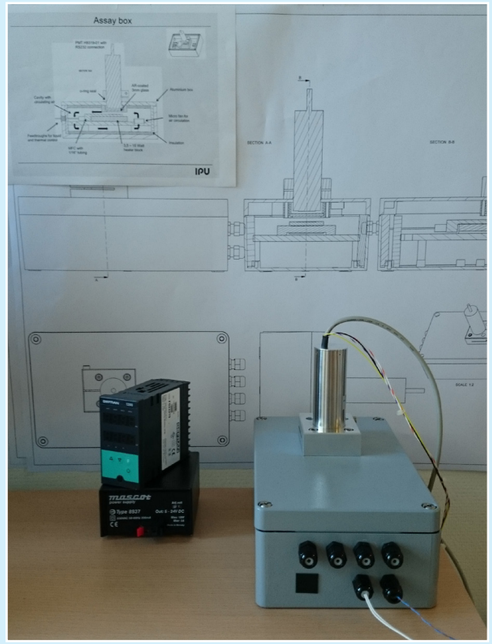


Figure 2 Automated detection of bacteria using monitoring of ATP levels in waste water.

The system employs microfluidic channels (channels that contain very small volumes at any time) to avoid contamination with foreign sources of ATP and allows easy automation. Briefly, the new system consists of a microfluidic cartridge on which the ATP assay is performed by mixing a sample with two separate reagent flows. The small fluidic channels allowed accurate control and reduced volume of the expensive chemicals. The light emitted by the reaction was recorded using a highly sensitive detector that is placed in a light excluding box together with the microfluidic cartridge (Figure 2). System sensitivity was estimated to be more than 2.5 pg/mL of ATP, which is good. Tap water samples and tap water spiked with untreated wastewater were analysed and showed a higher level of total ATP than free ATP concentration as expected. This was due to the release of ATP from microorganisms in the wastewater samples increasing the overall ATP concentration when adding the extraction reagent. **The microfluidic system is therefore able to measure ATP levels with high sensitivity, which is needed for drinking water testing**. However, further work is required to optimize analysis when particulate matter is present in the water sample.

Enhanced automatic optical imaging systems based on Fluorescent In-Situ Hybridization (FISH)

The existing commercial imaging system consists of a microscope, a camera, and translation stage table, which are automatically controlled via software. The software also automatically detects and counts bacterial cells. We developed an automatic optical imaging system based on Fluorescent In Situ Hybridization (FISH) technology (fluorescent probes bind to pathogen targets to allow visualisation) for the simultaneous detection and quantification of *E. Coli* and coliform bacteria, indicative of unsafe drinking water. This is novel because up until now, microscope

systems for the automatic detection of FISH stained fluorescent bacterial cells with automatic focus were not available. This limitation makes the time requirements for a complete filter scan tremendously high (> 66 h per reading), and limits the useful of FISH techniques for bacterial detection because the technique would be more time consuming than conventional culture based methods. Moreover, quantification methods for single cells are far from robust and still need skilled and well trained technicians, which limits its usefulness for routine detection of waterborne pathogens.

The system developed within the Aquavalens project overcame these issues and achieved tremendous improvement as the technique currently takes around 3h per scan, with an improved optical adapter (Figure 3). Additionally, the system uses modern pattern recognition techniques to detect and count cells automatically. This system also learned to automatically distinguish cells from the background by using a large number of "correct cells" examples provided by the experts, thereby allowing less skilled personnel to perform the analyses. The system can also be adapted to detect other types of cells, therefore allowing further development and applications. Another useful feature of our system is that the user can reposition the microscope after a scan, at the exact location where cells have been detected, and manually validate cell counting.

In summary, the Aquavalens project enabled the company Vermicon to improve the performance of their product.

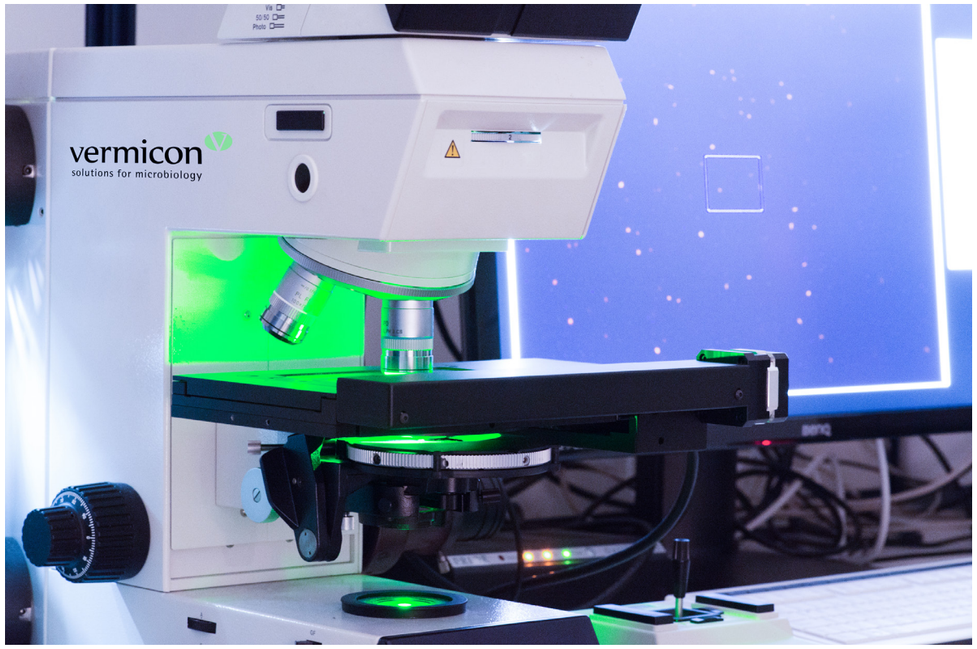


Figure 3 The Vermicon system capable of detecting and classifying bacterial cells.

The path to direct microbiology, the new Scan VIT® System: Knowledge transfer through an SME

The Scan VIT® System: Direct Microbiology

Vermicon AG provide solutions for microbial challenges that arise in industrial microbiology including the Aquavalens project. It developed VIT®, the industrial standard for fluorescent in situ hybridization (FISH) which is particularly useful for water testing within the Aquavalens project when determining the viability of microbes. Distinguishing between dead and alive pathogens is important as dead pathogens do not provide the same level of threat, FISH allows us to do this.

The Aquavalens project required the direct and fast detection of viable, single microbial cells using FISH, an area in which Vermicon specialises. The developed technology composes of a scanning system, a fluorescence microscope and the Scan VIT® test kits to produce the Scan VIT® System with the potential capability of detecting single Aquavalens targeted pathogen cells in a water sample.

The system allows for the direct, highly-specific identification and absolute quantification of vital, single microbial cells in a water sample. And by completely avoiding the use of enzymes, high robustness of the system could be achieved, important for industry standardisation. Currently the first applications have been developed for the detection of Escherichia coli in drinking water with other pathogen targets to follow.

Here, the typical working steps undertaken by Aquavalens partners include: (1) Filtration of the sample, (2) hybridization and (3) scanning of the filter (containing captured microbes), (4) specific detection of the single cells. Within only two hours the whole filter is scanned and the results are presented in a customer-specific report, with the likely customers being water industry professionals that can then make a decision on the safety of their treated water.

Demonstrating the reliability of fast molecular pathogen detection: standardisation and validation

Traditionally, the detection of pathogens in drinking water, surface water, and other environmental samples is done by cultivation (growth on medium in the laboratory). The cultivation methods often require several days of incubation time, this can be very challenging for some pathogens or even impossible for others.

In the framework of the Aquavalens project, new analytical methods and platforms have been developed for the detection of pathogens in different types of water. Validation of the techniques is essential for their acceptance and forms the basis for practical applications including routine testing. Therefore, validation experiments of newly developed commercial kits were performed: reproducibility, robustness, sensitivity, cross-reactivity, as well as potential for practical applications were assessed for both the methods and their analytical platforms.

Technique validation of the detection methods is essential for their acceptance and forms the basis for practical applications including routine testing

Validation of PCR based commercial kits

One group of detection methods are based on polymerase chain reaction (PCR), which allows specific and rapid detection without the need for a cultivation step. PCR results in the specific amplification of pathogen DNA in tested samples. Variations of PCR include real-time or quantitative PCR (qPCR) that provides information on pathogen concentration in the sample. Hence, qPCR enables microbiological analysis within a few hours, and thus is suitable for rapid water quality monitoring. Many specialised companies provide a number of qPCR kits for the detection of water pathogens, two of them are partners within the Aquavalens project.

The first step of the validation process was to test a selection of commercial qPCR kits for the detection of bacterial, viral and protozoan targets in one laboratory with respect to handling, robustness and reproducibility of results. Different concentrations of target organisms were added to tap water and various water concentrates. The initial concentrations of the added microorganisms were determined using flow cytometry (detection of pathogens as they pass a laser), microscopy and/or cultivation. The test kit results were compared with established qPCR techniques and theoretically spiked concentrations. The investigated commercial PCR detection kits proved to be suitable for detection of bacteria such as *Campylobacter jejuni* and *Escherichia coli* (see Figure 1), viruses such as adenovirus and mengovirus and protozoa such as *Cryptosporidium parvum*.

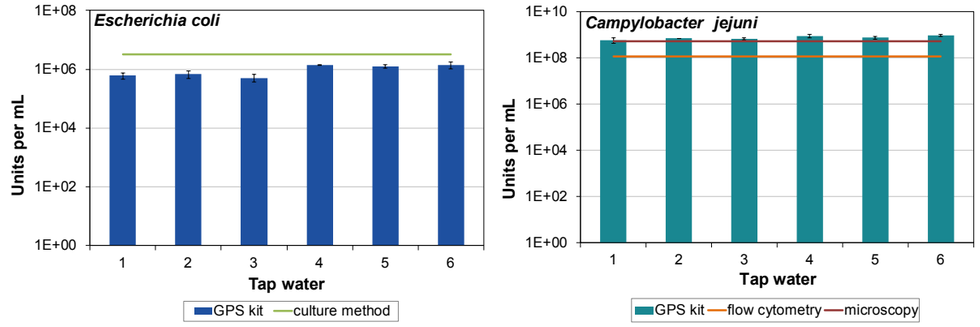


Figure 1 Comparing selected results for Escherichia coli and Campylobacter jejuni detection using commercial kits (GPS) and standards methods (culture, flow cytometry and microscopy). GPS= Genetic PCR Solutions.

The results of the molecular kit assays defined by previous groups within Aquavalens were consistent with current standard microbiological analysis, highlighting the potential of PCR methods for rapid and specific microbiological analysis of potable water.

Interlaboratory validation

In the next step, interlaboratory ring validation experiments with three to seven participants (depending on the target) were performed. For these experiments nucleic acids (building blocks of genetic material such as DNA or RNA but also play a role in many molecular pathways) were used as validation samples. In all tests, higher concentrations of spiked nucleic acids corresponded to higher gene copy numbers, as expected. No false positives for blank samples were observed and importantly the obtained results were very close to the expected values. PCR detection was highly specific for the target pathogen. Good reproducibility as well as low deviation of results was obtained in pure water, target/non-target mixtures (as harmless microorganisms will be mixed with potential pathogenic ones within natural sources) and in presence of PCR inhibiting substances (which may be present in collected samples) (see Figure 2). However, when the concentration of PCR inhibitors increased, the variability in results increased too. In nucleic acid extracts of surface water samples, the deviation of results increased and median values decreased. In conclusion, the PCR kits developed in the Aquavalens project proved to be very suitable for the detection of health related water microorganisms.

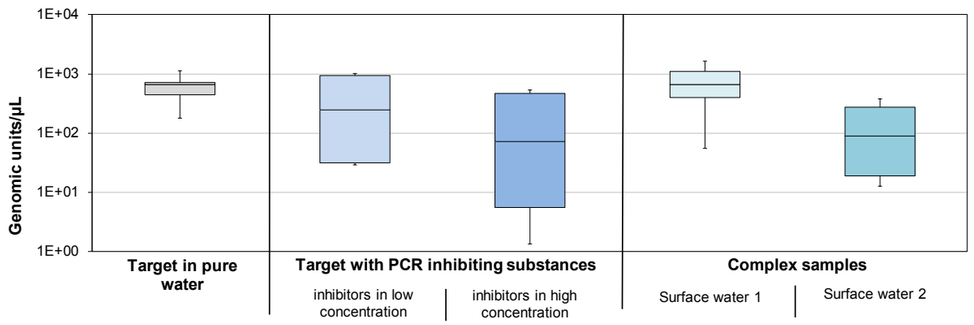


Figure 2 Results of interlaboratory ring trial for the commercial qPCR kit to detect hepatitis A virus in various conditions, likely to be encountered in field samples.

Validation of the automated ultra-filtration system for the concentration of pathogens from water samples

For the detection of pathogenic microorganisms in water samples, an enrichment of the microorganisms is necessary. Experiments within the Aquavalens project have shown that hollow fibre ultra-filtration with Rexeed-25A modules is a very good method for the concentration of microorganisms, whether bacteria, viruses or protozoa. The advantages of this system are: simple handling and disposable nature of the filters used, which minimizes the risk of cross contamination. Based on these results, an automated concentration system was developed.

Experiments using surface water, tap water and model microorganisms were carried out to validate this automated system. The process consisted of reducing the initial sample from 5 L of water to a volume of 250 mL and evaluating the recovery rates for a number of pathogens of interest. The ultra-filtration method showed good recovery for *Escherichia coli*, coliforms and *Enterococci* added to tap water (see Figure 3). Also for these complex matrix samples, a good average recovery of 53% was observed. Overall, the ultra-filtration with Rexeed-25A modules was successfully validated. These promising results allowed the validated platforms to be used in field testing, which was part of the Cluster 3 work of the Aquavalens project.

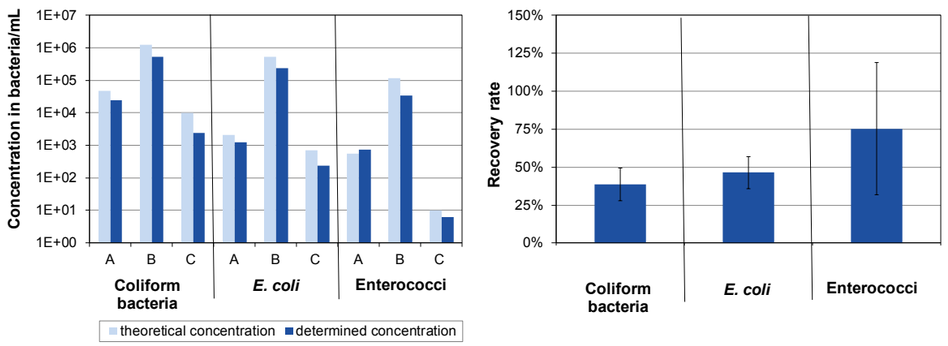
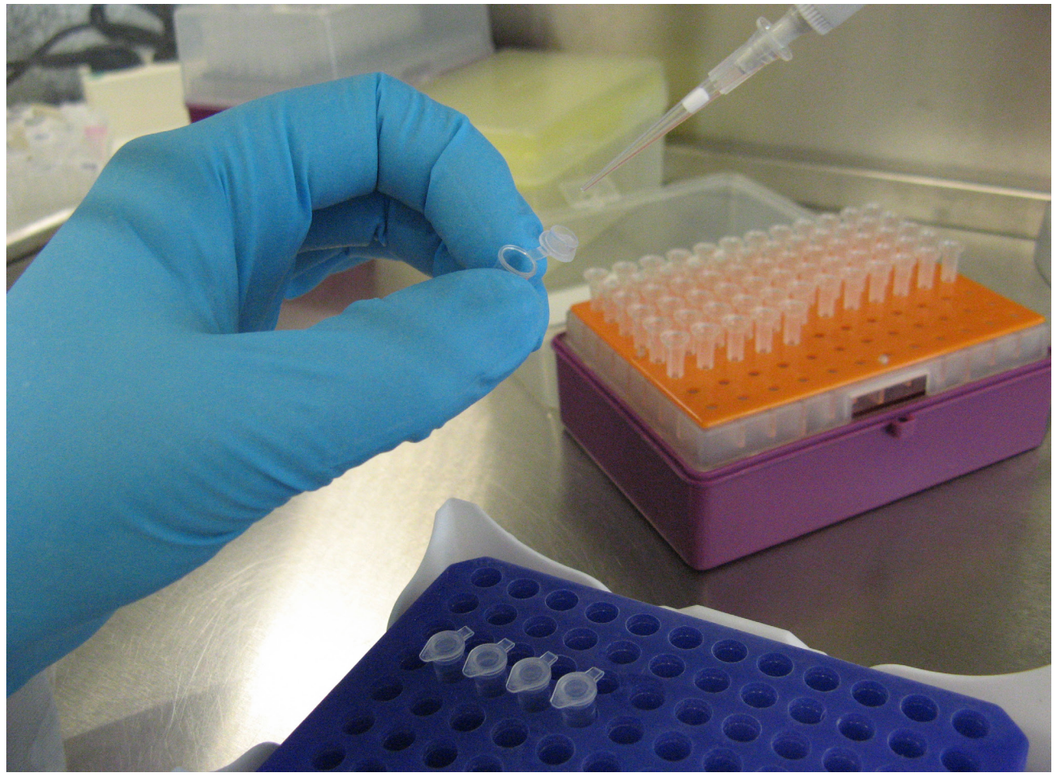


Figure 3 Validation of the automated ultra-filtration system of natural surface waters.



Evaluating the newly developed technologies to detect pathogens in large water systems across Europe

Why there is a need for testing the new technologies?

A rigorous evaluation is necessary to ensure that the technologies utilised by the Aquavalens project are robust and will provide reliable data on pathogen numbers in water supply systems. We organised and implemented an extensive programme of testing of the novel technologies such as concentration methods for large volumes, detection techniques for several different types of waterborne pathogens and integrated platforms in real water systems for online detection of indicators. A multidisciplinary team was put together that consists of universities, technological centres, Small and Medium Enterprises (SME) and large water systems (Figure 1). The activities undertaken included sampling and analysing water samples to detect waterborne pathogens at selected sites located in various large drinking water distribution systems across Europe. These sites included Drinking Water Treatment Plants (DWTPs) and Distribution Networks (DNs).

The main objectives of this part of the project were to demonstrate the potential use and applicability of these technologies in real systems, and to assess how they could help to manage water safety. This research also aims to give recommendations on the applicability of the different analytical tools in large water systems.

Figure 1 Research partners

Site selection

The project partners selected four sites, located in the United Kingdom, Spain, Germany and Denmark. These locations were chosen to give a representative range of water sources, treatment technologies, water distribution systems and weather conditions to test the performance of the developed technologies under different conditions.

The **UK site** (in an undisclosed location) supplies around 70,000 inhabitants and testing was coordinated by **WRc** and the **James Hutton Institute**. The **Spanish site** supplies water to about 2.8 million inhabitants and was coordinated by **Cetaqua** and **Aigües de Barcelona**. Testing at the **German site**, which supplies water to around 3 million inhabitants was coordinated by **Technologiezentrum Wasser**. The **Danish site** supplies drinking water to 200,000 inhabitants and work was coordinated by **Technical University of Denmark** and **Nordvand**.

Before sampling began, each of the large systems was fully characterised to obtain information on water supply systems and associated climatic conditions.

Scenarios investigated

A number of scenarios were selected because it was considered that the newly developed technologies would improve the assessment and management of the risks posed by waterborne pathogens. These scenarios were:

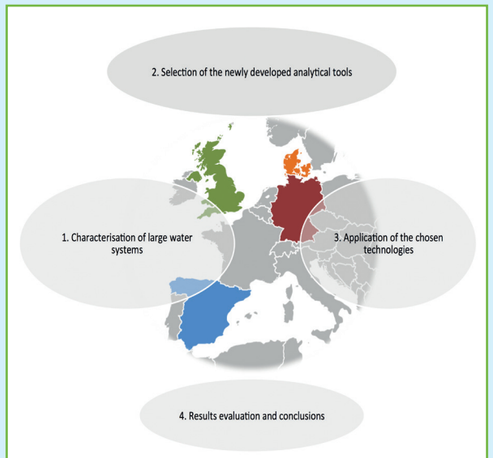


Figure 2 Location of large water systems and Aquavalens research work plan

- **Pathogen detection at source and at several stages throughout the treatment of drinking water.**

Knowledge of pathogen occurrence in sources of drinking water is necessary to be able to assess the robustness of water treatment for the production of microbiologically safe drinking water. It is a two-step process that involves (1) recovery and concentration (2) molecular detection of the target pathogen.

A detailed sampling programme was prepared that involved selecting the most appropriate technologies for a particular application in real large systems followed by an evaluation of the results (Figure 2). Field-testing campaigns were performed on a monthly basis at all selected sites for one year duration. In order to complement the microbiological data, physico-chemical parameters and meteorological data were also collected.

Knowledge of pathogen occurrence in sources of drinking water is necessary to be able to assess the robustness of water treatment.

- **Assessing the consequences of particular events on the microbiological safety of drinking water**

Events involving any situation that differs from the usual conditions, which could have an impact on the microbiological quality of drinking water were recorded. Examples of events at DWTPs included flood or drought episodes, a change of water sources and operational changes in the treatment process. Events at the DNs included regular cleaning procedures, stagnation at dead ends (sections without sufficient water circulation) and others such as pipe breaks and intrusion detection in tanks.

Figure 3 Pictures of different procedures during sampling campaigns. Left picture: On-site filtration in Germany, Right picture: Filter elution in the laboratory at Aigües de Barcelona

Technologies under evaluation

The technologies were selected after careful consideration of the work carried out by our Aquavalens partners, and selection was based on known performance and suitability to large water systems as illustrated in Figures 3 and 4.

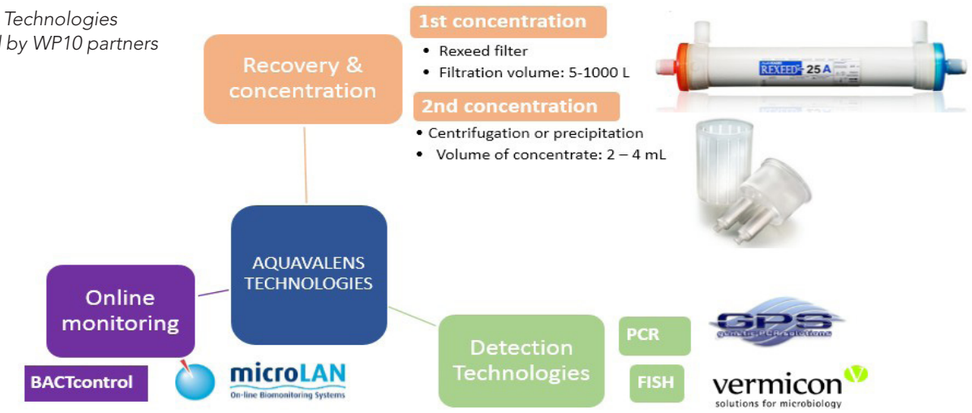
Recovery and concentration techniques

The first concentration step is done using a hollow fibre ultrafiltration filter (Rexeed). This filter **allows the simultaneous concentration of viruses, bacteria and protozoa**, which is advantageous over existing technologies that require separate filters for each kingdom. Moreover, this system allows **filtration of large volumes** (up to 1000 L of water). This can improve microbial detection due to the low abundance of some microorganisms in water, particularly pathogenic microorganisms. The second concentration step can then be carried out by centrifugation or precipitation.

Molecular detection technologies

The advanced detection tools are based on molecular methods. Two novel technologies developed by the Aquavalens project were tested: PCR (Polymerase Chain Reaction) kits (amplifies and measures genetic material of a specific target) and FISH (Fluorescence *in situ* hybridisation) (fluorescently tags pathogens for easy visualisation using optical devices) (Figure 5). The performance of these new methods was compared to conventional detection techniques: ISO standardised methods, to ensure their suitability for

Figure 4 Technologies selected by WP10 partners



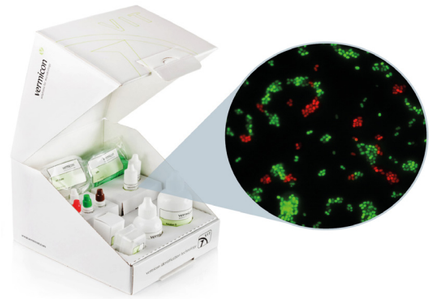


Figure 5 Molecular techniques Top picture: GPS kits, Bottom picture: VIT® kits (vermicon, FISH detection)

microbiological quality monitoring. PCR and FISH techniques reduced the time of analysis, allowing the provision of results within a few hours, which is superior to traditional culture-based methods that require days for the pathogen to grow and produce positive results.

All selected sites have agreed to test the following waterborne pathogens using the kits in parentheses:

Viruses: Norovirus genotype I and Norovirus genotype II (Ceeram kits)

Bacteria: *Campylobacter* spp (*Campylobacter jejuni*) and *E. coli*. Both parameters were measured by quantitative PCR (qPCR, GPS kits) and FISH (vermicon, VIT kits)

Protozoa: *Cryptosporidium* and *Giardia* species (GPS and Ceeram kits).

In addition, Hepatitis A viruses, Somatic coliphages, *Salmonella*, *Pseudomonas aeruginosa*, *Legionella pneumophila*, *Clostridium perfringens*, *Enterococci*, *Toxoplasma gondii* and *Giardia intestinalis* were additionally tested at some of the selected sites.

- **On line detection systems**

The BACTcontrol system from microLAN (Figure 6) is an automated system for the semi-continuous measurement of the bacterial indicators *E. coli* and coliforms, based on the enzymatic activities of ß-glucuronidase for *E. coli* and ß-galactosidase for coliform bacteria. In addition, the system can be used for the detection of total bacterial activity, based on the enzymatic measurement of alkaline phosphatase.

At the Spanish and Danish sites, the total activity module was tested, whereas at the German and UK sites, the *E.coli* module was tested. The results showed that the BACTcontrol device has a great potential as an early warning system for improved management of operation processes and microbiological quality monitoring in large water systems.

Figure 6 BACTcontrol from microLAN installed in the Sensor Platform in Aigües de Barcelona

Conclusions

Field-testing in large water systems demonstrated the capacity of these novel techniques to concentrate large volumes of water, potentially leading to improvement in waterborne pathogen detection. Results obtained so far show a great potential for the improvement of microbiological sampling and pathogen detection in terms of time, money and human resources. Furthermore, the implementation of these promising techniques could improve operational water safety plans and reduce the health risk of the exposed population.

Small Water Systems – Pathogen detection in small water supplies across Europe

Small water supply systems

Most people living in towns and cities in Europe have water supplied to their houses and apartments through pipes that are owned and managed by large water utilities (water companies). The quality of their water is assured by the vast resources of these organisations and the expertise of the people who work for them. Inevitably, problems do sometimes occur and the quality of the water received by the consumer is not optimal, but these events are uncommon and the water companies have the resources to quickly resolve most of them. In the last few years, new ways of inspecting large water supply systems have been used across Europe that are improving the safety of the water supplies and lowering the risk of people getting a disease by drinking substandard water.

If you live in a city or town you may not be aware that one third of people in Europe do not get their water from the large companies; instead, they rely on small water systems for their supplies. These are known as Small Water Supplies or Private Water Supplies across Europe and for the purpose of this introduction, we establish that small water systems serve relatively small populations (less than 5,000 people, but frequently just one or two households) and that they are not operated by large water utilities.

Is consuming water from small water systems a problem? It should not be, of course, but very often it is. Small water systems are managed by an individual, or a small group of people, who generally do not have the resources or the expertise to maintain a constant supply of safe drinking water. As a result, gastrointestinal disease can be relatively common amongst users of these small water systems.

The work that we are doing in the Aquavalens project is giving us a better understanding of water quality from small water supplies and how it affects the health of consumers. Our teams are working in Portugal, Serbia and Scotland. In each country, we are regularly taking samples from different small water supplies and testing them for disease-causing microorganisms (pathogens) using methods developed for the Aquavalens project. Additionally, the condition of the water source and supply, the local environment, and the weather on the day of sampling and on the five days before sampling were all recorded for further analysis. In particular, we are interested to find out if it has rained during the time of sampling or beforehand and whether the rain was heavy or light. All this information is important because it will allow us to see if there is a connection between the weather, the environment, and the condition of the water supply, and the presence or absence of different pathogens. From this, we can predict the potential for consumers to become unwell from drinking the water, and the particular set of circumstances when this risk is highest.

Although it is very useful to be able to measure the risk of ill health to the water consumers, we need to know if this measure of risk is accurate and meaningful in terms of people actually becoming ill. Some of the consumers of the water supplies being monitored have been asked to keep a daily record of their health for three months. In one country, we are comparing their responses to the health of people who drink water supplied by a large water utility. We hope that the information we collect from this survey will complete our understanding of the connection between the water source, the local environment, the condition and management of the water system, the likelihood of pathogen presence and the risk of producing disease that can ultimately lead to people becoming ill. We can then use this knowledge to advise people responsible for the small water systems on how to better manage their supplies and protect the health of the population consuming the water.

The presence of pathogens in Small Water Supplies can pose a significant health risk to those relying on untreated wells, boreholes or public fountains as their only source of drinking water

We believe that the methods and processes developed by the Aquavalens project will bring tangible benefits to the owners and users of small water systems. However, it is one thing for academics and industry experts to believe that their project has brought benefits to the community, but the perception of the users of small water supplies could be very different. To be certain that the methods developed in the Aquavalens project are relevant to small water supplies, we are asking the opinion of people who use and manage the small supplies we are monitoring. This work has just started and we are using questionnaires to find out how these water supplies are tested and managed, and the type of information that is being given to the consumers of the small water systems. From preparatory field visits, the users were made familiar with the aim and work plan of the Aquavalens project. As part of the evaluation and among other things, we are asking the users if it is advantageous to get the test results quicker than by using standard methods of testing, and if they would like to know if and when their water contains pathogenic microorganisms. The answers to these questions will be analysed to find out if further improvements/ modifications are needed to advance the Aquavalens' methods or their applications in the field.

Small water supply systems are, and will remain, an important source of drinking water in the EU. However, the characteristics that define small water supplies are what make them vulnerable to contamination and therefore, a risk to human health. By developing, testing, and analysing the impact of new methods for rapid detection of pathogenic microorganisms, the Aquavalens project hopes to bring measurable improvements to the quality of small water supplies.

Not just drinking water; water safety for food production

When considering the impact of water quality on public health, drinking water is generally the primary concern. However, water also plays an important role in food production, both as an ingredient, but also during production (irrigation) and processing (washing). Accordingly, contaminated water could pose a significant risk when considering food production, particularly for products which are not cooked prior to consumption such as salads. It was therefore an important aspect of research within the Aquavalens project.

As the consumption of fresh fruit and vegetables is increasing, the importance of water safety in food production intensifies

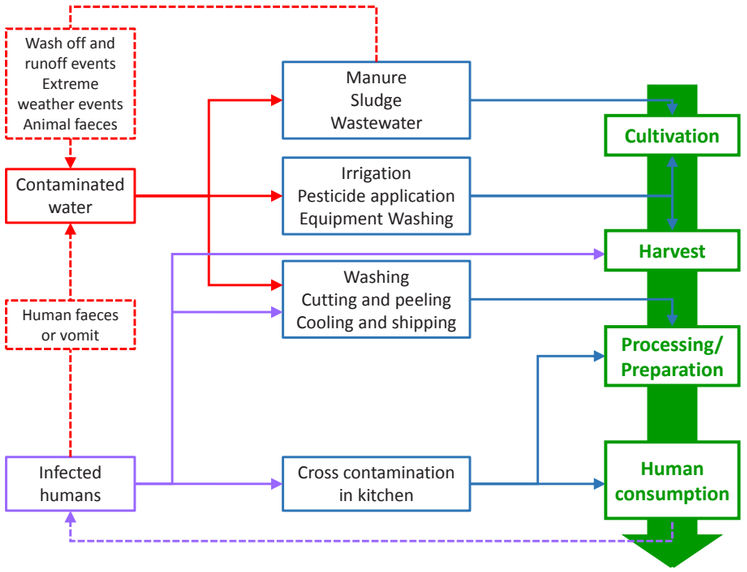
Food consumption patterns have changed in recent years, with an increasing drive towards the consumption of fresh fruit and vegetables. In many cases, these products are consumed without any processing step, which could inactivate any pathogenic microorganisms present in the sample.

Increased consumption, coupled with large scale production and ever greater globalisation of the market has led to longer distribution times and distances, which increases the complexity and importance of food safety management. Foodborne disease outbreaks linked to fresh produce have increased in recent years, indicating the importance of implementing measures to ensure the safety of these products. Contamination of fresh produce can occur at both pre and post-harvest stages of production, from a range of sources. As outlined in the figure below, contaminated water can be a risk factor in both stages, with irrigation water and processing water identified as potential contamination risks. The fruit and vegetable production sector therefore forms the ideal test bed for assessing Aquavalens developed methodologies.

Our aim was to ensure that the developed methodologies were assessed rigorously, focusing on sectors where they were likely to have the greatest impact. The Aquavalens team undertook a comprehensive analysis of 440 published reports of produce related disease outbreaks to identify a number of key pieces of information:

- The microorganisms most commonly associated with produce related disease outbreaks;
- The foods most commonly linked with produce related outbreaks;
- The source of contamination of the produce, if identified.

This analysis led to the selection of target microorganisms and food products, for a sampling study to be undertaken in Ireland, Portugal, Serbia and the United Kingdom. This study is currently ongoing, with results expected to be available in late 2017. The study is focusing on producers of salad leaves, soft fruit and sprouted seeds. The project team is testing irrigation water and processing water from each site for the presence of a range of bacterial, viral and protozoan pathogens using the Aquavalens developed tools, in parallel with testing by conventional methods.



Contaminated water could pose a significant risk for food production

Water testing technology allows for the rapid and reliable testing of water sources involved in the production and processing of food

A significant challenge is that these pathogens are often present in very low numbers, but this low concentration can be enough to contaminate foods and result in disease. One of the major advantages of the Aquavalens methods being employed in this study is that a single filtration step is utilised to concentrate bacteria, viruses and protozoa, as opposed to requiring different filtration steps for each kingdom. The optimised concentration technique has been coupled with polymerase chain reaction (PCR) (a technique that amplifies target DNA for easier detection), developed by small and medium sized enterprise (SME) partners within the project. These assays come ready- to-use, utilise internal controls to avoid false negative results, and include positive and negative controls. They are highly robust, specific and sensitive for their target microorganism.

The study aims to demonstrate the applicability and utility of the novel Aquavalens pathogen detection methodologies for implementation in food safety management systems. A vital output of this research has been the opportunity to actively engage with food producers across Europe. It has provided a forum for discussion and learning for SMEs, researchers and food producers alike and contributed to build valuable linkages for future collaborations. The use of molecular tools in food safety management is relatively new to some producers and is sometimes met with scepticism, but this project has enabled a meaningful demonstration of the potential benefits, and their ease of use in this important sector.

Management of water quality with improved detection techniques: applications for water safety plans

The systematic preventive approach to ensure drinking water safety, named "Water Safety Plan" (WSP) is now recognized as an important method in reducing health risks from contaminated drinking water. It is based on a continuous process of risk management for all stages encompassing the supply of drinking water. It was developed by the World Health Organization for large and small water supplies alike and builds on the Hazard Analysis Critical Control Points approach (HACCP) developed for the food industry some decades ago to secure food safety. It is now used in around 90 countries and it could even become a mandatory requirement. Recent research showed that using this approach has improved water management, water quality and public health. For example, in Iceland, non-compliance with microbial regulation is 3.7 times more likely in supplies that have not implemented a water safety plan. Conversely, there is 14% less risk of clinical cases of diarrohea, when a water safety plan is in use. Nevertheless, waterborne outbreaks still occur in Europe and even in water supplies with an existing water safety plan.

Ensuring water safety using modern molecular technologies

EU regulation on the quality of drinking water (EC, 98/83/EC) that has been implemented in all EU and EES countries, involves testing for indicators (markers of fecal contamination), as these are the most common cause of waterborne disease outbreaks. The main regulatory indicators for fecal contamination are the bacteria: *E.coli* and *Enterococci*. Survival of pathogens (microbes that cause disease) in the environment depends on many factors such as temperature, acidity and composition of the strata, and is not the same for all kingdoms of pathogens. For example, parasites live much longer than bacteria in water and viruses travel further, draining through the strata because they are much smaller in size. Therefore, it is important to develop techniques to test for pathogens instead of relying only on indicators. Currently, it is not practical to test for pathogens during routine surveillance, excluding when an outbreak is suspected, as routine testing kits are not commercially available.

All surveyed water suppliers had challenges with old infrastructure being most common.

Within the Aquavalens project, molecular techniques for fast routine detection of waterborne pathogens as well as some on-line technologies for measuring biomass and microbes in water have been developed, standardized and tested. These techniques have been tested in twenty water supplies around Europe, including five large supplies serving 12.2 million inhabitants and fifteen small supplies serving 1100 inhabitants. So the question is, can the new developed methods improve management of water safety and prevent contamination of water and thereby protect public health. To answer this, evaluation of the usefulness of the new techniques developed by Aquavalens in the process of water safety plan was undertaken, in addition to collecting information on risk factors and water quality at each test site. The value of the new techniques was also evaluated by questionnaires.

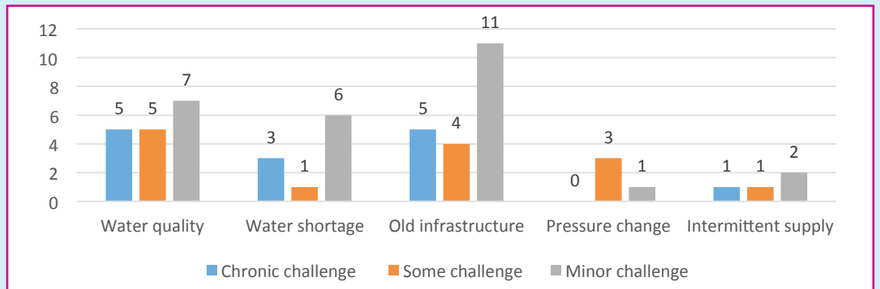


Figure 1 Main challenges in twenty European water supplies

Main challenges facing European water supplies

Current water suppliers face many challenges that are likely to impact the provision of safe drinking water. The information gathered from twenty participants revealed a whole range of issues ranging from old infrastructure to water shortages as shown in Figure 1. All surveyed water supplies had challenges with old infrastructure, with five supplies reporting this as a chronic challenge. This could be associated with a significant risk to water safety linked to, for example, leakage from broken pipes. Seventeen suppliers reported challenges with water quality, with five citing this as a chronic challenge. Ten supplies have water shortages and four supplies reported issues with pressure change and/or intermittent supply, all which are likely to decrease water quality.

Risk factors to water supplies

The main risk to water safety is from activity on the catchment and how this is managed. This survey showed that all five large supplies investigated had extensive activity on their catchment. Two of the supplies use groundwater, one surface water and two use both. All five supplies use disinfection and four implement pre-treatment procedures. Activity on catchments is extensive as is depicted in Figure 2. All have residential areas on their catchment, four have agriculture (with cultivation and livestock) and transport and three have sewage work or septic tanks and two have oil tanks. One supply has 50% of sewage in the same ditch as water pipes, which is a great risk to water safety not least when combined with aging infrastructure. All these risk factors have the potential to contaminate the water source and lead to disease.

Two of the five water supplies have had recent occasional bacterial incidents and one has experienced high turbidity and ammonia contamination during heavy rainfall. Two have had waterborne outbreaks in the last twenty years. One had legionella and the other *Cryptosporidium*. Measuring pathogens in raw water and after treatment would be an important step in securing water safety.

Impact of newly developed techniques on water quality

The main benefits from the Aquavalens newly developed technologies is that they will assist the identification of water quality problems and pollution sources. They will also help with risk assessment, which is the most important part of preventive and microbiological management and control. This should lead to fewer incidents of non-compliance and waterborne outbreaks and thereby have a positive impact on public health.

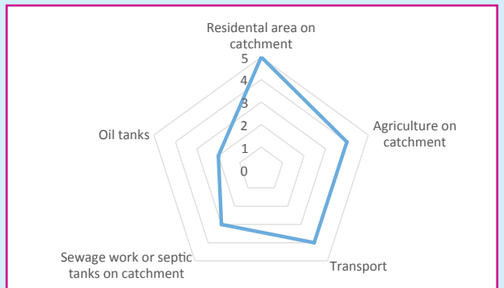


Figure 2 Activity on catchment at five large water supplies

Carbon Footprint of Aquavalens novel technologies

The hazardous impacts of human emissions of greenhouses gases (GHG) is nowadays one of the most important global environmental challenges that the world must face. Evidence for rapid climate change i.e. global temperature and sea level rise, extreme weather events, warming and acidification of oceans, decreased glacial and snow covers are well documented. This environmental concern was showcased by the Paris agreement, where 195 countries adopted the first-ever universal, legally binding global Climate Change mitigation deal. Governments agreed a long-term goal of keeping the increase in global average temperature below 2°C compared to pre-industrial levels.

The EU has long been concerned about climate change and has issued policies and provided guidance to the member states for their own domestic actions. In this respect, a comprehensive package of policy measures to reduce GHG emissions was initiated through the European Climate Change Programme (ECCP). Moreover, the Commission wants the EU to lead the clean energy transition through what is called "the winter package". For this reason the EU intends to cut CO_2 emissions by at least 40% by 2030, through the implementation of three main goals: putting energy efficiency first, achieving global leadership in renewable energies and providing a fair deal for consumers.

Environmental evaluation through Life Cycle Assessment

Life Cycle Assessment (LCA) is a quantification tool specifically designed to assess the environmental impacts of the whole production chain for a product or service. It covers the following aspects: extraction and processing of ancillary materials, manufacturing, distribution, use, maintenance and final disposal or recycling. This is much more comprehensive that the classical view, where for example, only the exhaust emissions from a car engine were considered, when evaluating the environmental impact of transport by car. With the LCA approach, all emissions along the whole life cycle should be accounted for, including a breakdown into reference unit of an activity, e.g. traveling one km by car. In practice, this requires us to calculate the amount of fuel consumed by the car and additionally, computing the impacts of petrol production, including all other aspects such as extraction, processing, refinement and distribution.

The carbon footprint (CFP) using the LCA approach accounts for the total emissions of GHG caused directly or indirectly by an activity or product. Therefore, it could be used as a measure of Climate Change impact, expressed in kg of carbon dioxide (CO_2) or equivalents. This would provide an indication of the warming effect based on established references for the potential impact of CO_2, using internationally recognized standards such as PAS 2050:2011 and ISO 14067:2013, Greenhouse Gas Protocol.

Environmental care in the Aquavalens project

As part of the objectives of the Aquavalens project, the environmental impact of the newly developed technologies/platforms for the detection of waterborne pathogens is evaluated through the analysis of their carbon footprint. This approach is novel because until now no CFP has been reported for analytical procedures used to determine water quality. To achieve this, an inventory of the expected emissions generated by novel technologies/platforms versus classical standard methods has been established.

The environmental impact of the newly developed technologies/platforms for the detection of waterborne pathogens is evaluated

The life cycle of the novel platforms was divided into two main stages: production and use (Figure 1). The first stage entails raw materials extraction, transport and manufacturing for platforms production. The evaluation of this phase is based on the information gathered from three Aquavalens partners that developed the three different platforms: two real time PCR (polymerase chain reaction) kits and one on-line detection platform. The second stage, platform use, comprises all the activities involved in the use of the three different platforms for waterborne pathogen detection. These activities include transport, materials and energy consumption during sampling and laboratory work. For the evaluation of this phase, we worked with Aquavalens partners in charge of testing the new platforms in the field, namely in large and small water systems and water used for food production. The information to build the GHG emissions inventory relied on the knowledge of relevant Aquavalens partners. Therefore, communication and mutual understanding was crucial in order to obtain representative and accurate data, and for technology providers to understand the importance of their role in reducing the CFP of their activities.

Carbon footprint of platform production

In order to calculate CFP, specific questionnaires were designed, which cover specific activities and consumption data depending on the combination of partner-platform assessed. For instance, to compute the CFP of one PCR platform produced, we have asked the manufacturer about materials and products used to produce it, with special attention to packaging material, energy consumption needed for assembly, and conditions for transport and storage.

Regarding the results of the platform production, the packaging turned out to be an important factor. Different formats of PCR kits were evaluated, these

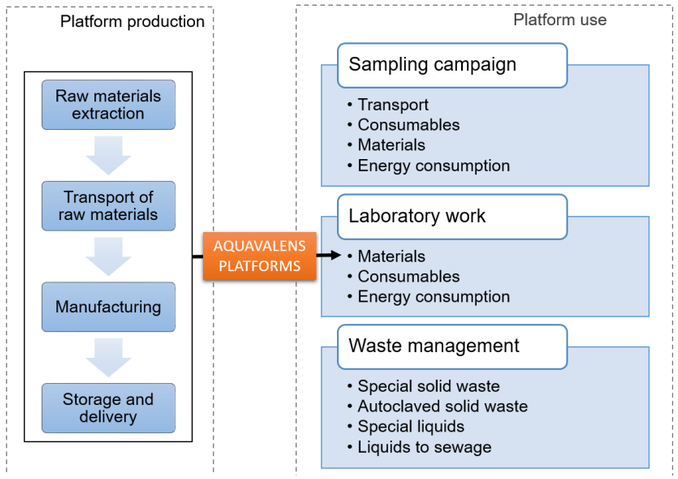


Figure 1 Flow diagram of the life cycle of the Aquavalens platforms.

varied with the main difference being single test or multiple test kits. As could have been expected, the CFP decreases with the number of tests allowed per individual product. This is logical as the amount of packaging material used will be lower per analysis in those products that enable more analyses per kit. Formats in which the substances provided for each analysis are more individually packed, have a higher rate of packaging materials per analysis, and so CFP. For example there are "monodose" kits that dispense the materials needed for one single PCR reaction separately. One-dose/single-dose/monodose formats. These are kits that allow a certain number of PCR reactions, with amounts of each substance needed for each reaction independently packed. While in other formats the amounts needed of each subtance for X reactions are packed together. However, with the obtained CFP results, the environmental implications can be considered when selecting the presentation of the platform, and can inspire the manufacturer in the design of greener formats.

Carbon footprint of platform use

Considering the whole life cycle of the platform, the activities needed to conduct the water analysis using the platform produce much more GHG emissions than the activities needed to produce the platform. As an illustration, the CFP calculated to carry out an analysis of a water sample using one of the Aquavalens platforms is on average around 4 kg CO_2 /analysis, which is approximately the same CFP produced by a fridge per day. Conversely, the CFP corresponding to the production of the same platform is only 3 g CO_2 /analysis, comparable to 10 minutes of 60W light bulb usage.

How do Aquavalens methods compare to traditional testing methods in terms of CFP?

In order to fairly compare the CFP obtained for the use of Aquavalens platforms and the corresponding conventional analytical procedures, an extended life cycle was considered. It included all activities needed to identify the presence of a set of selected pathogens belonging to the three kingdoms: bacteria, parasites and viruses.

To evaluate the pollution from activities of the use phase, we have asked the end users about the sampling campaigns, with particular reference to transport; materials and consumables used and equipment utilized during laboratory work; as well as management of the waste produced in all these tasks. The first conclusion from this analysis is that consumption patterns do not vary significantly from one partner to another, probably because of the use of preset common protocols. However, the sampling and sample transport patterns should be studied in detail since these would differ significantly in actual future applications.

For the activities carried out during the platform use, those with higher impact in the final CFP are related to laboratory materials, such as plastic consumables i.e. filters, gloves, pipette tips, caps, tubes, etc. (Figure 2). This is followed by activities involving equipment with significant energy consumption, such as a fridge, centrifuge, vortex, cycler, autoclave, etc. Therefore, these phases could be targeted for improvement opportunities, since the reduction of these consumptions could lead to a significant CFP reduction. In conclusion, the CFP analysis of the Aquavalens new technologies would allow producers and potential users to be conscious of the implications that their decisions and consumption habits may have on GHG emissions and climate change.

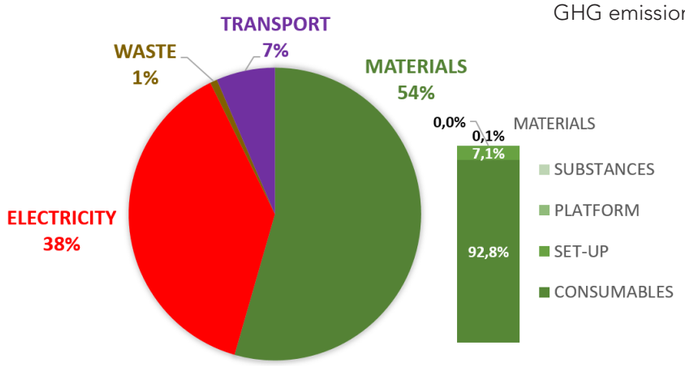


Figure 2 Distribution of the Carbon Footprint by different type of inputs for one water sample analysis using an Aquavalens platform.

Publications produced by the Aquavalens research project:

BEAZ-HIDALGO, R., HOSSAIN, M. J., LILES, M. R. & FIGUERAS, M.-J. 2015. Strategies to avoid wrongly labelled genomes using as example the detected wrong taxonomic affiliation for Aeromonas genomes in the GenBank database. PLoS One, 10, e0115813.

BOSCH, A., PINTÓ, R. M. & GUIX, S. 2016. Foodborne viruses. Current Opinion in Food Science, 8, 110-119.

BOUZID, M., ELWIN, K., NADER, J. L., CHALMERS, R. M., HUNTER, P. R. & TYLER, K. M. 2016. Novel real-time PCR assays for the specific detection of human infective Cryptosporidium species. Virulence, 7, 395-399.

BOUZID, M., HALAI, K., JEFFREYS, D. & HUNTER, P. R. 2015. The prevalence of Giardia infection in dogs and cats, a systematic review and meta-analysis of prevalence studies from stool samples. Veterinary parasitology, 207, 181-202.

CHALMERS, R. M., ELWIN, K., FEATHERSTONE, C., ROBINSON, G., CROUCH, N. & DAVIES, D. P. 2016. Clinical and Microbiological Investigation of Zoonotic Cryptosporidiosis in two Children by Routine Diagnostic Methods and Quantitative Polymerase Chain Reaction. Journal of Veterinary Medicine and Research, 3, 1054.

CHALMERS, R. M., ROBINSON, G., HOTCHKISS, E., ALEXANDER, C., MAY, S., GILRAY, J., CONNELLY, L. & HADFIELD, S. J. 2017. Suitability of loci for multiple-locus variable-number of tandem-repeats analysis of Cryptosporidium parvum for inter-laboratory surveillance and outbreak investigations. Parasitology, 144, 37-47.

FIGUERAS, M., LATIF-EUGENÍN, F., BALLESTER, F., PUJOL, I., TENA, D., BERG, K., HOSSAIN, M., BEAZ-HIDALGO, R. & LILES, M. 2017a. 'Aeromonas intestinalis' and 'Aeromonas enterica'isolated from human faeces,'Aeromonas crassostreae'from oyster and 'Aeromonas aquatilis' isolated from lake water represent novel species. New Microbes and New Infections, 15, 74-76.

FIGUERAS, M., LEVICAN, A., PUJOL, I., BALLESTER, F., RABADA QUILEZ, M. & GOMEZ-BERTOMEU, F. 2014a. A severe case of persistent diarrhoea associated with Arcobacter cryaerophilus but attributed to Campylobacter sp. and a review of the clinical incidence of Arcobacter spp. New microbes and new infections, 2, 31-37.

FIGUERAS, M., PÉREZ-CATALUÑA, A., SALAS-MASSÓ, N., LEVICAN, A. & COLLADO, L. 2017b. 'Arcobacter porcinus' sp. nov., a novel Arcobacter species uncovered by Arcobacter thereius. New Microbes and New Infections, 15, 104-106.

FIGUERAS, M. J., BEAZ-HIDALGO, R., HOSSAIN, M. J. & LILES, M. R. 2014b. Taxonomic affiliation of new genomes should be verified using average nucleotide identity and multilocus phylogenetic analysis. Genome announcements, 2, e00927-14.

FISHER, J. C., LEVICAN, A., FIGUERAS, M. J. & MCLELLAN, S. L. 2014. Population dynamics and ecology of Arcobacter in sewage. Frontiers in microbiology, 5, 525.

FUENTES, C., GUIX, S., PÉREZ-RODRIGUEZ, F. J., FUSTER, N., CAROL, M., PINTÓ, R. M. & BOSCH, A. 2014. Standardized multiplex one-step qRT-PCR for hepatitis A virus, norovirus GI and GII quantification in bivalve mollusks and water. Food microbiology, 40, 55-63.

FUSTER, N., PINTÓ, R. M., FUENTES, C., BEGUIRISTAIN, N., BOSCH, A. & GUIX, S. 2016. Propidium monoazide RTqPCR assays for the assessment of hepatitis A inactivation and for a better estimation of the health risk of contaminated waters. Water research, 101, 226-232.

GÓMEZ-DOÑATE, M., CASANOVAS-MASSANA, A., MUNIESA, M. & BLANCH, A. R. 2015. Development of new host-specific Bacteroides qPCRs for the identification of fecal contamination sources in water. Microbiology Open.

GUNNARSDOTTIR, M.J., GARDARSSON, S.M., ELLIOTT, M., SIGMUNDSDOTTIR, G., BARTRAM, J. 2012. Benefits of Water Safety Plans: Microbiology, Compliance and Public Health. Environ.Schi. Technol., 46(14) 7782-7789. Open access

HADFIELD SJ, PACHEBAT JA, SWAIN MT, ROBINSON G, CAMERON SJS, ALEXANDER JL, HEGARTY MJ, ELWIN K, CHALMERS RM. Generation of whole genome sequences of new Cryptosporidium hominis and Cryptosporidium parvum isolates directly from stool samples. BMC Genomics 2015, 16:650 DOI: 10.1186/s12864-015-1805-9

HJELMSØ MH, HELLMÉR M, FERNANDEZ-CASSI X , TIMONEDA N , LUKJANCENKO O , SEIDEL M, ELSÄSSER D, AARESTUP FM, LÖFSTRÖM C, BOFILL-MAS S, ABRIL JF, GIRONES R, SCHULTZ AC (2017). Evaluation of Methods for the Concentration and Extraction of Viruses From Sewage Water in the Context of Metagenomic Sequencing. PLoS ONE 12(1):e0170199, DOI: 10.1371/journal.pone.0170199.

HO, J., SEIDEL, M., NIESSNER, R., EGGERS, J. & TIEHM, A. 2016. Long amplicon (LA)-qPCR for the discrimination of infectious and noninfectious phix174 bacteriophages after UV inactivation. Water research, 103, 141-148.

HOSSAIN, M. J., BEAZ-HIDALGO, R., FIGUERAS, M. J. & LILES, M. R. 2014. Draft genome sequences of two novel Aeromonas species recovered in association with cyanobacterial blooms. Genome announcements, 2, e01181-14.

JIMENEZ, M. & BRIDLE, H. L. 2015. Angry pathogens, how to get rid of them: introducing microfluidics for waterborne pathogen separation to children. Lab on a Chip, 15, 947-957.

JOFRE, J., BLANCH, A. R., LUCENA, F. & MUNIESA, M. 2014. Bacteriophages infecting Bacteroides as a marker for microbial source tracking. Water research, 55, 1-11.

KERROUCHE, A., DESMULLIEZ, M. P. & BRIDLE, H. 2015. Megasonic sonication for cost-effective and automatable elution of Cryptosporidium from filters and membranes. Journal of microbiological methods, 118, 123-127.

KROG JS, FORSLUND A, LARSEN LE, DALSGAARD A, KJAER J, OLSEN P, SCHULTZ AC (2017). Leaching of viruses and other microorganisms naturally occurring in pig slurry to tile drains on a well-structured loamy field in Denmark. Hydrogeology Journal, special issue "Hydrogeology and Human Health", 25(4):1045–62.

LATIF-EUGENÍN, F., BEAZ-HIDALGO, R. & FIGUERAS, M. 2016. Evaluation of different conditions and culture media for the recovery of Aeromonas spp. from water and shellfish samples. Journal of applied microbiology, 121, 883-891.

LESNIK, R., BRETTAR, I. & HÖFLE, M. G. 2016. Legionella species diversity and dynamics from surface reservoir to tap water: from cold adaptation to thermophily. The ISME journal, 10, 1064-1080.

LEVICAN, A., ALKESKAS, A., GÜNTER, C., FORSYTHE, S. J. & FIGUERAS, M. J. 2013. Adherence to and invasion of human intestinal cells by Arcobacter species and their virulence genotypes. Applied and environmental microbiology, 79, 4951-4957.

LEVICAN, A., COLLADO, L. & FIGUERAS, M. J. 2016. The use of two culturing methods in parallel reveals a high prevalence and diversity of Arcobacter spp. in a wastewater treatment plant. BioMed Research International, 2016.

LEVICAN, A., COLLADO, L., YUSTES, C., AGUILAR, C. & FIGUERAS, M. J. 2014. Higher water temperature and incubation under aerobic and microaerobic conditions increase the recovery and diversity of Arcobacter spp. from shellfish. Applied and environmental microbiology, 80, 385-391.

LEVICAN, A. & FIGUERAS, M. J. 2013. Performance of five molecular methods for monitoring Arcobacter spp. BMC microbiology, 13, 220.

LEVICAN, A., RUBIO-ARCOS, S., MARTINEZ-MURCIA, A., COLLADO, L. & FIGUERAS, M. J. 2015. Arcobacter ebronensis sp. nov. and Arcobacter aquimarinus sp. nov., two new species isolated from marine environment. Systematic and applied microbiology, 38, 30-35.

MARTÍNEZ-MURCIA, A., BEAZ-HIDALGO, R., NAVARRO, A., CARVALHO, M. J., ARAVENA-ROMÁN, M., CORREIA, A., FIGUERAS, M. J. & SAAVEDRA, M. J. 2016. Aeromonas lusitana sp. nov., Isolated from Untreated Water and Vegetables. Current microbiology, 72, 795-803.

MILLER, B., JIMENEZ, M. & BRIDLE, H. 2016. Cascading and Parallelising Curvilinear Inertial Focusing Systems for High Volume, Wide Size Distribution, Separation and Concentration of Particles. Scientific reports, 6.

MOHAMMED, M.-I. & DESMULLIEZ, M. P. Y. 2014a. CO2 laser manufacturing of miniaturised lenses for lab-on-a-chip systems. Micromachines, 5, 457-471.

MOHAMMED, M., ABRAHAM, E. & DESMULLIEZ, M. 2013. Rapid laser prototyping of valves for microfluidic autonomous systems. Journal of Micromechanics and Microengineering, 23, 035034.

MOHAMMED, M. & DESMULLIEZ, M. 2013. The manufacturing of packaged capillary action microfluidic systems by means of CO2 laser processing. Microsystem technologies, 19, 809-818.

MOHAMMED, M. I. & DESMULLIEZ, M. P. Y. 2014b. Characterization and theoretical analysis of rapidly prototyped capillary action autonomous microfluidic systems. Journal of Microelectromechanical Systems, 23, 1408-1416.

MOORE, C. E., ELWIN, K., PHOT, N., SENG, C., MAO, S., SUY, K., KUMAR, V., NADER, J., BOUSFIELD, R. & PERERA, S. 2016. Molecular characterization of Cryptosporidium species and Giardia duodenalis from symptomatic Cambodian children. PLoS neglected tropical diseases, 10, e0004822.

ORISTO, S., RÖNNQVIST, M., AHO, M., SOVIJÄRVI, A., HANNILA-HANDELBERG, T., HÖRMAN, A., NIKKARI, S., KINNUNEN, P. M. & MAUNULA, L. 2017. Contamination by norovirus and adenovirus on environmental surfaces and in hands of conscripts in two finnish garrisons. Food and environmental virology, 9, 62-71.

PEREIRA, R. P. A., PEPLIES, J., BRETTAR, I AND M. G. HÖFLE (2017). Development of a genus-specific next generation sequencing approach for sensitive and quantitative determination of the Legionella microbiome in freshwater systems. BMC Microbiol. 17:79; DOI: 10.1186/s12866-017-0987-5.

PÉREZ-CORDÓN, G., ROBINSON, G., NADER, J. & CHALMERS, R. M. 2016. Discovery of new variable number tandem repeat loci in multiple Cryptosporidium parvum genomes for the surveillance and investigation of outbreaks of cryptosporidiosis. Experimental Parasitology, 169, 119-128.

PETERSEN TN, RASMUSSEN S, HASMAN H, CARØE C, BÆLUM J, SCHULTZ AC, BERGMARK L, SVENDSEN CA, LUND O, SICHERITZ-PONTÉN T, AARESTRUP FM (2015). Meta-genomic analysis of toilet waste from long distance flights; a step towards global surveillance of infectious diseases and antimicrobial resistance. Sci. Rep. 5, 11444; doi: 10.1038/srep11444.

RASMUSSEN-IVEY, C. R., FIGUERAS, M. J., MCGAREY, D. & LILES, M. R. 2016. Virulence Factors of Aeromonas hydrophila: in the Wake of Reclassification. Frontiers in Microbiology, 7.

RASMUSSEN, L. D., SCHULTZ, A. C., UHRBRAND, K., JENSEN, T. & FISCHER, T. K. 2016. Molecular Evidence of Oysters as Vehicle of Norovirus GII. P17-GII. 17. Emerging infectious diseases, 22, 2024.

RYZINSKA-PAIER, G., LENDENFELD, T., CORREA, K., STADLER, P., BLASCHKE, A., MACH, R., STADLER, H., KIRSCHNER, A. & FARNLEITNER, A. 2014. A sensitive and robust method for automated on-line monitoring of enzymatic activities in water and water resources. Water Science and Technology, 69, 1349-1358.

SABRIÀ, A., PINTÓ, R. M., BOSCH, A., BARTOLOMÉ, R., CORNEJO, T., TORNER, N., MARTÍNEZ, A., DE SIMÓN, M., DOMÍNGUEZ, A. & GUIX, S. 2014. Molecular and clinical epidemiology of norovirus outbreaks in Spain during the emergence of GII. 4 2012 variant. Journal of Clinical Virology, 60, 96-104.

SALAS-MASSÓ, N., ANDREE, K. B., FURONES, M. D. & FIGUERAS, M. J. 2016. Enhanced recovery of Arcobacter spp. using NaCl in culture media and re-assessment of the traits of Arcobacter marinus and Arcobacter halophilus isolated from marine water and shellfish. Science of The Total Environment, 566, 1355-1361.

SCHIWY, A., MAES, H. M., KOSKE, D., FLECKEN, M., SCHMIDT, K. R., SCHELL, H., TIEHM, A., KAMPTNER, A., THÜMMLER, S. & STANJEK, H. 2016. The ecotoxic potential of a new zero-valent iron nanomaterial, designed for the elimination of halogenated pollutants, and its effect on reductive dechlorinating microbial communities. Environmental Pollution, 216, 419-427.

STADLER, P., RYZINSKA-PAIER, G., LENDENFELD, T., VOGL, W., BLASCHKE, A.P., STRAUSS, P., STADLER, H., LACKNER, M., ZESSNER, M., FARNLEITNER, A.H. (2017). Automated near-real-time monitoring of enzymatic activities in water resources, in Handbook of Online and Near-Real-Time Methods in Microorganism Detection by Lackner, Stadler, Grabow, CRC Press, ISBN 9781498764025, Taylor and Francis Group, in press, to be published on October 1st 2017.

STEVERDING, D. & TYLER, K. 2015. Of mice and men: are billions victim to toxoplasma mind control? Microbiology Today, 52, 10-13.

TEUNIS, P. & FIGUERAS, M. J. 2016. Reassessment of the enteropathogenicity of mesophilic Aeromonas species. Frontiers in microbiology, 7.

UHRBRAND K, SCHULTZ AC, KOIVISTO J, NIELSEN U, MADSEN AM (2017). Assessment of airborne bacteria and noroviruses in air emission from a new highly-advanced hospital wastewater treatment plant in Denmark. Water Research. 112: 110-119. DOI: 10.1016/j.watres.2017.01.046.

VAN ALPHEN, L. B., DORLÉANS, F., SCHULTZ, A. C., FONAGER, J., ETHELBERG, S., DALGAARD, C., ADELHARDT, M., ENGBERG, J. H., FISCHER, T. K. & LASSEN, S. G. 2014. The application of new molecular methods in the investigation of a waterborne outbreak of norovirus in Denmark, 2012. PloS one, 9, e105053.

VEZZULLI, L., GRANDE, C., REID, P. C., HÉLAOUËT, P., EDWARDS, M., HÖFLE, M. G., BRETTAR, I., COLWELL, R. R. & PRUZZO, C. 2016. Climate influence on Vibrio and associated human diseases during the past half-century in the coastal North Atlantic. Proceedings of the National Academy of Sciences, 201609157.

VEZZULLI, L., GRANDE, C., TASSISTRO, G., BRETTAR, I., HÖFLE, M., PEREIRA, R., MUSHI, D., PALLAVICINI, A., VASSALLO, P. & PRUZZO, C. 2017. Whole-genome enrichment provides deep insights into Vibrio cholerae metagenome from an African river. Microbial ecology, 73, 734-738.

VEZZULLI, L., PEZZATI, E., BRETTAR, I., HÖFLE, M. & PRUZZO, C. 2015a. Effects of Global Warming on Vibrio Ecology. Microbiology spectrum.

VEZZULLI, L., STAUDER, M., GRANDE, C., PEZZATI, E., VERHEYE, H. M., OWENS, N. J. & PRUZZO, C. 2015b. gbpA as a novel qPCR target for the species-specific detection of Vibrio cholerae O1, O139, non-O1/non-O139 in Environmental, Stool, and Historical Continuous Plankton Recorder Samples. PloS one, 10, e0123983.

WELLS, B. 2015. Controlling Cryptosporidium in the environment. The Veterinary record, 176, 615-616.

WELLS, B., SHAW, H., HOTCHKISS, E., GILRAY, J., AYTON, R., GREEN, J., KATZER, F., WELLS, A. & INNES, E. 2015a. Prevalence, species identification and genotyping Cryptosporidium from livestock and deer in a catchment in the Cairngorms with a history of a contaminated public water supply. Parasites & vectors, 8, 66.

WELLS, B., SHAW, H., INNOCENT, G., GUIDO, S., HOTCHKISS, E., PARIGI, M., OPSTEEGH, M., GREEN, J., GILLESPIE, S. & INNES, E. A. 2015b. Molecular detection of Toxoplasma gondii in water samples from Scotland and a comparison between the 529bp real-time PCR and ITS1 nested PCR. Water Research, 87, 175-181.

WELLS, B., THOMSON, S., ENSOR, H., INNES, E. & KATZER, F. 2016. Development of a sensitive method to extract and detect low numbers of Cryptosporidium oocysts from adult cattle faecal samples. Veterinary Parasitology, 227, 26-29.

XIA, H., GRAVELSINA, S., ÖHRMALM, C., OTTOSON, J. & BLOMBERG, J. 2016. Development of single-tube nested real-time PCR assays with long internally quenched probes for detection of norovirus genogroup II. BioTechniques, 60, 28-34.

YAHYA, M., BLANCH, A. R., MEIJER, W. G., ANTONIOU, K., HMAIED, F. & BALLESTÉ, E. 2017. Comparison of the Performance of Different Microbial Source Tracking Markers among European and North African Regions. Journal of Environmental Quality.

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