

UNIVERSITÀ DEGLI STUDI DELLA TUSCIA DI VITERBO
DIPARTIMENTO DI
AGRICOLTURA, FORESTA, NATURA ED ENERGIA (DAFNE)

Corso di Dottorato di Ricerca in
BIOTECNOLOGIE VEGETALI - XXVIII Ciclo

ON LINE ROBOTIC SYSTEMS FOR CONTINUOUS MONITORING OF
TOXICITY AND CHEMICAL PARAMETERS IN IRRIGATION WATER

Settore Scientifico – Disciplinare
(s.s.d. BIO/13)

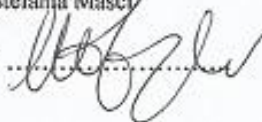
Tesi di dottorato di:

Dott. Annalisa Tortelli

Coordinatore del corso

Prof. Stefania Masci

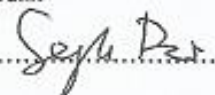
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Sergio Bodini

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Abstract

Global water scarcity caused an increased use of wastewater effluents as unconventional water sources for agriculture products. Since more than 10% of the world population consumes food produced by irrigation with wastewater, the integration of chemical and biological monitoring in wastewater effluents has now become a key issue for safeguarding the environmental quality and hygiene of water bodies.

The opportunity of rapidly achieving consistent results, in conjunction with improved analytical throughput, represents a remarkable technological breakthrough in this field. Being conventional methods expensive, time consuming and based on laborious procedures, they result ineffective to quickly monitor changes in the quality of water and wastewater samples. Technological developments in on-line, real time and early warning systems have facilitated the continuous audit of water for protecting environmental biota and human health.

A new line of discrete analysers, performing both biological and chemical continuous measurements in water samples, has been developed. Specifically, two original models of on-line analysers, respectively Easy Coli for continuously measuring *Escherichia coli* concentration in irrigation water and Easychem Tox On-line for performing chemical analysis along with toxicity tests, have been constructed and tested.

Easy Coli is an on-line automatic device for the analysis of *E. coli* in effluent wastewater equipped with autosampler, filtration unit, and detection apparatus. After the improvement of the automatic analytical cycle, a field campaign has been achieved for quantifying the bacteria load in samples coming from wastewater effluents.

Easychem Tox On-line is a random-access platform, which comprises: a refrigerated plate for storing bacteria; an illuminated compartment for microalgae; a plate for containing reagents, calibrants and controls; a mechanical arm for aspiration, transferring and dispensing operations; a thermostated reaction plate holding 80 positions, incorporated with a fluorimeter, a colorimeter, a luminometer and an automated cuvette washing station. Within Easychem Tox On-line, both luminescent bacteria and fluorescent algae have been selected as biomonitors for rapidly measuring acute toxicity in water samples. In parallel, the determination of some common chemical parameters allowed to perform an integrated chemical and biological control of water and wastewater, thus giving information about causes and effects of contamination within the same instrument.

1. Introduction

1.1 Water scarcity and water recycling

The Seventh World Water Forum in South Korea (April 2015) showed to the world public that water is the central issue of the 21st century since the life of billions of people depends on the management of this resource. The scarcity of water for human use, such as food and energy production, manufacturing, drinking water and ecosystem conservation, is a global problem whose solution merely goes beyond the preservation of freshwater sources.

Although three quarters of the Earth's surface is covered by water, most of this resource is either contained in oceans or confined in glaciers. Therefore, the volume of freshwater available for human activities results less than 1% and is unequally distributed throughout the globe; in some cases, this water is confined to the deep subsoil or, even worst, is polluted. Furthermore, the desertification of large areas, caused by climate changes, has intensified the lack of water sources in cities and rural areas throughout the world.

Water scarcity results in food insufficiency, since 70% of the water withdrawn for human activities goes to agriculture. It is estimated that in 2025 nearly one-third of the population of developing countries (some 2.7 billion people) will live in arid or semi - arid regions (FAO, 2007). This contingency places many municipalities in a precarious position, especially in the face of increasing water demand, increasing water supply costs and increasing competition for good-quality fresh water reserves. As a result, they will have to reduce the amount of water used in irrigation and transfer it to the domestic, industrial and environmental sector (De Silva, 2000).

In light of increasing concerns about water supplies availability, wastewater reclamation was found a good opportunity for augmenting water reserves, especially when compared to the alternatives, such as importing water through costly convenience systems or constructing dams or reservoir.

Effluents coming from wastewater treatment plants have become suitable and convenient water sources, since their supply is reliable and uniform, and it is constantly increasing due to population growth. Moreover, costs of this reclaimed water are low compared with those of other unconventional sources. So, wastewater has been proven as a reliable alternative water resource, which can play a vital role in integrated management simultaneously addressing a number of critical issues such as water demand and supply, wastewater disposal and environmental protection (Lazarova, 2001).

1.2 Wastewater reuse: water quality criteria and management

Since the volume of wastewater generated by domestic, industrial and commercial sources has increased with population, urbanization and economic development, its productive use has contemporarily improved. These increasing availability and use generated challenges for public agencies charged with minimizing potential impacts on public health and environment.

Water quality criteria, economic analyses and supervision projects are essential components in the context of wastewater reclamation. Proper and integrated planning in the reuse of reclaimed water may, in fact, provide sufficient flexibility to respond to short-term needs as well as to increase the long-term reliability of water supply.

One of the most important aspects that contributed to the development and the implementation of water recycling, is legislation. Within the EU, at least two major environmental directives raise the issue of wastewater reuse, as these directives lead towards two primary objectives. On one hand, the Urban Wastewater Treatment Directive (91/271/EEC) has the main objective to protect the environment from the adverse effects of wastewater discharges, so it requires that “treated wastewater shall be reused whenever appropriate”. On the other hand, the Water Framework Directive (WFD) (2000/60/EC) refers to “emission controls” and “efficiency measures, promotion of water efficient technologies in industry and water saving techniques for irrigation”, as two, non-exclusive, supplementary measures.

Upgraded wastewater used in various application categories shall respect specific conditions, described in the following:

- Reuse water shall be safe for its intended use and shall not jeopardise the safety of the product through the introduction of chemical, microbiological or physical contaminants in amounts that represent a health risk to the consumer.
- Reuse water should not adversely affect the quality (flavour, colour, texture) of the product.
- Reuse water shall be subjected to on-going monitoring and testing to ensure its safety and quality. The frequency of monitoring and testing are dictated by the source of the water or its prior condition.
- The selected water treatment system should be such to provide the level of reconditioning appropriate for the intended water reuse.
- Proper maintenance of water reconditioning systems is critical.
- Treatment of water must be undertaken with knowledge of the types of contaminants the water may have acquired from its previous use.

The choice of the right wastewater treatment technologies is fundamental in planning the recycling, because they represent the way of decreasing or eliminating the environmental risks, connected with

chemical or microbiological contamination potentially found in the upgraded waters. Because of the presence of organic, inorganic and microbial pollutants in wastewater, a prior step of depuration is necessary before reuse in irrigation, in order to avoid both the pollution of soil, crops and the nearby water sources, and the dissemination of waterborne diseases.

Primary treatment schemes, such as coagulation – flocculation, with sedimentation or aerobic / anaerobic stabilization ponds, are used for treating wastewater to irrigate crops that are not intended for human consumption, while secondary treatment, as biological treatment followed by disinfection, is recommended when unrestricted crops are irrigated. In developing countries, most or the whole volume of wastewater produced in cities is treated prior to irrigation, while in low income countries treatment is not a priority. Thus, untreated or partially treated wastewaters, or a mixture of them, are commonly used for agricultural purposes.

The World Health Organization estimates that nearly 20 million hectares throughout the world are irrigated using untreated wastewater (WHO, 2006). These applications pose a significant risk of pollution, not only to soil and crops but also to the surface and subterranean water sources surrounding the irrigated area.

1.3. Benefits and negative impacts of wastewater reuse in agriculture

Wastewaters coming from industry, agriculture or domestic sewage are either dumped on land or used for irrigation and fertilisation purposes, leading in every case both opportunities and problems. When wastewater is used for producing crops, this practice represents a method for providing supplement nutrients such as nitrogen, Phosphorus, potassium, and other elements in minimal concentrations (*Table 1*).

Table 1: Nutrient addition to soil, when irrigated with treated wastewater (Lazarova, 2005).

Nutrient	Concentration (mg · L ⁻¹)
Nitrogen	16 – 62
Phosphorus	4 – 24
Potassium	2 – 69
Calcium	18 – 208
Magnesium	9 – 110
Sodium	27 - 182

Since wastewater is constantly produced and, thus, is always available, its consistent use in irrigation may stabilize the content of nutrients in the soil, even in growing crops with high nutritional requirements. As these components are not required to be removed by treatments above

a certain level, the cost of wastewater use can be reduced, with benefits for economic sustainability of reclamation projects. Recycling nutrients by wastewater reclamation promotes savings in energy, which would otherwise be consumed in the production of fertilizers.

The positive impacts of reusing wastewater in agricultural irrigation, in all of its forms, are summarized in *Fig. 1*.

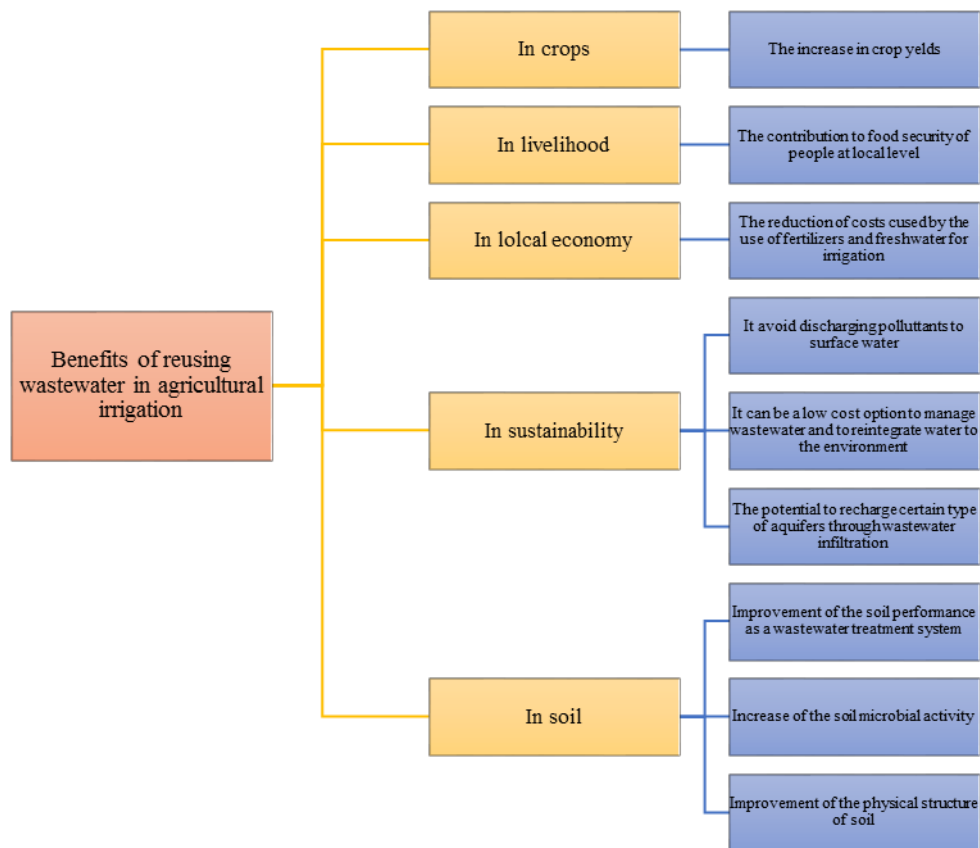


Figure 1: Beneficial impact of reusing wastewater in agriculture.

Although wastewater use in agriculture has substantial benefits towards crops production and water resources management, considerable risks to public health are posed, especially when untreated wastewater is employed. There can also be chemical risks to plant health, and risks to the environment in the form of soil and groundwater pollution. Even when soil acts as an efficient living filter to remove, inactivate and transform the pollutants contained in wastewater, it is not fully effective at eliminating some of them. The pollutants received by soil via wastewater may be various and with different origins. Examples of these include pathogenic microbial agents, inorganic and organic pollutants such as herbicides, nanomaterials and new-generation antibiotics. Pollution by pathogenic agents is the main cause of concern regarding the application of treated / untreated wastewater for irrigating crops. Due to the variety of microorganisms entering the soil, mainly via municipal wastewater, there is a high risk of enteric disease outbreaks for farmers and

consumers. The occurrence of antibiotic resistance in pathogens reaching soil via reclaimed waters is gaining the attention of scientists and health organizations around the world. Pathogenic organisms can be reduced by treatment processes and their level should be monitored regularly, in coordination with other disinfection methods to protect exposed population and crops.

Similarly, pollution of soil by organic substances has been a matter of concern to scientists and organizations regulating the quality of soil, water sources and food for several decades. Most of the dissolved and particulate organic matter contained in municipal wastewater is produced by the degradation of human and animal excreta, being the present organic matter mainly composed by saccharides, lipids, aminoacids and proteins. With regard to organic pollution, a current topic of interest is the entry to the soil of the so-called “contaminants of emerging concern”. These compounds, such as pharmaceutical and personal care products (PPCPs), are substances that have not previously been contemplated as pollutants since they are part of everyday products. However, due to the subtle but harmful effects that they may cause in a variety of aquatic and terrestrial organisms, concerns have risen, considering the continuous entry of these contaminants into the environment via wastewater.

Hazards from heavy metals do not seem high, because most agricultural wastewater irrigation is performed using municipal wastewater, which contains negligible amounts of heavy metals. The occurrence of these elements in wastewater irrigated soils is significantly lower than the maximum permissible concentrations established by international regulations. However, there are some cases in which it is necessary to be careful while reusing wastewater in irrigation, e.g. close to tanneries, metal processing or mining areas.

1.4 Monitoring wastewater quality: from static sampling to continuous records

Facing all risks and benefits, various governments and organizations have defined new approaches aiming to improve water quality on the basis of past legislation, which was often based on lists of known or suspected pollutants. Many of them persist in the environment, bioaccumulate and cause serious deleterious effects on organisms (e.g., carcinogenesis and endocrine disruption). Knowledge of the environmental fate of these substances in irrigated soils is important in order to perform more accurate risk assessment studies, and provides information to policy makers for promoting a responsible management of treated / untreated wastewater in agricultural irrigation. In particular, countries seeking to improve wastewater use in agriculture should pursue a ‘multiple barrier’ approach to risk management, including wastewater treatment together with post-treatment health-protection control measures. They can be used both singly and in combination, in particular when wastewater treatment alone cannot achieve the health-based targets.

Traditionally, the quality of treated wastewater is defined by the measurement of global parameters based on physical, organoleptic, chemical and biological measurements. Further to providing vital information on the quality of treated wastewater and treatment efficiency, the different measured parameters demonstrate if a wastewater treatment plant meets statutory laws.

The common methods used to monitor pollutants of concern involve taking spot water samples at specified intervals from the field back to the laboratory, for analysing them by classical chromatographic and spectroscopic techniques. In most cases, accredited methods (e.g., the various US Environmental Protection Agency Method Series) are used for the analyses of various classes of substances, and usually results have a high degree of confidence. On the other hand, less confidence is associated with the sampling procedures, since infrequent measures at a restricted number of sampling points may not provide a representative picture of water quality where levels of pollutants can vary both spatially and temporally.

As claimed by WFD, it has become necessary a new concept of water survey, from both a static approach, represented by discontinuous sampling, and a dynamic water control approach, represented by continuous records of quality variables.

1.5 Early warning systems and on-line monitoring

During recent years, concerns regarding the possibility that deliberate attacks on water could take place, or contaminant agents could reach the final consumer, leading in both cases to a crisis situation, have increased. In addition, considering the large number of potentially hazardous compounds entering the environment, the need for suitable analytical techniques along with toxicity monitoring to assess the interaction effects of mixtures on the biota, has become very important (Blasco, 2009).

As a consequence, the opportunity to rapidly detect sudden quality changes caused by pollution events, preferably by integrating chemical and physicochemical information (such as pH, conductivity, turbidity, trace organics, pesticides, heavy metals etc.), with biological data coming from biomonitors (algae, bacteria, mussels, daphnia, fish, etc.) in the same instrument, is the most optimal way to ensure an appropriate and timely response.

According to a US Environmental Protection Agency (EPA) research report, early warning systems (EWS) respond to the above mentioned requirements, being “integrated systems for monitoring, analysing, interpreting and communicating data, which can be used to make decisions that are protective for public health and minimize unnecessary concern and inconvenience to the public”. Both chemical screening and biological early warning systems ensure the possibility to identify low

probability/ high-impact contamination events in sufficient time to produce an alert and quickly respond to changes (Grayman, 2001).

To become a widely used, effective, and reliable part of a water distribution security and quality monitoring system, an ideal EWS should demonstrate a number of characteristics (Hasan, 2004):

- provide a rapid response;
- include a sufficiently wide range of potential contaminants that can be detected;
- exhibit a significant degree of automation, including automatic sample archiving;
- require low skill and training;
- identify the source of the contaminant and allow accurate prediction of the location and concentration downstream of the detection point;
- demonstrate sufficient sensitivity to detect contaminants;
- permit minimal false-positives / false-negatives;
- exhibit robustness and ruggedness to continually operate in a water environment;
- allow remote operation and adjustment;
- function continuously.

An essential criterion for the effectiveness of a EWS is its location: according to the definition, there should be sufficient time for action, once a contaminant has been detected. This implies that monitoring systems should be installed far enough upstream from the point of water abstraction to provide a timely warning. According to the relationship between the sampling procedure and the analytical process, both *in situ* and on-line EWS exist.

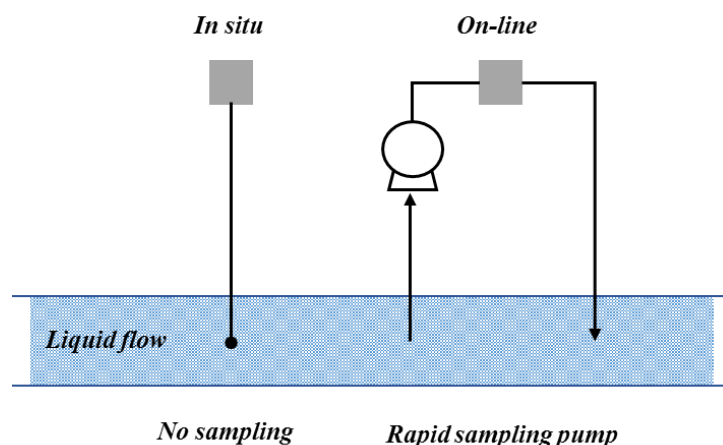


Figure 2: Main approaches to automated water monitoring.

The *in situ* monitoring systems generally involve the use of a sensing device directly immersed in the water body. Data are collected automatically, and either recorded for later retrieval or

transmitted telemetrically to a distant laboratory for interpretation. A good example is the deployment of multi-parameter probes that generally use electrochemical or spectroscopic techniques to acquire continuous data on characteristics of the matrix (e.g. pH, temperature, conductivity, dissolved oxygen, turbidity, and Chlorophyll content). Biological *in situ* early warning systems also exist and are generally used at sensitive sites to provide a rapid warning of deleterious changes in water quality.

On the other hand, on-line analysers are located near to the water body being monitored; in such way the sample is automatically drawn into the system, where the analysis is performed. Samples can be pretreated before analysis, by including in the system prediluting or filtering apparatus. Measurements are generally based on optical techniques (i.e. UV-visible or fluorescence spectroscopy) and often the system works in thermocontrolled conditions.

Depending on the analytical method employed, both discrete and continuous on line analysers exist. In contrast to continuous flow analysers, discrete systems are automated devices which perform tests on samples that are kept in discrete containers (Bonini *et al.*, 1988). Initially employed in clinical analysis, the discrete analysers were introduced into the field of environmental monitoring only recently but they rapidly developed, because of some operational advantages, such as working with small volumes of samples and reagents, producing less quantities of waste and simultaneously performing a consistent number of different parameters.

At the present both chemical and biological EWS are installed on-line for respectively providing quantitative information on the physicochemical characteristics of the matrix and qualitative information about the toxicity of pollutants respect to living organisms, even if only few examples of integrated systems, such as Bio-Sensor (Biological Monitoring), Biota Guard (Biota Guard AS) and TOXControl (microLAN B.V.) have been developed. In any case, on-line systems represent highly advantageous systems, by analysing samples at very frequent intervals, requiring only weekly or monthly maintenance operations and, often, providing operators of remote connection for controlling data.

1.6 Aim of the project

Real-time monitoring of wastewater quality remains an unresolved problem to the wastewater treatment industry. In order to comply with increasingly stringent environmental regulations, plant operators as well as instrument manufacturers have expressed the need for new standards and improved comparability and reliability of existing techniques. On the other hand, because of the health risks associated with wastewater reuse for agricultural practices, the integration of chemical

and biological monitoring tools is a key issue for safeguarding the environmental quality and the hygiene of water bodies (WHO, 2006).

First of all, the opportunity of obtaining fast and consistent results, in conjunction with improved analytical throughput, represents a remarkable technological advance in this field: on line water quality monitoring technologies have received attention and interest in order to provide accurate and continuous wastewater quality information.

In this context the project of this thesis was developed. Main purpose of this PhD project was the construction and testing of two original models of on line analysers, respectively Easychem Tox On-line, able to perform fully automated toxicity measurements integrated with chemical analyses and Easy Coli, for continuously measuring *Escherichia coli* concentration in irrigation water.

For the development and optimization of Easychem Tox On-line, two parallel lines were undertaken: on one hand, the automated and continuous monitoring of acute toxicity in sample waters, by using biomonitors, was studied and developed. Among the several types of living organisms employed in the monitoring field, luminescent bacteria and fluorescent algae were selected as test organisms. Starting from already existing toxicity assays, based respectively on bacteria and algae, the complete automation was improved within Easychem Tox On-line. Short term toxicity tests on environmental samples, such as drinking, surface and marine water, and wastewater effluents were performed, by combining specified volumes of the test sample with the test organism suspensions in a test tube and measuring the inhibition of both light emission (when bacteria were employed) or Chlorophyll a fluorescence (when algae were employed).

In parallel, part of the work focused on the applicability of the robotic system to the on-line monitoring of different chemical parameters. Starting from the standard methods for the analysis of water and wastewater, it was possible to fully automate the manual analytical procedures within the Easychem Tox On-line. A set of experiments was performed for measuring relevant chemical parameters such as nutrients or heavy metals, and the combination with toxicity tests in the same instrument was finally achieved, thus providing both chemical and biological on-line control of water and wastewater samples.

The second part of the PhD project was directed to the improvement of Easy coli, an on-line analyser for the rapid detection of *E. coli* in samples coming from wastewater treatment plants. Initially, a manual procedure for rapidly quantifying *E. coli* in water samples was improved with the scope to overcome the limits of culture-based standard techniques. The strategy applied in this study was based on the properties of the species-specific β -D-glucuronidase (GUD) enzyme, which hydrolyses the compound 4-methylumbelliferyl glucuronide (MUG) to yield a fluorogenic product that can be quantified and directly related to the number of *E. coli* cells present in the sample. After

validating the one-hour manual procedure, the full automation of analytical steps, namely sampling, filtration, addition of reagents, detection and data processing was achieved within a new robotic system, the Easy coli analyser. An initial laboratory campaign was performed, with the aim to verify the proper functioning of the system, set-up parameters and check out detection limits. Finally, a field trial on a real wastewater treatment plant (WWTP) was conducted in order to demonstrate the real potential of the analytical system. Being able to monitor almost instantaneously the level of bacterial abatement, Easy coli could both function as early warning system in case of unexpected failures and provide a feedback tool to the disinfection process whose parameters, especially the amount of oxidizing agent, were finely regulated according to the level of disinfection required.

2. Easychem Tox On-line:

Automated Toxicity Tests and Chemical Measurements

2.1. Early warning systems for water quality monitoring

2.1.1. Wastewater irrigation and health

It is a fact that wastewater has become a valuable non-conventional resource for agricultural practices. For this reason, the treatment of sewage requires proper management to minimize the environmental and health risks related to its use in agriculture. In view of this, there is an urgent need for the development and validation of cost-effective technologies and methodologies that can be widely adopted for the routine monitoring of wastewaters and receiving surface waters. In particular, there is general agreement that the best way to assess the environmental quality of the recycled wastewater with respect to hazardous substances is to use a suite of chemical and biological measurements in an integrated fashion (Quevauviller, 2008).

2.1.2. Integrated biological and chemical measurements for water quality monitoring

In the past, water quality monitoring has heavily relied on spot sampling followed by instrumental analytical measurements to determine pollutant concentrations. Despite a number of advantages, these procedures have considerable limitations in terms of (i) temporal and spatial resolution that may be achieved at reasonable cost, and (ii) the information on bioavailability that may be obtained. Successful implementation of the Water Framework Directive (2000/60/EC) across EU member states has required the establishment and use of emerging and low-cost tools as part of monitoring programmes. These techniques may complement monitoring already in place by providing additional information, with the aim to obtain a more representative picture of the quality of a water body. In order to fully assess the quality control of wastewater, questions about the bioavailability of hazardous substances and their impact on organisms are now being posed: biological effects techniques have, therefore, become increasingly important. In particular, an integrated approach to monitoring is based on the simultaneous measurement of contaminant concentrations (in biota, sediments, and, in some cases, water or passive samplers), biological effects parameters, and a range of physical and other chemical measurements so as to permit normalization and appropriate assessment.

A similar integrated programme, which involves both chemical and biological effect measurements primarily contributes to the improvement of the interpretative value for the individual

measurements. Traditional physical and chemical measures of water quality are useful to determine sources of water contamination, but they only indirectly measure the health of the aquatic ecosystem because they don't look directly at biological responses to pollution. On the other hand, biological effects' measurements complete the information about concentrations of contaminants in biota or sediments. When biological effects measurements are carried out in combination with chemical measurements, this provides an improved assessment allowing identification of the substances contributing to the observed effects (Allan, 2006).

2.1.3. Monitoring the causes of water pollution

Monitoring the chemical quality of water involves the analysis of chemical substances classified as important. In most legislation there are lists of priority pollutants that are regarded as being harmful or potentially harmful. These are normally classified under three major categories: nonpolar (some pesticides and industrial chemicals such as PAHs) and polar (some pesticides and pharmaceuticals) organic compounds, inorganic pollutants (mineral acids, inorganic salts, trace elements) and heavy metals (e.g. mercury and cadmium), and the methods for monitoring these are very different in detail.

The best established monitoring methods in terms of quality assurance and control are based on chromatography (gas and liquid) coupled with a sensitive detector (e.g. flame ionization, electron capture, mass spectrometer, fluorescence and UV spectrometer), whereas for metals detection, methods such as inductively coupled plasma mass spectrometry, or graphite oven atomic absorption spectrometry are generally employed. These methods are very accurate and reproducible within low limits of detection, but they are time consuming and expensive in performing target analysis and require qualified personnel. However, a range of other methods (based on chemical, optical, electrochemical or biological techniques) is available for carrying out the analytical step, depending on the chemical to be measured.

A series of rapid analytical methods that can be used in the laboratory or, in some cases, in the field, is available. These protocols are generally based on specific complexation reactions whose products, usually coloured, can be quantified in a colorimeter or a spectrophotometer. Some can be measured directly on the basis of modification of an electromagnetic beam to produce a characteristic spectrum (e.g. UV and IR spectroscopy), or through emission of a characteristic spectrum (e.g. fluorescence and luminescence spectroscopy). Electrochemical techniques are also available; they are based on either the measurement of changes in a potential or a current generated between two electrodes placed in the analyte solution.

Chemical parameters analyses commonly employed for a rapid pollution monitoring are limited and only indicate the nature of pollutants, whereas do not yield any information about the biological effects. Chemical concentrations can be measured with an instrument but only living material can be used to measure toxicity. Measurements of biological responses to the levels of contaminants present in the environment can provide an alternative or complementary approach to measurements for the identification of trends in quality. Biomonitoring has been used for several decades in environmental analysis, and it assesses the effect of biologically harmful or biologically stimulating substances on selected test organisms.

Toxicity tests are one of the most frequently used biomonitoring tools and they may be used to demonstrate the probability of toxicity to the biota within a receiving water body. The basis assumption is that an effluent which results toxic to one or more species in a test system, is likely to be toxic to important components of the ecosystem, and therefore capable of causing adverse environmental impact. In general, there is a proportional relationship between the concentration of an analyte in the water and its impact on a living organism. Toxicity tests are based on the use of whole organisms, including a range of animals, algae and bacteria, or of isolated parts of organisms (cells, tissues or enzyme systems) which can be used to detect or quantify levels of organic and inorganic pollutants and to measure the general toxicity of water samples. Since there is generally a complex mixture of contaminants in the water, the former is the most common application of bioassays. Some assays use a range of species of organisms with varying sensitivities to the different types of pollutant in order to provide maximum sensitivity across the range of pollutants encountered. Toxicity depends in part on the bioavailability of the toxicants, and this is affected by factors such as the presence of suspended matter or high concentration of dissolved organic carbon. It is also affected by physicochemical variables such as pH, temperature, water hardness, oxygen tension, and salinity. Bioassays usually depend on the use of spot sampling and are laboratory-based. However, it is difficult to add preservatives to samples for transport such that, in some cases, they would markedly affect the outcome of the assay.

Therefore, some assays can be conducted in situ or on-line by deploying organisms in retaining systems directly in the environment, or by placing them in a flow of water coming from the water body being monitored. Changes in metabolism, behaviour or physiological parameters induced by sub-lethal effects are measured using automated detection systems. The employment of continuous biotests for the monitoring of inlet and outlet points offers the possibility of constantly monitoring water in relation to time. In the monitoring of inlets, biotests work primarily as warning systems which, for instance, indicate elevated concentrations caused by accidental discharges above the usual background concentration of the water course. In conjunction with chemical analysis and

more detailed biological examinations, they assist in securing evidence of illegal discharges and accidents. On the other hand, when applied to the monitoring of emissions, toxicity tests can supplement the monitoring with standardised static biotests and evaluate the treatment effectiveness.

In the case of biological early warning systems (BEWS), the changes in the behaviour or metabolism are linked to an alarm system so that personnel responsible for the management of water quality are aware of any pollution events and can take appropriate action to safeguard resources such as drinking water production plants or aquaculture facilities from pollution (Kramer and Botterweg, 1991). Most of these assays provide a measure of acute toxicity only, and do not provide information on chronic toxicity produced by long-term exposure to toxicants (possibly present in very low concentrations). BEWS are particularly useful for detecting intermittent toxic events in the environment.

Continuous, real time information on time-variable toxicity levels is important to environmental managers who need to understand point source and non-point source impacts in a watershed, evaluate whether surface water is of suitable quality for use in a water treatment plant, or consider whether an effluent from a wastewater treatment plant is suitable for discharge. Neither traditional toxicity tests nor chemical-specific sensors can provide comprehensive, real-time information on toxic events in an aquatic system.

2.1.4. Types of bioindicators: organism categories

Since different organisms respond differently to toxicants, bioassays employing diverse organisms are needed. Many biological early warning systems (Kramer 1991, van der Schalie 2001) have been developed to monitor physiological responses of whole organisms such as fish, protozoa, bacteria and algae to toxic chemicals in wastewater and soils (*Table 2*).

2.1.4.1. Fish-based bioassays

Fish bioassays have been applied in toxicity evaluation for decades. It is known that fish show distinct behavioural and physiological responses to quite low levels of contaminants. The assays often rely on fish mortality, as they are exposed to toxic compounds. The results are reported as concentration of the compound that is lethal to 50% of the fish within the prescribed time (LC₅₀). In general, fish biotests demonstrate good sensitivity and, under certain circumstances, allow real-time analysis. However, they have some disadvantages, such as standardization problems and require ethical issues, long maintenance time and special equipment.

Table 2: Organisms employed in Biological Early Warning Systems.

Organism	Stimuli
Freshwater fish	Swimming capacity behaviour
Zebra fish	Movement rapidity
Daphnia	Locomotor behaviour
Mussel	Valve movement
	Respirometry, oxygen production and consumption
Algae	Photosynthetic activity
	Growth inhibition
	Respirometry, oxygen production and consumption
Bacteria	Natural bioluminescence inhibition

2.1.4.2. Invertebrate-based bioassays

Invertebrate are commonly used as test species in toxicity bioassays, being the primary consumers of algae or herbivores. *Daphnia magna* is the most frequently tested invertebrate for acute and chronic toxicity tests. The application of daphnids has many advantages for routine toxicity tests, such as short reproductive cycle and high sensitivity to toxins.

2.1.4.3. Algae-based bioassays

Algae-based biosensors can be utilized for monitoring water toxicity by means of algae metabolic pathway: growth rate, fluorescence induction and photosynthetic activity are the most popular endpoints studied. The growth-inhibition test is sensitive to a great number of contaminants, such as insecticides, metals, industrial organic compounds and herbicides. The photosynthetic-activity inhibition test has proved efficient for herbicides, oil products and surfactants. The most widely developed algal biosensors employ *Chlorella vulgaris*, a green alga belonging to the Chlorophyceae group. The Chlorophyll fluorescence emitted from its photosynthetic activity enables herbicide detection while inhibition of its alkaline phosphatase and esterase allows determination of heavy metals and organoPhosphorus insecticides respectively.

2.1.4.4. Bacteria-based bioassays

The widest applied category of organisms in toxicity assessment comprises bacteria, which belong to the trophic level of decomposers, along with fungi, since they degrade dead organisms. Bacterial bioassays tend to fall into one of the five categories: population growth, substrate consumption, respiration, ATP luminescence and bioluminescence inhibition assays. The test species used for bioluminescence inhibition assays include *Vibrio fischeri*, *Photobacterium phosphoreum* and *Vibrio harveyi*. The growing interest in these tests is due to the fact that, despite the existence of different toxicity mechanisms for various organisms of different species, a substance that is toxic for an

organism often demonstrates similar toxic effects on other organisms (Kaiser 1998). Thus, luminescence inhibition in a bacterium can effectively indicate toxic effects on higher organisms. Bacteria toxicity test has been reported as a particularly sensitive assay while testing a range of chemicals and it is applicable to a wide range of samples, including complex effluents, surface water and groundwater, wastewater coming from industry and municipal waste effluent or sediments.

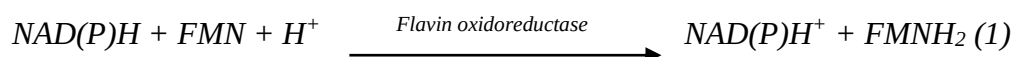
Many researchers have studied the development of rapid and sensitive bioassays to assess aquatic toxicity measurements by using higher organisms such as crustacean, fish and *Daphnia*: most of the tests, however, require long-term observation and time-consuming propagation of test organisms. As contrasted with higher organisms-based BEWS, biosensors and toxicity evaluation systems based on bacteria or unicellular algae have been widely used in acute toxicity tests because they are easy to use, non-invasive, costing little, rapid and reproducible (D'souza, 2001). Both of these tests are well explained in the following chapters.

2.1.5. Bioluminescent Bacteria Toxicity Test

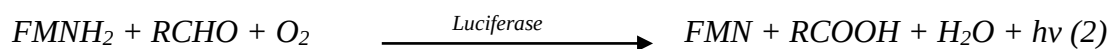
The application of luminescent bacteria on toxicity assays captured the researchers' attention and was quickly developed. In vivo luminescence is a sensitive indicator of xenobiotic toxicity because it is directly coupled to respiration by the electron transport chain and thereby reflects the cell's metabolic status: luminescence decreases in presence of noxious substances.

2.1.5.1 Biochemical mechanism in luminescent bacteria

Luminescent bacteria can emit blue-green light in the process of the normal metabolism. They are ubiquitously distributed in nature, e.g. seawater, sediment or skin of fishes, and the major part of them is marine species. The light emitted by luminescent bacteria principally relies on the bioluminescent enzyme system which consists of a NAD(P)H:FMN oxidoreductase and a luciferase. Firstly, FMN reduces to FMNH₂ upon the reaction catalysed by NAD(P)H:FMN oxidoreductase enzyme in the presence of a reduced NAD(P)H and a H⁺ (Equation 1).



Then bacterial luciferase catalyses the oxidation of reduced FMNH₂ to produce oxidized FMN in the presence of long chain aldehyde (RCHO) and O₂ with the light emission (Equation 2) (Inouye, 1994). The intensity of produced light is proportional to the amount of reagents involved in the chemical reaction. The spectral range of the bioluminescence is between 420 nm and 660 nm.



2.1.5.2. Bioluminescence inhibition assay

The light emission is closely related to cellular metabolism, and thus light intensity reflects the metabolic status of the bacteria. When the luminescent bacteria are exposed to toxic substances, the bacterial luciferase could be inhibited and the light intensity decreases rapidly. By measuring the light intensity of the bacteria exposed to testing sample and comparing with that of control, the inhibition can be calculated for quantifying the toxicity to luminescent bacteria. That is the principle of traditional luminescent bacteria inhibition assay which has been employed as the standard in many countries and regions.

2.1.5.3. EN ISO 11348 - Determination of the inhibitory effect of water samples on the light emission of *Vibrio fischeri* (Luminescent bacteria test)

The measurements specified in EN ISO 11348 (2009) can be carried out using freshly prepared bacteria, as well as freeze-dried or liquid-dried bacterial preparations.

Specifically, Part 3 concerns a method using freeze-dried bacteria. According to the principle of the method, the inhibition of light emission by cultures of *V. fischeri* is determined by means of a batch test. Initially, lyophilized bacteria are reconstituted by pouring 1 mL of distilled water in the vial and transferring water and bacteria in a glass test tube to 4 °C, in order to prepare a luminescent bacteria suspension (cell concentration about 10⁸ cells per millilitre) as stock suspension. After a waiting time of 10 min, test suspensions are prepared, by combining specified volumes of the test sample or the diluted sample with the luminescent bacteria stock suspension in a test tube maintained at 15 °C. Diluted samples, reference controls or blanks can be added instead of the sample. A conditioning time of 15 min is required before determining the initial luminescence intensity, *I*₀, by means of a luminometer. Immediately after the *I*₀ measurement, the suspension is made up to a total volume of 1 ml with the analysed test sample (undiluted, diluted, reference or blank sample), and test tubes are stored in the thermo-block at 15 °C. Light measurements are so performed in all test tubes, generally at 5, 15 and 30 min after exposure to test samples, at intervals of 5 s to 20 s.

The test criterion is the inhibitory effect of each test sample, calculated from the final luminescence values, taking into account a correction factor (*f_{kt}*), which is a measure of intensity changes of control samples during the exposure time.

The most widely used index for bioassay is EC₅₀, corresponding to the concentration of toxicant which causes the inhibition value of 50 % at a certain exposure time. For the convenience of

comparison, the toxicity units (TU) and toxicity impact index (TII50) defined as the reciprocal of EC_{50} are also used to express the toxicity.

Some important criteria must be complied with for accepting the test results as valid:

- the f_{kt} value for 15 min or 30 min incubation ranges between 0.6 and 1.8;
- the parallel determinations do not deviate from their mean by more than 3 % for the control samples;
- for the test samples which determine the LID lb-value or the EC_{20}/EC_{50} -values, respectively, the deviation from their mean in “percent points” does not exceed 3 % points;
- for the batch of bacteria delivered, the three reference substances cause 20 % to 80 % inhibition after 30 min of contact time at the following concentrations in the final test suspension: 3.4 mg / L 3,5-dichlorophenol, 2.2 mg / L Zn(II) (equivalent to 9.67 mg / L zinc sulfate heptahydrate) and 18.7 mg / L Cr(VI) (equivalent to 52.9 mg / L potassium dichromate).

2.1.5.4. State of the art

Until now, the vast majority of reported studies were performed by using laboratory instruments. Bioluminescent bacteria were used to measure toxicity of several types of environmental samples like wastewater, seawater, surface and ground water, tap water, soil and sediment extracts, aerosol and air particulate (Girotti, 2008).

Various bacteria toxicity assays exploited the properties of the gram negative bacterium *Vibrio fischeri* and, in particular, the specific strain NRRL B-11177 on which the previously described European standard was also based. Among all, the most popular product is the Microtox® test commercialized by Beckman Instruments, Inc. U.S.A. since 1978 (Flokstra, 2008).

Microtox® test is based on monitoring changes of light emission by *V. fischeri* when exposed to a toxic substance or sample containing toxic material. The test is performed by rehydrating freeze dried cultures of the organism, supplied as the *Microtox Reagent* and containing about 10^8 bacteria / vial. First of all, the initial light output of homogenized bacterial suspensions is determined; then, appropriate aliquots of osmotically adjusted samples and sample dilutions are added to the bacterial suspension and light measurements are performed at specific intervals (generally at 5 and 15 minutes) after exposure to test samples. The blank is used to correct time-dependant change in light output.

The test endpoint is measured as the EC_{50} , which is calculated by log linear plotting the Concentration (C) vs. % Light Decrease (% Δ), or, more precisely, by plotting the corrected ratio of the amount of light lost to the amount of light remaining, namely Gamma (Γ), versus Concentration on a log-log graph. In practice, various computer programs and data reduction systems are used for calculating Gamma and corresponding EC_{50} values. Given the high sensitivity of *V. fischeri* towards

a large spectrum of toxic substances, the EC₅₀ values of metals and non-metals were commonly obtained by using the Microtox® and also other similar products like ToxAlert® and Lumistox® bioassays (Jennings, 2001).

Besides that, earlier works investigated the effects of heavy metals and organic pollutants on the light emission of luminescent bacteria other than *V. fischeri* (Deheyn, 2004). Several of these bacterial strains showed exclusive performances in terms of luminescence, life-time, and sensitivity (Tsybulskii, 2010). These findings supported the idea to effectively use alternative strains, especially for on-line applications.

In accordance with this alternative trend, a long term industrial research and development program was carried out in Syntea laboratories, in order to identify, characterize, stabilize and lyophilize an environmental strain of *Photobacterium phosphoreum*, to be used in the Easychem Tox On-line toxicity assay. This strain is also the reference organism for Asian standards (GB/T 15441 1995).

2.1.6. Fluorescent Algae Toxicity Test

The use of unicellular micro-organisms like algae and bacteria has already proven useful in evaluating water toxicity. In particular, algae have shown to be very sensitive to pesticides and herbicides, even those found in small concentrations. Herbicides represent a specific class of pesticides employed in the chemical weeding of various crops. The most commonly used in agriculture are urea derivatives, triazines, diazines and phenols, acting as inhibitors of photosynthesis. In particular, triazines (atrazine, simazine, terbuthylazine etc.) and ureas (linuron, diuron, etc.) from agricultural runoff can contaminate soils, surface and ground waters with severe toxic effects on humans. Moreover, their widespread use may lead to the natural selection of resistant weed species, in turn requiring higher use of herbicides in agriculture. Since the determination of these contaminants has been a growing concern of agriculture and health care professionals and regulatory agencies, and since the commonly used high-performance-liquid chromatography (HPLC) or gas chromatography coupled with mass spectrometry (GC-MS) techniques require time-consuming methods and expensive equipment for preconcentration, algae-based biosensors could serve as an easy and cost efficient way to perform a qualitative pre-screen test.

Owing to their ubiquity, short life cycles, easiness of culture and high sensitivity to a number of pollutants, unicellular algae are frequently employed in ecotoxicological screening of contaminated freshwater (Sanchez-Ferandin, 2013), environment and agrifoods (Lavecchia, 2013).

Specifically, considering that a large number of pollutants are capable of affecting photosynthetic activity, Photosystem II (PSII)-based biosensors are reported to be able to detect herbicides in the

environment (Giardi, 2001). Photosynthesis inhibition is an interesting indicator that rapidly reflects the toxic effect of pollutants: about 30% of herbicides, including derivatives of phenylurea, triazine, and phenolic herbicides, are targeting the vegetal PSII (Moreland, 1980 and Draber, 1991); these substances inhibit photosynthetic electron flow by blocking the PSII quinone binding site and thus modify the emitted Chlorophyll fluorescence, by giving responses which vary in a concentration-dependent manner.

2.1.6.1. Photosynthetic activity – inhibition test

When photosynthetic organisms are illuminated, the absorbed radiation energy is transformed, through several physical and chemical processes, into chemical energy. However, not all absorbed energy follows this fate, the excess being dissipated in a radiationless manner as heat or reemitted as fluorescence. Fluorescence spectrometry allows the measurement of the fraction of the absorbed photosynthetically active radiation not used for charge separation in the reaction centre; therefore, it is usually considered an inverse measure of the activity and functional state of the whole photosynthetic process.

In particular, for biosensor-based analyses it is important to consider the modification in the Chlorophyll a fluorescence curve due to the presence of the analyte. Changes in the yield of Chlorophyll fluorescence were first observed as early as 1960 by Kautsky and co-workers (Kautsky *et al.*, 1960). They found that upon transferring photosynthetic material from the dark into the light, an increase in the yield of Chlorophyll fluorescence occurred over a time period of around 1 second (Fig. 3a). According to Kautsky's effect, when a dark-adapted photosynthetic sample is illuminated by continuous light, PSII reaction centres are progressively closed. This gives rise to an increase in the yield of Chlorophyll fluorescence, which rises rapidly from the initial minimal level (O) to a maximum (P). Following on from this, the fluorescence level typically starts to fall again (S) over a log time-scale of a few minutes, and finally reaches a terminal steady-state level (T), (Fig. 3b).

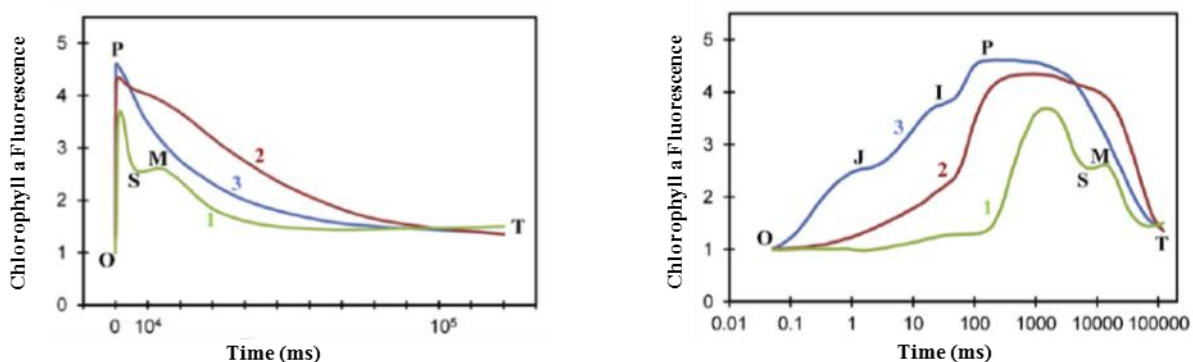


Figure 3: Chlorophyll a fluorescence induction transients on a linear (a) and logarithmic time scale (b) (Stirbet, 2011).

The fast increasing part of the curve appears to be an essential tool to study activity and integrity of the photosynthetic apparatus under different stress conditions, being very sensitive to any changes in the electron-transfer reactions on both the donor and the acceptor side of PSII. When considering the *O-J-I-P* polyphasic transient, there is a sub-effect on the Fluorescence/Time curve caused by the interference of herbicides (*Fig. 4a*). This effect can be normalized with respect to valley point *O* and peak point *P* to produce the curves shown in *Fig. 4b*, whose features can be measured.

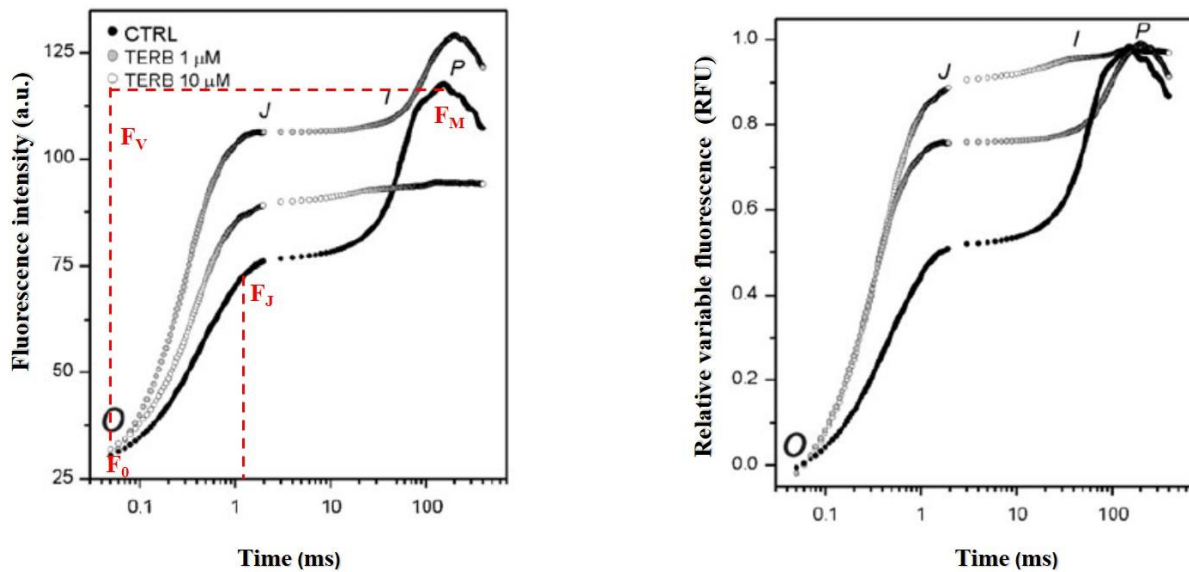


Figure 4: The *OJIP* transients of Chlorophyll *a* fluorescence both in absence (black circle) and in presence of herbicide (grey and white circles) (a) and Normalized Fluorescence transient respect to F_0 (b) (Giardi, 2009).

In order to study fluorescence changes, some important parameters have to be evaluated:

- F_0 : minimal fluorescence, related to the activity of the PSII light harvesting structure. The F_0 level is achieved by dark adaptation of the sample and it is reached after some nanoseconds from the start of light excitation.
- F_M : maximal fluorescence, the maximum intensity of the fluorescence transient, measured when all reaction centres of PSII are closed. It is obtained by a pulse of saturating light and normally it is reached after hundreds of milliseconds to several seconds from light excitation.
- F_J : fluorescence at the J region of the curve at about 2ms.
- F_v : variable fluorescence, the difference between the fluorescence coming from closed and open reaction centres ($F_v = F_M - F_0$). It represents a relative measure of the photochemically active reaction centres.

- F_v / F_m : maximum quantum yield of the PSII photochemical reaction. It is calculated in dark-adapted samples and it is useful on plant vitality analysis since its decline indicates a loss of photochemical efficiency, which is, in turn, symptomatic of the effect of various environmental stresses.
- V_j : relative variable fluorescence, calculated as $V_j = (F_j - F_0) / (F_m - F_0)$. It is for a parameter that allows estimating the inhibitor strength and consequently detecting the presence of herbicides acting on QA pocket binding site.

2.1.6.1. ISO 8692: Freshwater algal toxicity assay

The European standard for algal tests is based on monospecies green algae belonging to the order of *Sphaeropleales* (*Desmodesmus subspicatus* or *Pseudokirchneriella subcapitata*). This strain is cultured for several generations in a defined medium containing a range of concentrations of the test sample, prepared by mixing appropriate quantities of growth medium, test sample, and an inoculum of exponentially growing algal cells. The test batches are incubated for a period of 72 ($\pm$ 2) h during which the cell density in each test solution is measured at least every 24 h. Inhibition is measured as a reduction in specific growth rate relative to control cultures grown under identical conditions.

2.1.7. **New trend: On-line biomonitoring systems**

Both algal and bacterial assays are highly relevant as they measure the combined toxicity of all the toxic compounds present in a complex sample. They have been applied frequently to monitor toxicity in several environmental applications like wastewater, seawater, surface and ground water, tap water, soil and sediments (Wang, 2014, for bacteria and Buonasera, 2010 for algae) and they were both selected for this research project.

However, being based on a strategy including sampling on site, transport to the laboratory, and then manual analysis, this offline scheduling is still not effective in timely detection of unpredictable, sudden quality changes as a result of pollution events. Moreover, laboratory methods are based on discontinuous sampling, so they provide only a partial response in terms of compliance with WFD and related legislations. These performances can only be successfully achieved by operating with on-line monitoring systems. Automated, standardized and well validated tests employing bacteria or algae as substrates are therefore good candidates to be used as an initial screening test that enables timely recognition of highly toxic samples.

On-line biomonitors are the latest technological advancements in the field of environmental toxicity monitoring. They run continuously and in real time, and usually the user has remote connection for attending the instrument and watching the data. Furthermore, the maintenance should be performed only once per week, or, in some cases, less frequently.

2.1.7.1. On-line bacteria – based biomonitors

Automated systems which continuously monitor water toxicity by means of luminescence measurements already exist. Analysers such as TOXcontrol (Microlan B.V.) or Microtox® CTM (Modern Water) are the most recent early warning systems, which employ *V. fischeri* as biomonitor. The former includes two chambers where luminescent bacteria are separately mixed with both samples and water reference. The luminescence is measured in both cases and toxicity is quantified as comparison between blank and sample. Microtox® CTM is a flow system, including a bioreactor for cultivating and storing bacteria in refrigerated conditions. In the TOXcontrol configuration, the use of a dual-channel apparatus suffers from limited analytical frequency since sample analysis is intermittent and give only one test result in typically 15 - 30 minutes whereas, in the case of CTM, the use of continuous flow technology poses the risk of cross-contamination and bio fouling episodes.

2.1.7.2. On-line algae - based biomonitors

Some examples of algae-based on-line systems are Algae Toximeter II (BBE-Moldaenke) and Fluotox (Ifetura-eu). The former is based on cultures of green alga *Chlorella vulgaris*, which are continuously grown in a fermenter, regulated by means of a second fluorescence measurement. Since Chlorophyll a photosynthetic activity is measured within 10 minutes, the system functions as early warning, by activating an alarm above a pre-defined threshold. Differently, Fluotox analyser employs microscopic Chlorophyllian algae which emit fluorescence, when they are excited. The presence of pollutants is linked to the reduction of the emitted fluorescence, caused by the inhibition of photosynthesis. These systems result very sensitive to trace of herbicides, even if both of them are based upon flow technologies, thus requiring up to 30 mL of sample and constant maintenance.

2.1.8. **On-line chemical measurements in wastewater**

A sensitive, accurate and rapid analysis of major inorganic substances and metals in water distribution systems and municipal and industrial wastewater facilities, is essential for monitoring and maintaining healthy aquatic environments.

High concentration of nutrients in water bodies can potentially cause eutrophication, stimulating excessive plant growth and so reducing the concentration of dissolved oxygen. Nutrients can come from several sources, such as fertilizers applied to agricultural fields, golf courses and suburban lawns, erosion of soil and wastewater treatment plant discharges. Nutrients found in wastewater generally include nitrate, nitrite, ammonia, organic nitrogen and phosphate. Traditional methods for quantifying these nutrient concentrations often require laborious techniques or expensive specialized equipment, making these analyses difficult. Many online probes able to measure

chemical-physical properties as turbidity, temperature, chlorine or pH are commonly used; others, such as those for nitrate, Phosphorus or other nutrients analyses, have been also validated.

Heavy metals are common industrial by-products and generally these substances enter the environment as a result of wastewater discharges from cleaning, plating and other industrial operations or after being leached from industrial waste piles. Several heavy metals such as chromium, copper, lead, mercury and zinc are listed as priority pollutants by EPA (EPA 2014) and require monitoring. Although inductively coupled plasma atomic emission spectroscopy (ICP-AES) is the typical method of metal analysis required for compliance reporting and other official purposes, this method is not practical for continuous on-line monitoring in multi-constituent media due to sample preparation requirements and apparatus that can handle only a limited number of samples. The development of simple, inexpensive, reliable and rapid methods for measuring metals in water samples is highly desirable: in this respect, photometric methods are convenient, attractive, and also rely upon more simple principles, avoiding the aid of any advanced instrument.

The original idea supporting this part of the thesis is that, similarly to what is commonly done in clinical analysis, also environmental photometric determinations can be automated within a discrete analysis system. With one single analyser, a large number of different parameters can be simultaneously determined. The colour reactions take place in reaction cells, which usually are cuvettes, within an incubator. For each determination, a separate reaction cell is used. Preset volumes of the sample and the reagents are pipetted into the cells and mixed. After expiry of the incubation period, the absorbance of the solution is measured at the specific wavelength. This is done by passing the cuvettes through the photometer or by transferring the measuring solution from the reaction cells to a photometer with a flow-through cell.

2.1.9 Aim

Within these premises, one of the main scopes of this thesis was the improvement of new monitoring tools in order to rapidly and autonomously detect any alteration in water quality, by combining analytical chemistry characterization and ecosystem safety evaluation within the same device. The research was focussed to the development and testing of a new robotic system, Easychem Tox On-line, able to automatically perform both chemical measurements and toxicity tests, namely for determining causes and effects of contamination.

2.2 Materials and Methods

2.2.1. Apparatus: EasychemTox On-line analyser

Easychem Tox On-line (*Fig. 5*) is a multi-parameter random access monitoring system based on a robotic technology, commonly housed in an industrial cabinet and equipped with colour touchscreen LCD and a mechanical arm for aspiration, transferring, and dispensing of reagents and samples. The arm is provided with liquid level sensing, automated probe washing and sample pre- and post-dilution utilities. Aspiration volume ranges from 2 to 330 μL with 1 μL increment and ± 0.1 μL maximum errors.



Figure 5: Final assembly of Easychem Tox On-line.

The main body (*Fig. 6*) is a 71 (W) x 50 (H) x 40 (D) cm aluminium plate comprising: (i) on the left, a 36 position refrigerated reagent compartment; (ii) in the right centre, a sampling area containing five overflow wells; (iii) in the front left, an automated opener of bacterial vials; (iv) in the left centre, a sampling arm and related washing well; (v) in the front middle left, a 3-position cooled bacterial compartment; (vi) in the middle-back, a thermostated and lighted algal

compartment; (vii) in the front right, a Blank Position for dilution water; (viii) on the right, a reaction plate incorporated with an automated cuvette washing station, a luminometer, a fluorimeter and a colorimeter.

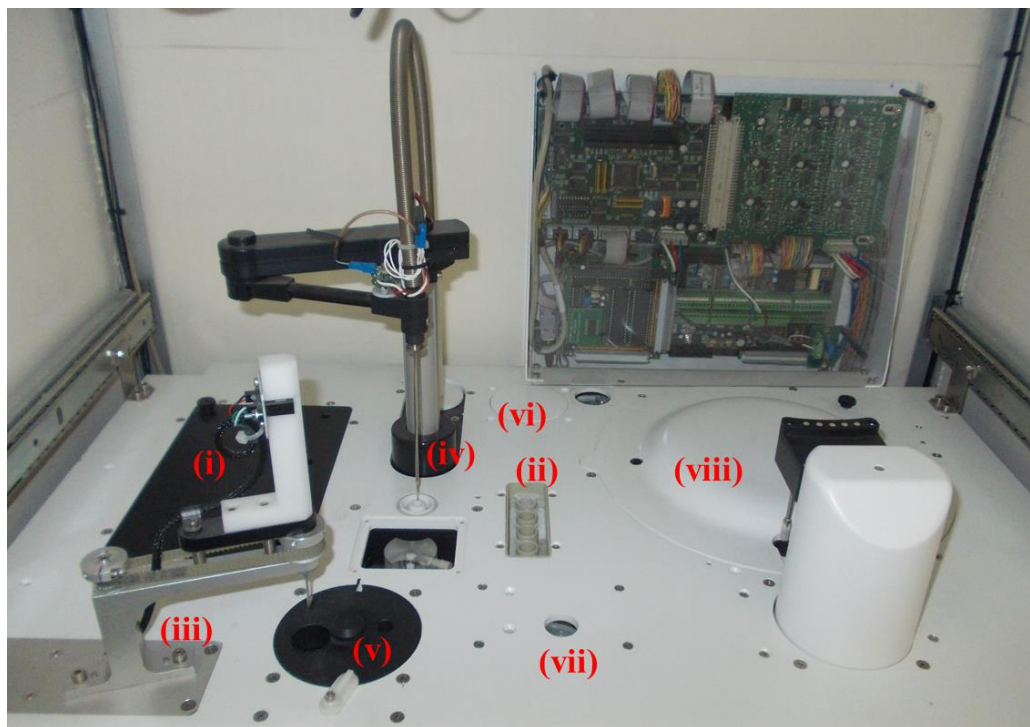


Figure 6: Main body of Easychem Tox On-line: aluminium plate comprising: (i) on the left, a 36 position refrigerated reagent compartment cooled to 10 - 15 °C; (ii) in the middle, a sampling area containing five overflow wells; (iii) in the front left, an automated opener of bacterial vials; (iv) in the left centre, a sampling arm and related washing well; (v) in the front left side of the centre, a 3-position cooled bacterial compartment; (vi) in the back side of the centre, a thermostated and lighted algal compartment; (vii) in the front right side of the centre, a Blank Position for dilution water; (viii) on the right, a 80-position thermostated cell reaction plate incorporating a luminometer, a fluorimeter, a colorimeter and an automated cuvette washing station.

(i) The reagent plate is a refrigerated plate consisting of two reagent racks which are able to turn around their own axis through different stepper motors, which provide high precision rack positioning. The two reagent racks are universal racks for standard, control and reagent positioning containing up to eight 50-mL positions or sixteen 20-mL positions each. Four extra peripheral fixed positions for 50-mL bottles are located on the right side for containing extra-toxicity buffers, if necessary.

(ii) The sampling area consists of five overflow wells to which samples are flowed via a peristaltic pump. Optionally, any of the wells can be directly connected with different pre-treatment options covering most of common applications, including homogenization, high temperature and UV digestion, filtration, distillation and dialysis.

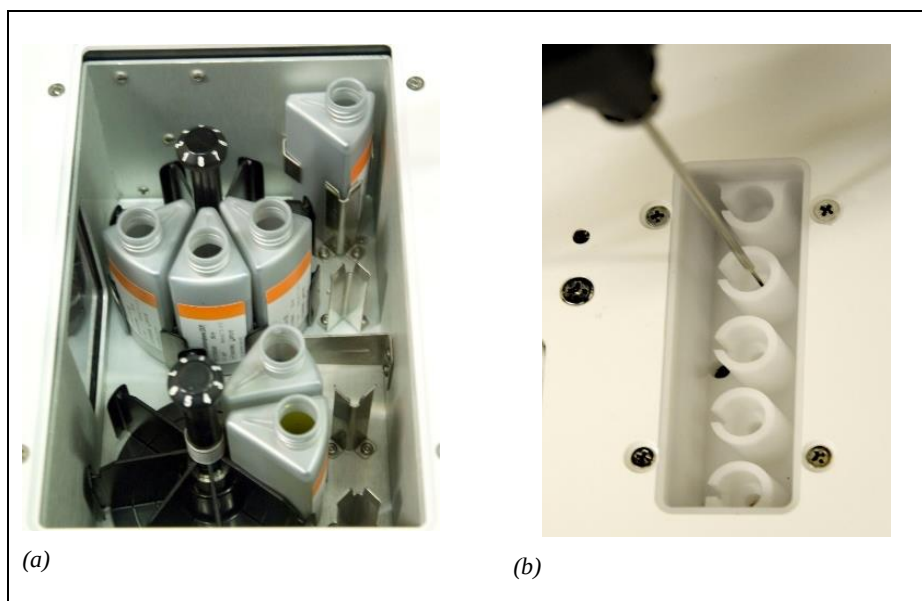


Figure 7: Refrigerated reagent plate (a) and sampling area consisting of five overflow wells (b) in the analyser.

(iii) The bottle opener, familiarly known as the “corkscrew”, is a mechanical arm ending in a sharp harpoon to allow automated opening of bacterial vials through a vertical movement into the bacteria plate. The opener can allocate up to two vial stoppers.

(iv) The sampling arm is a programmable robotic mechanical arm able, by means of two stepper motors, to execute extending and rotary horizontal and vertical movements, with respect to the analyser. It performs all sampling operations, including aspiration, transferring and dispensing of reagents, microorganisms and samples, by moving in and out from the reagent plate (6 sampling positions), the sample plate, the bacteria or algae plate, the blank position, the reaction plate and the washing well. Aspiration volume ranges from 2 μL to 330 μL with 1 μL increment and $\pm 0.1 \mu\text{L}$ maximum errors. The device is actuated through the movement of a unique central piston for the operations of liquid aspiration and delivery.



Figure 8: Automated bottle opener in the left part and sampling arm in the back.

- (v) The bacterial compartment is refrigerated at 4 - 6 °C by a Peltier cell and can contain up to three different 25-mL bacterial vials positioned on a motorized rotor.
- (vi) The algal compartment is a thermo controlled and lighted reactor for the supporting of algal solutions. It can contain a 100-mL test tube which is constantly lighted with LED diodes and maintained at 25 °C.
- (vii) The blank position is dedicated to contain milliQ-grade DI reference water for performing blank measurements or sample dilutions in toxicity tests.

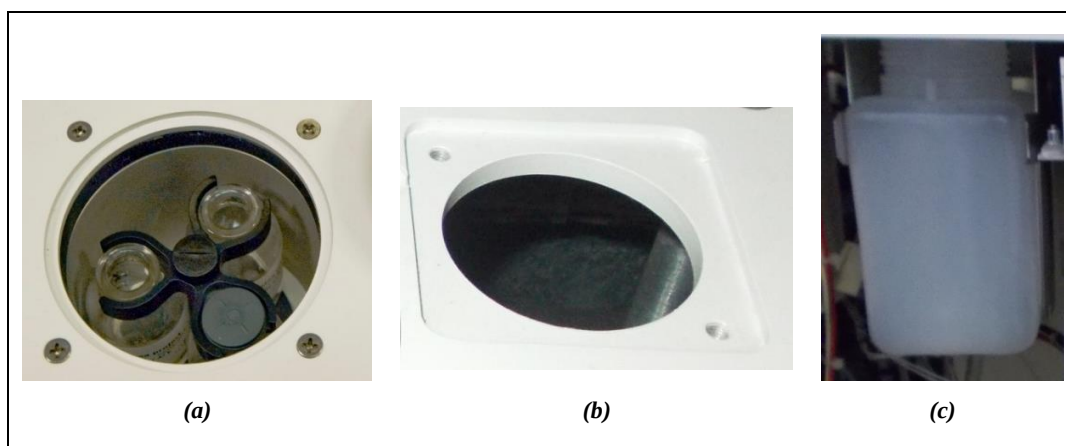


Figure 9: Refrigerated bacteria compartment (a), thermocontrolled algae plate (b) and DI grade water container for blanks run (c) within Easychem Tox On-line.

- (viii) The reaction plate contains four disposable racks with 20 reaction cuvettes each, for a total of 80 cuvettes. Each of them has an optical path of 6 mm. The plate can rotate through a stepper motor which assures a high precision of positioning, superior to $\pm 0.1^\circ$. The incubation temperature

(from 15 to 45 °C according to necessity) is controlled by an external Refrigeration Module and an internal Heating System. The reaction plate includes a luminometer, a fluorimeter and a colorimeter integrated with a washing station.

The luminometer is a direct reading device, encompassing a photomultiplier, which allows the measurement of the luminescence emitted by the bacteria in any cuvette of the Reaction Plate.

The fluorimeter comprises three excitation light sources with different wavelengths and the respective detectors are placed at 90 ° with respect to the light sources.

The spectrophotometer is a direct reading, dual channel device supplied with 9 narrow band automatically selectable interferential filters and providing linear results from 0.0001 to 4.2000 Absorbance units. The direct reading of the Optical Density (O.D.) is allowed for any reaction cuvette. The light source is a halogen lamp (6 V / 10 W) powered by a Photometer Lamp Board. The device contains a 9-position Filter Wheel for optical measurements at different wavelengths controlled by a home sensor that guarantees high-precision positioning. Equipped with dark position, the Filter Wheel is controlled by a stepper motor to allow the filter selection.

The washing station allows to empty, wash and dry the reaction cuvettes, after any measurement. The apparatus consists in a series of five small probes, including a drying pad, each of which enters a different cuvette for performing a cleaning step (empty, wash or dry). The vertical motion is allowed by an arm connected to a stepper motor.

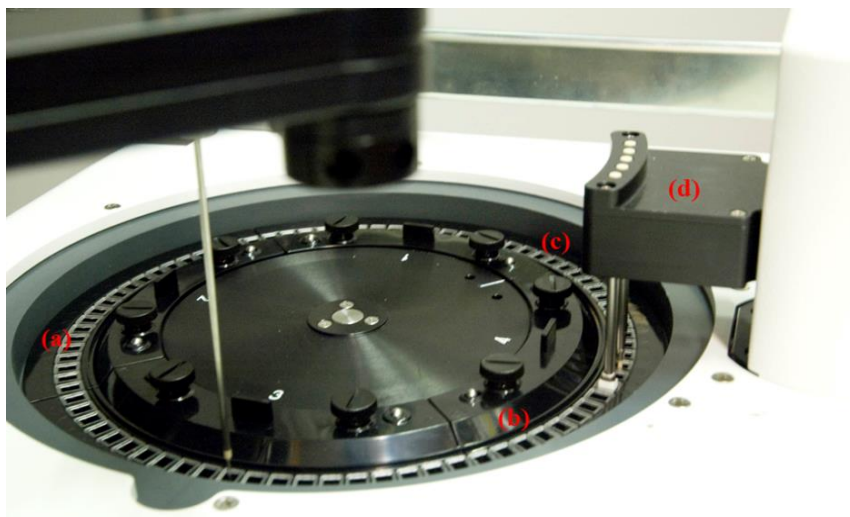


Figure 10: Reaction plate, consisting of 80 500-μL cuvettes and comprising luminometer (a), fluorimeter (b), spectrophotometer (c) and washing station (d).

The user interacts with the analyser, in laboratory or in the field, by using Windows based software on the PC. The software has specifically been designed for Easychem TOX on-line operations using the most recent version of Visual Basic, and allows the complete remote control and management

by a GSM / GPRS device. The instrument is equipped with local data storage, serial RS-232 communication, separated analog outputs 4-20 mA, remote data storage on a web server and remote data transmission via ftp, http and SMS. An alert service is at the user's disposal for giving immediate notice of detected non-compliances.

2.2.2. Bioluminescent Bacteria Toxicity Test performed within Easychem Tox On-line

2.2.2.1. Bacteria

The vials containing freeze-dried *P. phosphoreum*, an environmental marine strain, directly isolated from the Mediterranean Sea, were prepared by Systea laboratories, according to proprietary protocols. The freeze-drying process was developed by using a freeze dryer according to the following procedure: the bacterial culture was centrifuged and resuspended in a specific suspending fluid, including for the 96 %, sugars and pH stabilizers. Then 1 mL of the suspension was placed in small vials and kept at -80°C for at least 3 h. The first freeze-drying step required that the sample be brought to -60°C for approximately 24 h under vacuum, whereas the second dehydration step took 24 h.

In order to increase the production of freeze-dried bacteria, the conditions were set up to perform preparations on an industrial scale (*Fig. 11*). The culture conditions were the same as for the laboratory scale, except that the volume was increased to 10 L by using a 20-L bioreactor with the following operating conditions: temperature 22°C, 400 rpm, 12 L / min air. When light emission reached the maximum level, the biomass was collected by centrifugation at 4000 $\times g$, and the wet pellet of cells (3.5 g / L) was suspended in the suspension fluid at the ratio 1 g : 20 mL. One millilitre of bacterial suspension was dispensed into vials and freeze dried for 36 hours. The vial format used was 25 mL vial.



Figure 11: 20-L bioreactor for producing *P. phosphoreum* on industrial scale (a) and continuous greenish-light, coming from the cultivated light-emitting bacteria (b)

Artificial sea water for the reconstitution and dilution of lyophiles was prepared as suggested by ISO Standard 11348-2009 (*Table 3*), whereas the osmotic protection of the marine organisms was assured by the addition to non saline samples of Acute Test Buffer, prepared at the Systea laboratories. This information is classified.

Table 3: Composition of Artificial sea water.

Composition	Amount (g/L)
NaCl	22
KCl	0.65
CaCl ₂	1.0
Na ₂ SO ₄	3.7
MgCl ₂ *6 H ₂ O	9.7
NaHCO ₃	0.20
MgSO ₄ *7 H ₂ O	0.35
H ₃ BO ₃	0.023

2.2.2.1. Quality control solutions

Chemicals used as standard reference compounds, zinc sulphate and chromium dichromate, were purchased from Sigma-Aldrich (St. Louis, MO, USA) and were of the highest purity grade available; 99 % pure 3,5-dichlorophenol (DCP) was purchased from Acros Organics (Basel, Switzerland).

Zinc sulphate was used as reference standard in all assays. A nominal stock solution containing a known weight of 51.56 mg / L of zinc sulphate heptahydrate, corresponding to 11.73 mg / L of zinc ion, was prepared and stored at 4 °C in the reagent plate within the analyser. The pH of this standard solution was not adjusted and it was used as quality control at a final [Zn²⁺] of 2.2 mg / L, expected to cause approximately 50% inhibition after 30 min contact time in each assay.

A nominal stock solution containing a known weight of 282.19 mg / L of potassium dichromate, namely 99.70 mg / L of chromium (VI) ion was prepared and stored at 4°C in the reagent plate within the analyser. The pH of this standard solution was not adjusted and it was used as quality control at a final [Cr⁶⁺] of 18.69 mg / L, expected to cause approximately 50% inhibition after 30 min contact time in each assay.

A nominal stock solution containing a known weight of 18.13 mg / L of 3,5-dichlorophenol was prepared and stored at 4°C in the reagent plate within the analyser. The pH of this standard solution was not adjusted and it was used as quality control at a final [DCP] of 3.38 mg / L, expected to cause approximately 50% inhibition after 30 min contact time in each assay.

2.2.2.3. Automated cycle of analysis

The process for automatically analysing and monitoring aqueous samples within Easychem® TOX on-line, comprises the following steps.

- The aqueous samples are transferred from a water supply or drainage network into the sample wells of the analyser, by means of a peristaltic pump.
- The *P. phosphoreum* vial, previously located within the refrigerated bacteria compartment by the user, is automatically opened by the corkscrew and immediately rehydrated. The User can select the rehydration volume, which is generally between 15 and 22 mL. The needle samples small aliquots of reconstitution solution from a bottle stored in the reagent plate and releases them within the vial which is externally agitated after every addition, in such a way to mix well the bacterial suspension. When the rehydration step ends, bacteria are ready to use for toxicity tests. They can keep a measurable luminescence emission for ten days, if stored at 4 °C.
- According to the analytical cycle, an equal volume of blank water or sample is mixed with the Test Buffer within the reaction cuvettes in order to osmotically adjust them. Blanks are always performed at the beginning and the end of the toxicity test. All samples are performed in duplicate.
- Bacteria (20 µL) are added to blanks and samples in a final volume of 320 µL after 5 minutes from the test beginning. The amount of bacteria added per run is about 10^6 cells / mL (*Fig. 12*). Temperature in the reaction plate can be set at any value comprised between 15 and 25 °C according to ISO 11348-3 2009 and GB/T 15441 1995 respectively.
- The test system measures the emitted light every 40 seconds during the exposure time and compares it to the luminescence of a blank control (bacteria plus deionized water, osmotically adjusted by the sodium chloride buffer). Owing to this, it is possible to follow, in real time, all luminescence kinetics.
- At the end of the analysis, all cuvettes were automatically emptied, washed out three times and dried out.
- The percent of inhibition at the time t (H_t) is obtained by calculating, for the two replicates, the average percent decrease with respect to blank control average luminescence.
- Samples can also be analysed in dilution mode: in such case, the system automatically performs the sample dilutions within the sampling arm, by aspirating volumes of sample and blank water according to the suitable dilution ratio.
- Concurrently, a Quality Control (QC) cycle is commonly run with the test. Since the bacterial light emission normally changes with time, for reasons other than the bioreactivity of the test sample, by using the QC it is possible to keep bacterial sensitivity and luminescence under

control. In the QC cycle, blank water and reference standard are mixed with the Test Buffer within the reaction cuvettes in order to osmotically adjust them. The analysis starts and ends with a blank run such to minimize the influence of bacterial variability, and in between blanks, every control is performed in duplicate. As for the analytical cycle, in the quality control cycle, bacteria are added in reaction cuvettes after 5 minutes and bioluminescence is measured every 40 seconds during the 15 or 30-minute incubation period.

- Required maintenance operations normally include loading of bacterial vials, refill of cleaning and dilution water tanks, replacement of buffer and standard solutions.

Chemistry Definition

Description : Tossicita acuta
Unit :
Decimals :
LIS Code :
Unit Factor :
Slope :
Intercept :

Reaction Type : Toxicity
LED power : ☐ Fluorim.
Direction :
E.P. Limit(Abs) :
Depl.Limit(Abs) :
First Limit(Abs) :
Linear. Factor :
Fit :
RBL Min (Abs) :
RBL Max (Abs) :
RBL Replicates :

Volumes and Time

	Predilution	C+Water	Buffer	Bacteria	Incubation	Read	Special Wash
Times (Sec):				280	1800	40	
Dil./Rgt Code:		ACQUA	ACTOX	LOT44			
Ratio/Vol(μl):	1/1		90	20			0
Rinse (μl):							
Sample (μl):		210					

RBL Stab. (Days) :
Calib. Stab. (Days) :
RBL Max CV% :

Calculation Model
Factor :
Sample Blank :
Min Cal. Factor :
Max Cal. Factor :

Figure 12: Method window of the Easychem Tox On-line dedicated software: reagents, volumes and times are set up for performing automated bacteria toxicity tests.

2.2.3 Fluorescent Algae Toxicity Test performed within Easychem Tox On-Line

2.2.3.1. Algae

Wild type strains of *C. reinhardtii* were grown and stabilized at the Biosensor laboratories (Formello, RM, Italy) using a well-defined protocol. Being a soil organism, it can be grown in laboratory either in liquid culture or on agar in simple mineral salts.

In particular, for this thesis, liquid cell cultures of *C. reinhardtii* were grown in Tris-acetate-phosphate (TAP) medium, pH 7.0÷7.2, with white-light neon lamps at $50 \mu\text{mol m}^{-2}\cdot\text{s}^{-1}$, 25°C temperature, and 150-rpm agitation. Cells were harvested in the mid-exponential growth phase (up to $2\text{-}3\cdot 10^7$ cells per milliliter), and growth curves were performed at regular intervals. At a specific point of the growth curve, corresponding to a 0.5 optical density (OD) measured by UV at 750 nm, whole cells were collected and utilized at the specific concentration (Fig.13).



Figure 13: *C. reinhardtii* liquid culture performed in Tris-acetate-phosphate (TAP) medium.

2.2.3.2. Reference toxic substances

Analytical-grade Linuron [3-(3,4-Dichlorophenyl)-1-methoxy-1-methylurea], MW 249.09 g / mol, was purchased from Sigma-Aldrich (Steinheim, Germany).

A nominal stock solution, containing a known weight of 0.0623g Linuron, was prepared in a 1000 mL of 50 mM ethanol solution and used as reference toxic for the automatic analyses.

Analytical-grade Diuron [3-(3,4-Dichlorophenyl)-1,1-dimethylurea], MW 233.09 g / mol, was purchased from Sigma-Aldrich (Steinheim, Germany).

A nominal stock solution, containing a known weight of 0.0233 g Diuron, was prepared in 1000 mL of 50 mM ethanol solution and further diluted for preparing reference toxic solutions.

2.2.3.3. Manual herbicide test with freshwater *C. Reinhardtii* suspension

Being the standard freshwater algal toxicity assay based on long-lasting procedure, and keeping in mind the need of rapid-responses methods, a research group working in National Council of Research in Rome (Department of Agrofood, CNR, Institute of Crystallography, Rome) developed a new line of fluorescence-based biosensors. In particular, photosynthetic living sensors such as the green microalgae *Chlamydomonas reinhardtii* and thylakoids of spinach were selected for monitoring pollutant presence in water, environment and agrifood products (Buonasera et al., 2010). Among the photosynthetic microorganisms, *C. reinhardtii* is commonly used as sensing element for the development of optical and amperometric biosensors. It is a 10- μ m diameter, unicellular green

alga, which swims by two flagella. It has a cell wall made of hydroxyproline-rich glycoproteins, a large cup-shaped chloroplast, a large pyrenoid, and an "eyespot" that senses light. Although widely distributed worldwide in soil and fresh water, *C. reinhardtii* is primarily employed as a model organism in biology and in the bio-sensoristic field, since it can grow both under illumination in media lacking organic carbon and chemical energy sources, or in dark when supplied with these. (Nickelsen, 2005).

A new rapid assay based on *C. reinhardtii* was, therefore, improved by the CNR research group: Chlorophyll a fluorescence was measured on *C. reinhardtii* wild-type strain after 10 min of incubation with white light at $50 \mu\text{mol m}^{-2}\cdot\text{s}^{-1}$, 25 °C temperature, and 150 rpm agitation, in the absence and in the presence of herbicides. Prior to each measurement, samples were kept in the dark for 10 min to reach the steady dark adapted state of PSII. The fluorescence excitation was carried out for 5 s at 650 nm wavelength by LED sources (1,500-mcd intensity) and the fluorescence emission was recorded at 720 nm by a photodiode. The total fluorescence measurement was completed in 20 s, resulting significantly faster than the laboratory chromatographic methods.

2. 2.3.4. Automated analytical cycle within ECT OL

For performing algal toxicity tests within the robotic system, *C. reinhardtii* IL strains were grown and stabilized offline according to the defined protocol.

In the automated algal toxicity assay, within Easychem Tox On-Line, the following steps take place.

- Water samples are transferred from a water supply into the sample wells of the analyser by a peristaltic pump.
- 75 mL of freshwater algal suspension is stored in the 100 mL appropriate container within the algal compartment and maintained at 25 °C under constant illumination.
- 300 μL of reference water and 100 μL of *C. reinhardtii* suspension are simultaneously added in a reaction cuvette to run "Blank 1".
- 300 μL of sample and 100 μL of *C. reinhardtii* suspension are simultaneously added in a reaction cuvette to run "Sample 1".
- 300 μL of sample and 100 μL of *C. reinhardtii* suspension are simultaneously added in a reaction cuvette to run "Sample 2".
- 300 μL of reference water and 100 μL of *C. reinhardtii* suspension are simultaneously added in a reaction cuvette to run "Blank 2".
- All blanks and samples are incubated in the dark for 15 min at 24°C (ISO 8692, 2012).

- The test system sequentially measures Chlorophyll a fluorescence induction transients of blanks and samples under 650 nm excitation wavelength. Each fluorescence transient is formed by 2,000 points measured from 0.01 to 10,000 milliseconds after excitation (*Fig. 14*).
- At the end of the analysis, all cuvettes are automatically emptied, washed out three times and dried out.
- After curve normalization, the software converts the photosynthetic efficiency loss due to algal exposition to samples, in inhibition values via a specially designed algorithm.
- Samples can be analysed in dilution mode: in such case, the system automatically performs the sample dilutions within the sampling arm, by aspirating volumes of sample and dilution water according to the opportune dilution ratio.
- Required maintenance operations include algae substitution, refill of cleaning and dilution water tanks and replacement of standard solutions.

Chemistry Definition

Description : Algal Toxicity
Unit :
Decimals :
LIS Code :
Unit Factor :
Slope :
Intercept :

Reaction Type : Fast Fluorimetry
LED power : ☐ Fluorim.
Direction :
E.P. Limit(Abs) :
Depl.Limit(Abs) :
First Limit(Abs) :
Linear. Factor :
Fit :
RBL Min (Abs) :
RBL Max (Abs) :
RBL Replicates :

Volumes and Time

	Predilution	C+Water	Buffer	Algae	Incubation	Read	Special Wash
Times (Sec):					900	30	
Dil./Rgt Code:		ACQUA		ALGHE			
Ratio/Vol(μl):	1/1			100			0
Rinse (μl):							
Sample (μl):		300					

RBL Stab. (Days) :
Calib. Stab. (Days) :
Automatic QC interval : Never
RBL Max CV% :

Calculation Model

Factor :
Sample Blank :
Min Cal. Factor :
Max Cal. Factor :

Figure 14: Method window of the Easychem Tox On-line dedicated software: reagents, volumes and times were set up for performing automatic algae toxicity tests.

2.2.4 Chemical parameters within Easychem Tox On-Line: nutrients and heavy metals

2.2.4.1. Chemicals substances

All chemicals were of reagent grade and were used without further purification. Potassium dichromate, 1,5-Diphenylcarbazide, Cupric sulfate pentahydrate, Trisodium citrate dehydrate, Bathocuproinedisulfonic acid disodium salt, Hydroxylammonium chloride, Ammonium sulphate, Sodium citrate dihydrate, Sodium phosphate dibasic, Sodium hydroxide, Sodium salicylate, Sodium

nitroprusside dihydrate, Sodium hypochlorite, Sodium nitrite anhydrous, Sodium nitrate anhydrous, Hydrazine sulfate, Sulfanilamide, *N*-(1-Naphthyl)ethylenediamine·2HCl, Potassium hydrogen phosphate anhydrous, Sodium molybdate dehydrate, Antimony potassium tartrate, Sodium dodecyl sulphate, Ammonium molybdate tetrahydrate and Oxalic acid dihydrate, 95 % Ethanol, 37% Hydrochloric acid and 97% Sulphuric acid and 85% ortho-Phosphoric acid were purchased from Merck (Darmstadt, Germany) and were of A.C.S grade; analytical grade Ascorbic acid was purchased from Prolabo (Milano, Italy).

2.2.4.2. Working reagents and working calibrants

All reagents and calibration solutions were freshly prepared prior to their use in automatic analyses, according to standard protocols. All the employed solutions and the corresponding preparation protocols are listed per parameter (*Table 4*).

Unless otherwise specified, solutions were made in Milli-Q grade DI water.

Table 4: List of working reagents and working standard employed for automated measurement of chemical parameters.

PARAMETER	REAGENTS AND STANDARDS
<u>Chromium Hexavalent as Cr⁶⁺</u>	R1: Sulphuric acid Solution R2: 1,5-Diphenylcarbazide Reagent Stock Standard: 100 mg Cr ⁶⁺ / L 0.283 g / L of Potassium dichromate Working Standard: 1 mg Cr ⁶⁺ / L
<u>Copper (Dissolved) as Cu²⁺</u>	R1: Hydroxylammonium chloride Reagent R2: Trisodium citrate Solution R3: Bathocuproine Reagent Stock Standard: 1000 mg Cu ²⁺ / L 3.929 g / L of Copper (II) sulfate pentahydrate Stock Standard: 50 mg Cu ²⁺ / L Working Standard: 500 µg Cu ²⁺ / L
<u>Ammonia as N-NH₃</u>	R1: Nitroprusside reagent R2: Oxidation Chlorine reagent Stock Standard: 1000 mg / L as N-NH ₃ 4.717 g / L of Ammonium sulphate Working Standard: 1 mg / L as N-NH ₃
<u>Nitrate as N-NO₃</u>	R1: Cupric sulfate Reagent R2: Hydrazine sulfate Reagent R3: N-(1-Naphthyl) ethylenediamine dihydrochloride Reagent Stock Standard: 1000 mg / L as N-NO ₃ 6.068 g / L of Sodium nitrate anhydrous Working Standard: 50 mg / L as N-NO ₃ ⁻
<u>Nitrite as N-NO₂</u>	R1: N-(1-Naphthyl) ethylenediamine dihydrochloride Reagent Stock Standard: 1000 mg / L as N- NO ₂ 4.93 g / L of Sodium nitrite Working Standard: 0.5 mg / L as N- NO ₂
<u>Phosphate as P-PO₄</u>	R1: Sodium molybdate and Antimony potassium tartrate Solution R2: Ascorbic acid Solution Stock Standard: 1000 mg / L of PO ₄ ³⁻ as P-PO ₄ ³⁻ 4.394 g / L of Potassium dihydrogen phosphate Working Standard: 5 mg / L of PO ₄ ³⁻ as P-PO ₄ ³⁻
<u>Silicon as Si</u>	R1: Ammonium molybdate Reagent R2: Oxalic acid Reagent R3: Ascorbic acid Reagent Working Standard (Merck): 10 mg / L of SiO ₄ ⁴⁻ as Si- SiO ₄ ⁴⁻

2.2.4.3. Colorimetric standard methods

The following standard methods were applied for the determination of chemical parameters.

The metals Cr (VI) and Cu (I+II) were analysed in compliance with APHA 1999. All nutrients were analysed in compliance with ISO 15923-1 2013.

Detection of Chromium(VI) according to SM 3500-Cr

Chromium may exist in water supplies in both the hexavalent and the trivalent state, although the trivalent form rarely occurs in potable water. The hexavalent chromium is determined colorimetrically by reaction with diphenylcarbazide in acid solution. A red violet colour of unknown composition is produced. The reaction is very sensitive, the molar absorptivity based on chromium being about $40,000 \text{ L g}^{-1} \text{ cm}^{-1}$ at 540 nm.

Detection of Copper(II) according to SM 3500-Cu E

Copper (II) is reduced to Cu (I) by hydroxylammonium chloride and analyzed using bathocuproine, which is a Cu (I)-specific sequestering agent. Bathocuproine binds specifically to the reduced form of copper, Cu (I), but not to the oxidized form, Cu (II). The Cu (I)–bathocuproine complex exhibits an absorption maximum at 480 nm.

Detection of P-Phosphorus according to ISO 15923-1

Phosphate reacts with molybdate and potassium antimony oxytartrate in an acid environment to produce the α -Keggin-type heteropolyoxometalate $\text{PMo}_{12}\text{O}_{40}^{3-}$. The complex thus formed is converted into a compound with a blue colour by reduction with ascorbic acid, and the absorbance at 880 nm is a measure of the orthophosphate content.

Detection of N-NO₂ according to ISO 15923-1

In an acid environment, nitrite reacts with sulfanilamide and naphthyl ethylenediamine (NED) to form a diazide compound with a red colour, whose absorbance at 540 nm measures the content of nitrite.

Detection of N-NO₃ according to ISO 15923-1

Nitrate is reduced to nitrite by an alkaline solution of hydrazine sulphate containing copper as a catalyst. Both the nitrite produced by this reaction and that present in the sample react with sulfanilamide and *N*-(1-naphthyl)-ethylenediamine dihydrochloride (NED dihydrochloride) to produce a reddish-purple azo dye at pH 2.0 to 2.5. The complex absorbance measured at 540 nm is a measure of the sum of the amount of present nitrite and nitrate.

Detection of N-NH₃ according to ISO 15923-1

Ammonium reacts with hypochlorite and salicylate at a pH of approximately 12,6, in the presence of sodium nitroprusside as a catalyst, to produce a compound with a blue colour. The reagent contains citrate to mask interference by cations, such as calcium and magnesium ions. The absorbance at 660 nm is a measure of the ammonium content.

Detection of Silica according to ISO 15923-1

The most popular method for the measurement of silica is the heteropoly blue colorimetric method, which is based on the reaction of ammonium molybdate with silica and any phosphate present to produce heteropolyacids, at pH approximately 1.2. Oxalic acid is added to counter interference by phosphate, by destroying the molybdophosphoric acid but not the molybdosilicic acid. The formed greenish-yellow α -Keggin type heteropolyoxometalate $\text{SiMo}_{12}\text{O}_{40}^{4-}$ is converted to a blue complex by reduction with ascorbic acid, to form the blue coloured β -keggin ion which is measured at 880 nm.

Chromophores and reading wavelengths above mentioned are resumed in the *Table 5*.

Table 5: Chromophores and absorption wavelengths in the tested chemical methods.

PARAMETER	CHROMOPHORE	λ (nm)
<u>Chromium Hexavalent as Cr⁶⁺</u>	Diphenylcarbazide	550
<u>Copper (Dissolved) as Cu²⁺</u>	Bathocuproine	480
<u>Ammonia as N-NH₃</u>	Blue indophenol complex	660
<u>Nitrate as N-NO₃</u>	SAA+NED Complex	550
<u>Nitrite as N-NO₂</u>	SAA+NED Complex	550
<u>Phosphate as P-PO₄</u>	Molybdenum Blue	880
<u>Silicon as Si</u>	Molybdenum Blue	880

2.2.4.3. Automated analysis of chemical parameters: method set up

For performing automated analyses of chemical parameter within Easychem Tox On-Line the above mentioned standard methods were adapted to be used in a discrete analyser. For each parameter, an automated method was set up in the dedicated software, by choosing absorbance wavelengths, reaction times, volumes of reagents and samples according to the required range of analysis.

Table 6: Method set up for chemical parameters analysed in automated mode.

PARAMETER	METHOD SET UP																																																																																		
Chromium Hexavalent as Cr^{6+}	Description: Chromium as EC200 Unit: ppb Decimals: 1 LIS Code: Cr6-EC Unit Factor: 1.00 Slope: 1.00 Intercept: 0.00 Reaction Type: End Point LED power: ☐ Fluorim. Direction: Direct E.P. Limit(Abs): 0.0200 Depl.Limit(Abs): First Limit(Abs): Linear. Factor: Fit: RBL Min (Abs): RBL Max (Abs): RBL Replicates: 0 Filter 1: 543 Filter 2: None Bichr.Factor:	Sample <table border="1"> <tr> <th colspan="4">LOW VALUES</th> <th colspan="4">HIGH VALUES</th> </tr> <tr> <td>0.0</td><td>0.0</td><td>0.0</td><td>0.0</td> <td>100.0</td><td>200.0</td><td>500.0</td><td>1000.0</td> </tr> <tr> <td>Low Alert</td><td>Very Low</td><td>Low</td><td>Normal Values</td> <td>High</td><td>Very High</td><td>High Alert</td><td></td> </tr> <tr> <td>No</td><td>No</td><td></td><td></td> <td>No</td><td>No</td><td></td><td></td> </tr> </table> Rerun When Over: No Volumes and Time <table border="1"> <tr> <th>Predilution</th> <th>S+R1</th> <th>Reag. 2</th> <th>Reag. 3</th> <th>Incubation</th> <th>Read</th> <th>Special Wash</th> </tr> <tr> <td>36</td> <td></td> <td></td> <td></td> <td></td> <td></td> <td></td> </tr> <tr> <td>Times (Sec):</td> <td></td> <td></td> <td></td> <td></td> <td></td> <td></td> </tr> <tr> <td>Dil./Rgt Code:</td> <td>R1-CR6</td> <td>R2-CR6</td> <td></td> <td></td> <td></td> <td></td> </tr> <tr> <td>Ratio/Vol(μl):</td> <td>10</td> <td>20</td> <td>0</td> <td></td> <td></td> <td></td> </tr> <tr> <td>Rinse (μl):</td> <td>0</td> <td>0</td> <td>0</td> <td></td> <td></td> <td></td> </tr> <tr> <td>Sample (μl):</td> <td>290</td> <td></td> <td></td> <td></td> <td></td> <td></td> </tr> </table> RBL Stab. (Days): Calib. Stab. (Days): 0 Automatic QC interval: Never RBL Max CV%: Calculation Model: Linear Factor: 1.00 Sample Blank: Yes Min Cal. Factor: 0.00 Max Cal. Factor: 1000.00	LOW VALUES				HIGH VALUES				0.0	0.0	0.0	0.0	100.0	200.0	500.0	1000.0	Low Alert	Very Low	Low	Normal Values	High	Very High	High Alert		No	No			No	No			Predilution	S+R1	Reag. 2	Reag. 3	Incubation	Read	Special Wash	36							Times (Sec):							Dil./Rgt Code:	R1-CR6	R2-CR6					Ratio/Vol(μl):	10	20	0				Rinse (μl):	0	0	0				Sample (μl):	290					
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Copper (Dissolved) as Cu^{2+}	Description: Total Copper Unit: ppb Decimals: 1 LIS Code: TCu Unit Factor: 1.00 Slope: 1.00 Intercept: 0.00 Reaction Type: End Point LED power: ☐ Fluorim. Direction: Direct E.P. Limit(Abs): 0.0100 Depl.Limit(Abs): First Limit(Abs): Linear. Factor: Fit: RBL Min (Abs): RBL Max (Abs): RBL Replicates: 0 Filter 1: 480 Filter 2: None Bichr.Factor:	Sample <table border="1"> <tr> <th colspan="4">LOW VALUES</th> <th colspan="4">HIGH VALUES</th> </tr> <tr> <td>0.0</td><td>0.0</td><td>0.0</td><td>0.0</td> <td>100.0</td><td>250.0</td><td>400.0</td><td>500.0</td> </tr> <tr> <td>Low Alert</td><td>Very Low</td><td>Low</td><td>Normal Values</td> <td>High</td><td>Very High</td><td>High Alert</td><td></td> </tr> <tr> <td>No</td><td>No</td><td></td><td></td> <td>No</td><td>No</td><td></td><td></td> </tr> </table> Rerun When Over: No Volumes and Time <table border="1"> <tr> <th>Predilution</th> <th>S+R1</th> <th>Reag. 2</th> <th>Reag. 3</th> <th>Incubation</th> <th>Read</th> <th>Special Wash</th> </tr> <tr> <td>90</td> <td></td> <td></td> <td></td> <td></td> <td></td> <td></td> </tr> <tr> <td>Times (Sec):</td> <td></td> <td></td> <td></td> <td></td> <td></td> <td></td> </tr> <tr> <td>Dil./Rgt Code:</td> <td>CU-A</td> <td>CU-B</td> <td>CU-C</td> <td></td> <td></td> <td></td> </tr> <tr> <td>Ratio/Vol(μl):</td> <td>13</td> <td>57</td> <td>35</td> <td></td> <td></td> <td></td> </tr> <tr> <td>Rinse (μl):</td> <td>0</td> <td>0</td> <td>0</td> <td></td> <td></td> <td></td> </tr> <tr> <td>Sample (μl):</td> <td>228</td> <td></td> <td></td> <td></td> <td></td> <td></td> </tr> </table> RBL Stab. (Days): Calib. Stab. (Days): 0 Automatic QC interval: Never RBL Max CV%: Calculation Model: Linear Factor: 1.00 Sample Blank: Yes Min Cal. Factor: 0.00 Max Cal. Factor: 1000.00	LOW VALUES				HIGH VALUES				0.0	0.0	0.0	0.0	100.0	250.0	400.0	500.0	Low Alert	Very Low	Low	Normal Values	High	Very High	High Alert		No	No			No	No			Predilution	S+R1	Reag. 2	Reag. 3	Incubation	Read	Special Wash	90							Times (Sec):							Dil./Rgt Code:	CU-A	CU-B	CU-C				Ratio/Vol(μl):	13	57	35				Rinse (μl):	0	0	0				Sample (μl):	228					
LOW VALUES				HIGH VALUES																																																																															
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Rinse (μl):	0	0	0																																																																																
Sample (μl):	228																																																																																		
Ammonia as N-NH_3	Description: Ammonium Ion Unit: ppb Decimals: 1 LIS Code: NH4 Unit Factor: 1.00 Slope: 1.00 Intercept: 0.00 Reaction Type: End Point LED power: ☐ Fluorim. Direction: Direct E.P. Limit(Abs): 0.0200 Depl.Limit(Abs): First Limit(Abs): Linear. Factor: Fit: RBL Min (Abs): RBL Max (Abs): RBL Replicates: 0 Filter 1: 660 Filter 2: None Bichr.Factor:	Sample <table border="1"> <tr> <th colspan="4">LOW VALUES</th> <th colspan="4">HIGH VALUES</th> </tr> <tr> <td>0.0</td><td>0.0</td><td>0.0</td><td>0.0</td> <td>100.0</td><td>110.0</td><td>400.0</td><td>500.0</td> </tr> <tr> <td>Low Alert</td><td>Very Low</td><td>Low</td><td>Normal Values</td> <td>High</td><td>Very High</td><td>High Alert</td><td></td> </tr> <tr> <td>No</td><td>No</td><td></td><td></td> <td>No</td><td>No</td><td></td><td></td> </tr> </table> Rerun When Over: No Volumes and Time <table border="1"> <tr> <th>Predilution</th> <th>S+R1</th> <th>Reag. 2</th> <th>Reag. 3</th> <th>Incubation</th> <th>Read</th> <th>Special Wash</th> </tr> <tr> <td>36</td> <td></td> <td></td> <td></td> <td></td> <td></td> <td></td> </tr> <tr> <td>Times (Sec):</td> <td></td> <td></td> <td></td> <td></td> <td></td> <td></td> </tr> <tr> <td>Dil./Rgt Code:</td> <td>NH4-A</td> <td>NH4-B</td> <td></td> <td></td> <td></td> <td></td> </tr> <tr> <td>Ratio/Vol(μl):</td> <td>40</td> <td>40</td> <td>0</td> <td></td> <td></td> <td></td> </tr> <tr> <td>Rinse (μl):</td> <td>0</td> <td>0</td> <td>0</td> <td></td> <td></td> <td></td> </tr> <tr> <td>Sample (μl):</td> <td>210</td> <td></td> <td></td> <td></td> <td></td> <td></td> </tr> </table> RBL Stab. (Days): Calib. Stab. (Days): 0 Automatic QC interval: Never RBL Max CV%: Calculation Model: Linear Factor: 1.00 Sample Blank: Yes Min Cal. Factor: 0.00 Max Cal. Factor: 100.00	LOW VALUES				HIGH VALUES				0.0	0.0	0.0	0.0	100.0	110.0	400.0	500.0	Low Alert	Very Low	Low	Normal Values	High	Very High	High Alert		No	No			No	No			Predilution	S+R1	Reag. 2	Reag. 3	Incubation	Read	Special Wash	36							Times (Sec):							Dil./Rgt Code:	NH4-A	NH4-B					Ratio/Vol(μl):	40	40	0				Rinse (μl):	0	0	0				Sample (μl):	210					
LOW VALUES				HIGH VALUES																																																																															
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Ratio/Vol(μl):	40	40	0																																																																																
Rinse (μl):	0	0	0																																																																																
Sample (μl):	210																																																																																		

Nitrate as
N-NO₃

Description : None Nitrate Unit : ppm Decimals : 2 LIS Code : NO3 Unit Factor : 1.00 Slope : 1.00 Intercept : 0.00	Sample <table border="1"> <tr> <th colspan="4">LOW VALUES</th> <th colspan="4">HIGH VALUES</th> </tr> <tr> <td>0.00</td><td>0.00</td><td>0.00</td><td>0.00</td> <td>10.00</td><td>25.00</td><td>40.00</td><td>50.00</td> </tr> <tr> <td>Low Alert</td><td>Very Low</td><td>Low</td><td>Normal Values</td> <td>High</td><td>Very High</td><td>High Alert</td><td></td> </tr> <tr> <td>No</td><td>No</td><td></td><td></td> <td></td><td>No</td><td>No</td><td></td> </tr> </table> Rerun When Over : No	LOW VALUES				HIGH VALUES				0.00	0.00	0.00	0.00	10.00	25.00	40.00	50.00	Low Alert	Very Low	Low	Normal Values	High	Very High	High Alert		No	No				No	No											
LOW VALUES				HIGH VALUES																																							
0.00	0.00	0.00	0.00	10.00	25.00	40.00	50.00																																				
Low Alert	Very Low	Low	Normal Values	High	Very High	High Alert																																					
No	No				No	No																																					
Reaction Type : End Point LED power : ☐ Fluorim. Direction : Direct E.P. Limit(Abs) : 0.0200 Depl.Limit(Abs) : First Limit(Abs) : Linear. Factor : Fit : RBL Min (Abs) : RBL Max (Abs) : RBL Replicates : 0 Filter 1 : 543 Filter 2 : None Bichr.Factor :	Volumes and Time <table border="1"> <tr> <th>Predilution</th> <th>S+R1</th> <th>Reag. 2</th> <th>Reag. 3</th> <th>Incubation</th> <th>Read</th> <th>Special Wash</th> </tr> <tr> <td>Times (Sec):</td> <td></td> <td>108</td> <td>450</td> <td>308</td> <td>0</td> <td></td> </tr> <tr> <td>Dil./Rgt Code:</td> <td>NO3-A</td> <td>NO3-B</td> <td>NO3-C</td> <td></td> <td></td> <td></td> </tr> <tr> <td>Ratio/Vol(μl):</td> <td>1/1</td> <td>184</td> <td>92</td> <td>108</td> <td></td> <td>0</td> </tr> <tr> <td>Rinse (μl):</td> <td>0</td> <td>0</td> <td>0</td> <td></td> <td></td> <td></td> </tr> <tr> <td>Sample (μl):</td> <td>16</td> <td></td> <td></td> <td></td> <td></td> <td></td> </tr> </table> RBL Stab. (Days) : Calib. Stab. (Days) : 0 Automatic QC interval : Never RBL Max CV% :	Predilution	S+R1	Reag. 2	Reag. 3	Incubation	Read	Special Wash	Times (Sec):		108	450	308	0		Dil./Rgt Code:	NO3-A	NO3-B	NO3-C				Ratio/Vol(μl):	1/1	184	92	108		0	Rinse (μl):	0	0	0				Sample (μl):	16					
Predilution	S+R1	Reag. 2	Reag. 3	Incubation	Read	Special Wash																																					
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Rinse (μl):	0	0	0																																								
Sample (μl):	16																																										
	Calculation Model : Linear Factor : 1.00 Sample Blank : Yes Min Cal. Factor : 0.00 Max Cal. Factor : 1000.00																																										

Nitrite as
N-NO₂

Description : Nitrite Unit : ppb Decimals : 1 LIS Code : NO2 Unit Factor : 1.00 Slope : 1.00 Intercept : 0.00	Sample <table border="1"> <tr> <th colspan="4">LOW VALUES</th> <th colspan="4">HIGH VALUES</th> </tr> <tr> <td>0.0</td><td>0.0</td><td>0.0</td><td>0.0</td> <td>100.0</td><td>250.0</td><td>400.0</td><td>500.0</td> </tr> <tr> <td>Low Alert</td><td>Very Low</td><td>Low</td><td>Normal Values</td> <td>High</td><td>Very High</td><td>High Alert</td><td></td> </tr> <tr> <td>No</td><td>No</td><td></td><td></td> <td></td><td>No</td><td>No</td><td></td> </tr> </table> Rerun When Over : No	LOW VALUES				HIGH VALUES				0.0	0.0	0.0	0.0	100.0	250.0	400.0	500.0	Low Alert	Very Low	Low	Normal Values	High	Very High	High Alert		No	No				No	No											
LOW VALUES				HIGH VALUES																																							
0.0	0.0	0.0	0.0	100.0	250.0	400.0	500.0																																				
Low Alert	Very Low	Low	Normal Values	High	Very High	High Alert																																					
No	No				No	No																																					
Reaction Type : End Point LED power : ☐ Fluorim. Direction : Direct E.P. Limit(Abs) : 0.0200 Depl.Limit(Abs) : First Limit(Abs) : Linear. Factor : Fit : RBL Min (Abs) : RBL Max (Abs) : RBL Replicates : 0 Filter 1 : 543 Filter 2 : None Bichr.Factor :	Volumes and Time <table border="1"> <tr> <th>Predilution</th> <th>S+R1</th> <th>Reag. 2</th> <th>Reag. 3</th> <th>Incubation</th> <th>Read</th> <th>Special Wash</th> </tr> <tr> <td>Times (Sec):</td> <td></td> <td>36</td> <td>0</td> <td>362</td> <td>0</td> <td></td> </tr> <tr> <td>Dil./Rgt Code:</td> <td>H2ON02</td> <td>NITRIT</td> <td></td> <td></td> <td></td> <td></td> </tr> <tr> <td>Ratio/Vol(μl):</td> <td>1/1</td> <td>30</td> <td>85</td> <td>0</td> <td></td> <td>0</td> </tr> <tr> <td>Rinse (μl):</td> <td>0</td> <td>0</td> <td>0</td> <td></td> <td></td> <td></td> </tr> <tr> <td>Sample (μl):</td> <td>210</td> <td></td> <td></td> <td></td> <td></td> <td></td> </tr> </table> RBL Stab. (Days) : Calib. Stab. (Days) : 0 Automatic QC interval : Never RBL Max CV% :	Predilution	S+R1	Reag. 2	Reag. 3	Incubation	Read	Special Wash	Times (Sec):		36	0	362	0		Dil./Rgt Code:	H2ON02	NITRIT					Ratio/Vol(μl):	1/1	30	85	0		0	Rinse (μl):	0	0	0				Sample (μl):	210					
Predilution	S+R1	Reag. 2	Reag. 3	Incubation	Read	Special Wash																																					
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Dil./Rgt Code:	H2ON02	NITRIT																																									
Ratio/Vol(μl):	1/1	30	85	0		0																																					
Rinse (μl):	0	0	0																																								
Sample (μl):	210																																										
	Calculation Model : Linear Factor : 1.00 Sample Blank : Yes Min Cal. Factor : 0.00 Max Cal. Factor : 1000.00																																										

Phosphate
as P-PO₄

Description : PPO4 Unit : ppm Decimals : 2 LIS Code : PPO4-S Unit Factor : 1.00 Slope : 1.00 Intercept : 0.00	Sample <table border="1"> <tr> <th colspan="4">LOW VALUES</th> <th colspan="4">HIGH VALUES</th> </tr> <tr> <td>0.00</td><td>0.00</td><td>0.00</td><td>0.00</td> <td>5.00</td><td>6.00</td><td>10.00</td><td>20.00</td> </tr> <tr> <td>Low Alert</td><td>Very Low</td><td>Low</td><td>Normal Values</td> <td>High</td><td>Very High</td><td>High Alert</td><td></td> </tr> <tr> <td>No</td><td>No</td><td></td><td></td> <td></td><td>No</td><td>No</td><td></td> </tr> </table> Rerun When Over : No	LOW VALUES				HIGH VALUES				0.00	0.00	0.00	0.00	5.00	6.00	10.00	20.00	Low Alert	Very Low	Low	Normal Values	High	Very High	High Alert		No	No				No	No											
LOW VALUES				HIGH VALUES																																							
0.00	0.00	0.00	0.00	5.00	6.00	10.00	20.00																																				
Low Alert	Very Low	Low	Normal Values	High	Very High	High Alert																																					
No	No				No	No																																					
Reaction Type : End Point LED power : ☐ Fluorim. Direction : Direct E.P. Limit(Abs) : 0.0100 Depl.Limit(Abs) : First Limit(Abs) : Linear. Factor : Fit : RBL Min (Abs) : RBL Max (Abs) : RBL Replicates : 0 Filter 1 : 880 Filter 2 : None Bichr.Factor :	Volumes and Time <table border="1"> <tr> <th>Predilution</th> <th>S+R1</th> <th>Reag. 2</th> <th>Reag. 3</th> <th>Incubation</th> <th>Read</th> <th>Special Wash</th> </tr> <tr> <td>Times (Sec):</td> <td></td> <td>36</td> <td>90</td> <td>506</td> <td>0</td> <td></td> </tr> <tr> <td>Dil./Rgt Code:</td> <td>R1-PO4</td> <td>SDS</td> <td>R2-PO4</td> <td></td> <td></td> <td></td> </tr> <tr> <td>Ratio/Vol(μl):</td> <td>1/1</td> <td>170</td> <td>10</td> <td>160</td> <td></td> <td>0</td> </tr> <tr> <td>Rinse (μl):</td> <td>0</td> <td>0</td> <td>0</td> <td></td> <td></td> <td></td> </tr> <tr> <td>Sample (μl):</td> <td>50</td> <td></td> <td></td> <td></td> <td></td> <td></td> </tr> </table> RBL Stab. (Days) : Calib. Stab. (Days) : 0 Automatic QC interval : Never RBL Max CV% :	Predilution	S+R1	Reag. 2	Reag. 3	Incubation	Read	Special Wash	Times (Sec):		36	90	506	0		Dil./Rgt Code:	R1-PO4	SDS	R2-PO4				Ratio/Vol(μl):	1/1	170	10	160		0	Rinse (μl):	0	0	0				Sample (μl):	50					
Predilution	S+R1	Reag. 2	Reag. 3	Incubation	Read	Special Wash																																					
Times (Sec):		36	90	506	0																																						
Dil./Rgt Code:	R1-PO4	SDS	R2-PO4																																								
Ratio/Vol(μl):	1/1	170	10	160		0																																					
Rinse (μl):	0	0	0																																								
Sample (μl):	50																																										
	Calculation Model : Linear Factor : 1.00 Sample Blank : Yes Min Cal. Factor : 0.00 Max Cal. Factor : 1000.00																																										

Silicon as Si

Description : Si-SiO2 Unit : ppb Decimals : 0 LIS Code : Si-1 Unit Factor : 1.00 Slope : 1.00 Intercept : 0.00	Sample <table border="1"> <tr> <th colspan="4">LOW VALUES</th> <th colspan="4">HIGH VALUES</th> </tr> <tr> <td>0</td><td>0</td><td>0</td><td>0</td> <td>500</td><td>750</td><td>1000</td><td>2000</td> </tr> <tr> <td>Low Alert</td><td>Very Low</td><td>Low</td><td>Normal Values</td> <td>High</td><td>Very High</td><td>High Alert</td><td></td> </tr> <tr> <td>No</td><td>No</td><td></td><td></td> <td></td><td>No</td><td>No</td><td></td> </tr> </table> Rerun When Over : No	LOW VALUES				HIGH VALUES				0	0	0	0	500	750	1000	2000	Low Alert	Very Low	Low	Normal Values	High	Very High	High Alert		No	No				No	No											
LOW VALUES				HIGH VALUES																																							
0	0	0	0	500	750	1000	2000																																				
Low Alert	Very Low	Low	Normal Values	High	Very High	High Alert																																					
No	No				No	No																																					
Reaction Type : End Point LED power : ☐ Fluorim. Direction : Direct E.P. Limit(Abs) : 0.0200 Depl.Limit(Abs) : First Limit(Abs) : Linear. Factor : Fit : RBL Min (Abs) : RBL Max (Abs) : RBL Replicates : 0 Filter 1 : 880 Filter 2 : None Bichr.Factor :	Volumes and Time <table border="1"> <tr> <th>Predilution</th> <th>S+R1</th> <th>Reag. 2</th> <th>Reag. 3</th> <th>Incubation</th> <th>Read</th> <th>Special Wash</th> </tr> <tr> <td>Times (Sec):</td> <td></td> <td>72</td> <td>72</td> <td>848</td> <td>0</td> <td></td> </tr> <tr> <td>Dil./Rgt Code:</td> <td>R1-Si</td> <td>H2O-R1</td> <td>R2-Si</td> <td></td> <td></td> <td></td> </tr> <tr> <td>Ratio/Vol(μl):</td> <td>1/1</td> <td>10</td> <td>5</td> <td>20</td> <td></td> <td>0</td> </tr> <tr> <td>Rinse (μl):</td> <td>0</td> <td>0</td> <td>0</td> <td></td> <td></td> <td></td> </tr> <tr> <td>Sample (μl):</td> <td>230</td> <td></td> <td></td> <td></td> <td></td> <td></td> </tr> </table> RBL Stab. (Days) : Calib. Stab. (Days) : 0 Automatic QC interval : Never RBL Max CV% :	Predilution	S+R1	Reag. 2	Reag. 3	Incubation	Read	Special Wash	Times (Sec):		72	72	848	0		Dil./Rgt Code:	R1-Si	H2O-R1	R2-Si				Ratio/Vol(μl):	1/1	10	5	20		0	Rinse (μl):	0	0	0				Sample (μl):	230					
Predilution	S+R1	Reag. 2	Reag. 3	Incubation	Read	Special Wash																																					
Times (Sec):		72	72	848	0																																						
Dil./Rgt Code:	R1-Si	H2O-R1	R2-Si																																								
Ratio/Vol(μl):	1/1	10	5	20		0																																					
Rinse (μl):	0	0	0																																								
Sample (μl):	230																																										
	Calculation Model : Linear Factor : 1.00 Sample Blank : Yes Min Cal. Factor : 0.00 Max Cal. Factor : 1000.00																																										

2.3 Results and Discussion

2.3.1. Luminescent Bacteria Toxicity Test

For performing luminescent bacteria toxicity test, the analyser was typically configured to run a sample analysis per hour and a control check per day, even if timing and frequencies of samples and controls can be programmed according to needs.

As previously mentioned, the parameter commonly used to define an acute toxicity measurement is the percentage inhibition, which represents the variation in the light emitted by the microorganisms, caused by the sample presence, expressed in percentage points. The light emission of water blank, performed by using water with null toxicity instead of the sample, is used as reference value.

The percentage inhibition at time t ($H_t(\%)$) could be determined as shown in *Equation 3*:

$$H_t(\%) = [1 - RLU_{S(t)} / RLU_{B(t)}] \times 100 \quad (3)$$

where:

- $H_t(\%)$ is the bioluminescence percentage inhibition at time t induced by the sample S ;
- $RLU_{S(t)}$ is the luminescence measurement of sample S at time t expressed in the Luminescence Relative Units;
- $RLU_{B(t)}$ is the luminescence measurement of the blank B at time t expressed in Luminescence Relative Units;

Toxicity data are usually expressed in terms of effective concentration (EC). In this particular case, the EC_{20} and EC_{50} values (mg / L) resulting in 20 and 50 % inhibition of the bioluminescence of *P. phosphoreum*, were obtained after 15 and / or 30 min contact time.

First of all, the percentage inhibition data obtained in each experiment were converted to Gamma (Γ) values according to *Equation 4*:

$$\Gamma = H_t(\%) / [100 - H_t(\%)] \quad (4)$$

In order to generate a dose - response curve, Gamma values were plotted against their corresponding chemical concentrations, after first transforming all data to logarithmic values (*Eq. 5*):

$$\text{Log}(\Gamma) = a + b \times \text{Log}(C) \quad (5)$$

where:

- I is the response;
- C is the dose ;
- a and b are respectively the intercept and the slope.

Values falling within the 5 - 95 % inhibition range were used to fit Log–transformed data by linear regression, and the resulting equations were used to calculate the EC_{20} and EC_{50} .

Inhibition and EC_{20} / EC_{50} values were automatically calculated by the statistical package and reported in a “Table of results”, which also showed the mean percentage deviations for the both blanks and samples replicates, and the correction factor. The latter was calculated as:

$$f_k = RLU_{B(t)} / RLU_{B(0)} \quad (6)$$

where:

- f_k is the correction factor for the contact time of 5 min, 15 min or 30 min;
- $RLU_{B(t)}$ is the luminescence intensity of the *Blank* after the contact time of 5 min, 15 min or 30 min, in Relative Luminescence Units;
- $RLU_{B(0)}$ is the luminescence intensity of the *Blank*, measured immediately after the addition of bacteria, in Relative Luminescence Units.

An automated alarm was associated with a light reduction threshold and could be generated even after few seconds. In such a case, the analyser immediately replicated the analysis and achieved a check on bacterial status and performances, by testing standard solutions as quality control, in order to minimize the occurrence of false positives.

Furthermore, the detection of a toxicity event was followed by the determination of inhibition values H_t (%) at different sample dilutions in order to calculate the EC_{50} value and assess the concentration-effect relationship at a given exposure time (t).

2.3.1.1. Laboratory trial

A laboratory testing campaign was performed with the objective to test if the Easychem Tox On-line system could be effectively used as a one month fully autonomous on line toxicity biomonitor. Three main goals characterized the experimental trial: (i) to estimate the bacterial life-time, (ii) to assess the reproducibility of the results, and (iii) to test the sensitivity of the automatic biomonitor with regard to international standards.

Three cycles, each one lasting ten days, were performed on non-toxic groundwater samples spiked with a final concentration of 2.2 mg / L of zinc ion. When the first ten days had expired, the second

vial was automatically rehydrated and employed for the successive ten days in order to perform the second automated cycle of analyses. The possibility of introducing 3 vials of lyophilized bacteria in the instrument allowed extending to one month the time of unattended activity.

For every measurement cycle, 240 tests were executed at a temperature of 15 °C and data collected were used to check how both bacterial luminescence and percentage inhibition varied. The final percentage inhibition was automatically calculated by the software, according to *Equation 3*.

For the whole measurement cycle, bacteria preserved a measurable luminescence, which decreased more rapidly during the first three days, reaching a quasi-constant value in the following. About the sensitivity to samples, a mean 30-minute inhibition value of $80 \pm 7 \%$ was calculated, in agreement with the higher limit of sensitivity accepted by the ISO standard regulating the *V. fischeri*-based toxicity test. (Fig. 15).

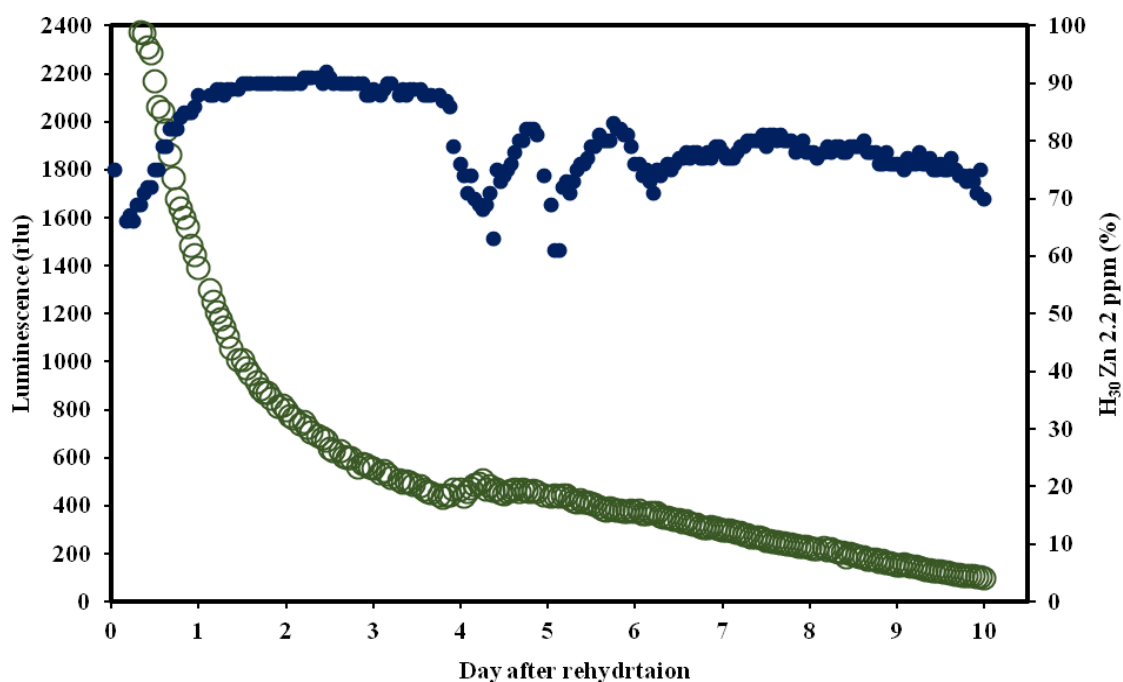


Figure 15: *P. phosphoreum* luminescence monitoring. The lyophilized was rehydrated and kept within Easychem Tox On-line instrument for the whole measurement cycle. Luminescence was measured every hour for 10 days.

All results, namely the mean value of final luminescence for Blanks and Samples and percentage inhibitions at time *t* were listed in the “Table of results”, together with the graphical view (Fig. 16).

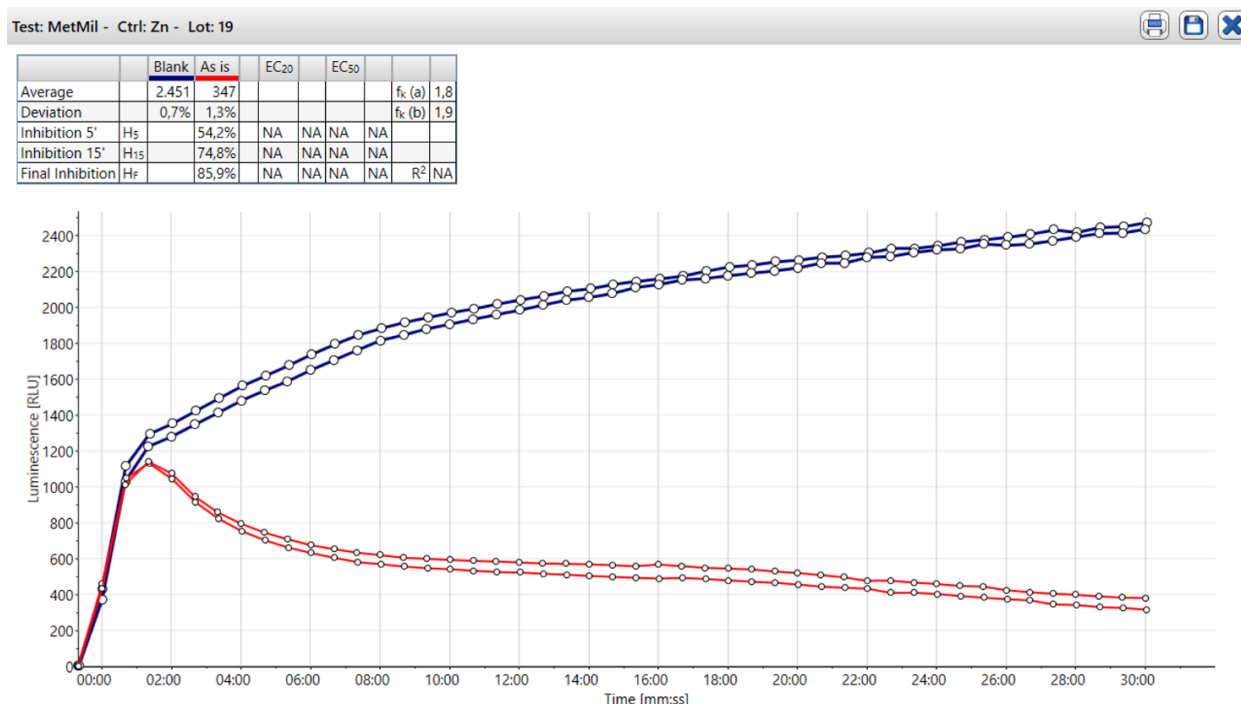


Figure 16: On-line monitoring of *P. phosphoreum* sensitivity to zinc ion; in the graph blank replicates (blue lines) were compared with positive control runs performed in the presence of 2.2 mg / L zinc ion as reference standard (red lines).
y axis: Luminescence (rlu); x axis: time (min; sec).

The compliance with the ISO requirements was completed by determining at 15 °C the EC₅₀ values of reference compounds 3,5-dichlorophenol and Cr(VI) which, after 30-minute test, were calculated to be, respectively, 4.1 ± 0.7 and 17.8 ± 4.9 mg / L (Fig. 17 and Fig. 18). Table 7 resumes the comparison between standard EC₅₀ values and that achieved by Easychem Tox On line, expressed in mg / L. Tests performed on-line resulted slightly less sensitive than laboratory-based standards for Zn (II) and DCP, but all of them were highly reproducible, as the Coefficient of variation of reproducibility (CV_R) confirmed.

Table 7: EC₅₀ values of reference substances determined in automated mode compared with values obtained in the International standard.

Toxicant	EN ISO 11348-3		Easychem Tox on line	
	EC ₅₀ (mg / L)	CV _R (%)	EC ₅₀ (mg / L)	CV _R (%)
Zn (II)	2.17 ± 0.73	± 33.6	1.15 ± 0.13	± 11.3
DCP	3.36 ± 0.32	± 9.6	4.12 ± 0.71	± 16.9
Cr (VI)	18.71 ± 6.17	± 32.9	17.8 ± 4.9	± 27.5

	Blank	As is	1/2	1/4	1/8	1/20	EC ₂₀	EC ₅₀		f ₁ (a)	f ₁ (b)
Average	1.473	443	679	973	1.113	1.327				1,9	1,9
Deviation	2,7%	0,5%	0,7%	1,9%	0,4%	2,8%					
Inhibition 5'	H _s	42,9%	31,3%	20,5%	12,5%	2,9%	24,1%	(21,7+26,5) %	NA	NA	
Inhibition 15'	H ₁₅	57,2%	41,0%	24,1%	12,7%	1,9%	20,2%	(18,3+22,0) %	65,8%	(59,7+73,7) %	
Final Inhibition	H _r	70,0%	53,9%	33,9%	24,4%	9,9%	10,9%	(8,5+13,3) %	40,6%	(33,6+51,0) %	R ² 0,991

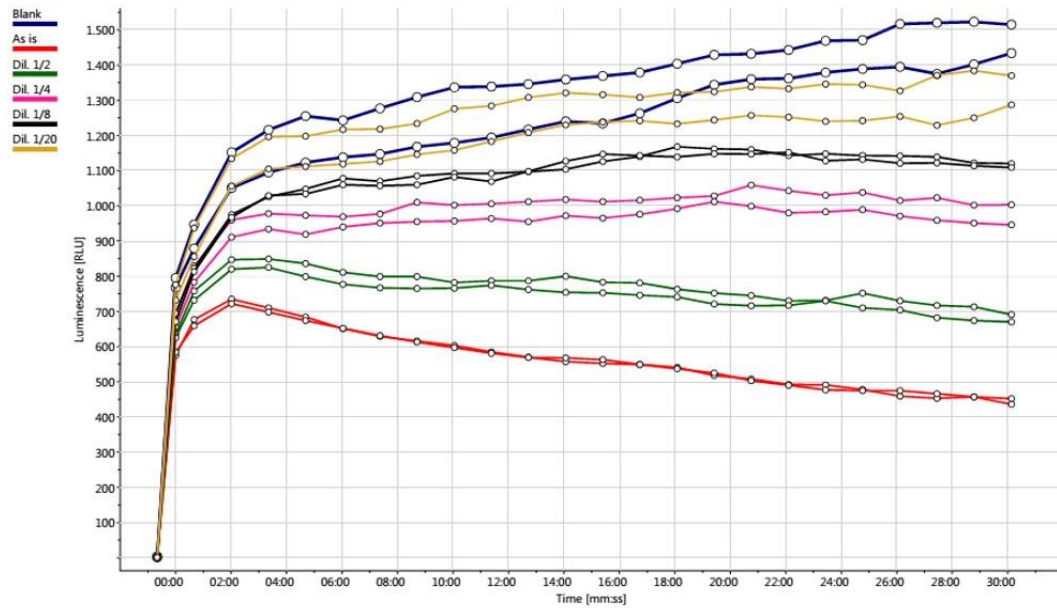


Figure 17: Inhibition effects of different dilutions of a 99.7 mg / L Chromium(VI) solution and EC₅₀ value at 30 min.

	Blank	As is	1/2	1/4	1/5	1/8	EC ₂₀	EC ₅₀		f ₁ (a)	f ₁ (b)
Average	1.168	257	662	1.000	1.080	1.109				1,7	1,7
Deviation	0,5%	0,3%	0,4%	0,9%	0,3%	2,3%					
Inhibition 5'	H _s	53,8%	29,5%	12,8%	8,6%	6,2%	33,5%	(28,1+40,6) %	81,7%	NA	
Inhibition 15'	H ₁₅	70,4%	41,4%	16,0%	10,2%	6,6%	27,4%	(23,0+32,2) %	56,6%	(46,7+74,3) %	
Final Inhibition	H _r	78,0%	43,4%	14,4%	7,6%	5,1%	28,4%	(22,9+34,9) %	52,2%	(41,8+72,4) %	R ² 0,974

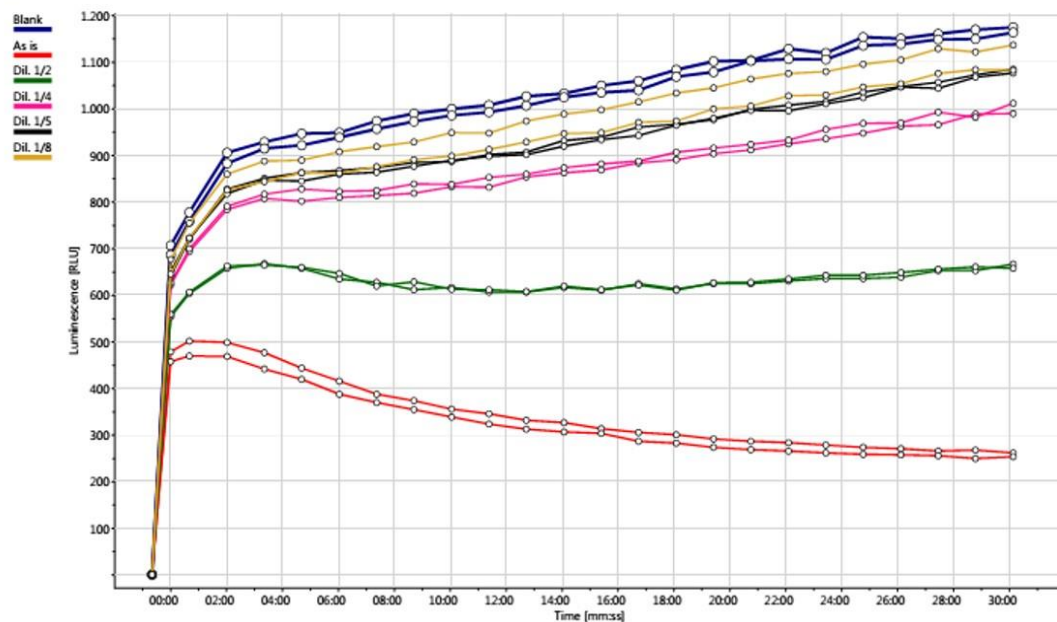


Figure 18: Inhibition effects of different dilution of 18.3 mg / L 3,5-dichlorophenol solution and EC₅₀ value at 30 min.

2.3.1.2. Field campaign in Metropolitana Milanese and EXPO

Finally, a long term luminescent bacteria toxicity test campaign was conducted in field with the installation of the Easychem Tox On-line by the water distribution network of Metropolitana Milanese SpA in EXPO 2015, the universal exposition held in Milano from May to October 2015. The scope was to monitor acute toxicity in potable water intended for human use after chlorination and immediately before distribution.

To exploit the maximum potential offered by *P. phosphoreum* in terms of sensitivity to toxic substances, the toxicity assays were executed at 25 °C. By means of the user interface, a 15-min toxicity test composed by two blank and two sample runs was performed every 90 minutes. Every day at midnight, a control check tested the bacterial viability using 2.2 mg / L of zinc ion as quality control standard solution (Fig. 19).

The system was programmed to automatically generate an early warning alarm just few seconds after the light reduction threshold of 50 % was exceeded without waiting for the end of the test. In such case, the analyser was configured to immediately replicate the analysis and perform a check on bacterial status and performance by using the reference standard, such to minimize the occurrence of false positives (Fig. 20). The confirmation of a toxicity event was meant to be finalized by the determination of inhibition values H_i at different sample dilutions, with the aim to assess the concentration - effect relationship at a given exposure time.

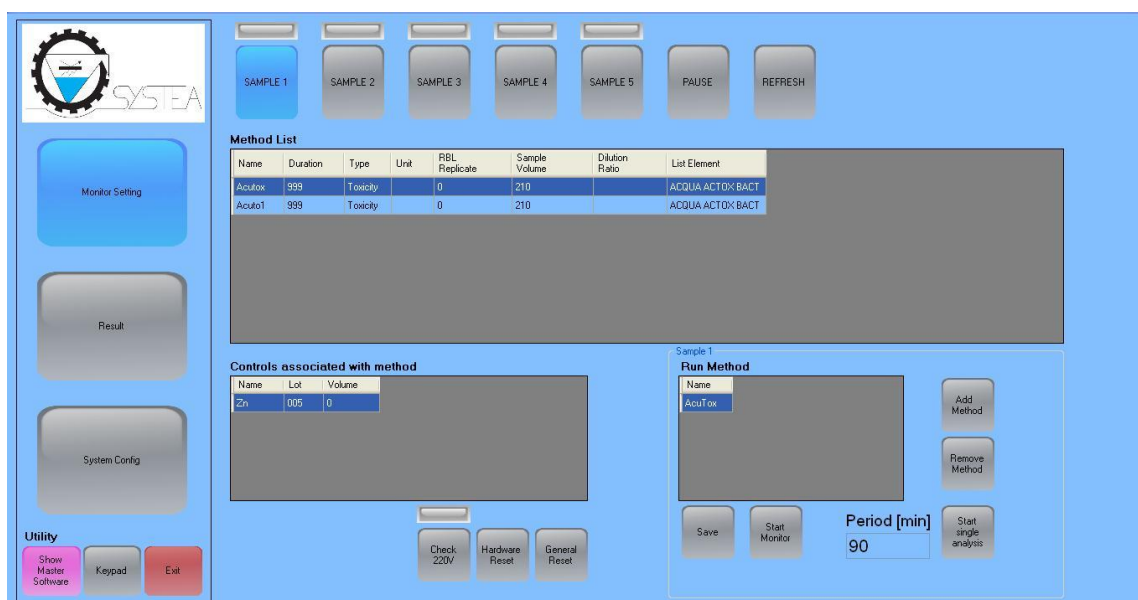


Figure 19: User interface for programming timing and frequency of both analytical cycles and quality controls.

Sample

SAMPLE 1 SAMPLE 2 SAMPLE 3 SAMPLE 4 SAMPLE 5

Control

Name	Lot	Volume
Zn	005	0

Save

Check Method

Name	Duration	Type	Unit	RBL Replicate
Acutox	999	Toxicity		0
Acuto1	999	Toxicity		0

Pre Inhibition Value > 50 %

Method

Name	Duration	Type	Unit	RBL Replicate
Acutox	999	Toxicity		0
Acuto1	999	Toxicity		0

Dilution Ratio: 1 None
Dilution Ratio: 2 None
Dilution Ratio: 3 None
Dilution Ratio: 4 None

Alarm Txs

Blank > 50 RLU
Inhibition Value > 50 %
Control Value > 20 %

Utility: Show Master Software Keypad Exit

Figure 20: User interface for programming early warning in the case that the inhibition value threshold is exceeded.

Daily laboratory analyses performed by Metropolitana Milanese SpA allowed to characterize the physicochemical sample profile with nitrate (EPA 1997), chromium (EPA 1994), total volatile organic carbon (EPA 2009), free chlorine and conductivity (APHA 1999) measurements.

Preliminary tests showed that bacterial luminescence was not affected by the amount of free chlorine contained in the supplied water which, in accordance with Italian normative, was calculated to range from 0 to 0.2 mg / L with a mean value during EXPO of 0.13 ± 0.05 mg / L (Fig. 20). For this reason, contrary to other cases, the usually recommended chlorine reduction with sodium thiosulphate was found not necessary.

Table 8: Physicochemical parameters daily measured by Metropolitana Milanese S.p.A. on supplied water.

	Average	Standard deviation
Free Chlorine (mg/L)	0.13	0.05
Nitrate (mg/L)	32.40	0.88
Cr (µg/L)	9.24	1.09
Total VOC (µg/L)	7.70	1.76
Conductivity 20°C (µS/cm)	540	12.79

Directly installed on the water inlet of EXPO 2015 from April to November 2015, the analyser was able to perform about 2,500 toxicity assays. The supplied water was characterized by electrical conductivity, at 20°C, of $540 \pm 13 \mu\text{S} / \text{cm}$, chromium and total VOC concentration of 9.24 ± 1.09 and $7.70 \pm 1.76 \mu\text{g} / \text{L}$ and nitrate content of $32.40 \pm 0.88 \text{ mg} / \text{L}$ (Table 8).

Despite the incidence of a combination of different conflicting effects including seasonal temperature, time of the day, water dilution and time occurred after vial reconstitution, the result repeatability fluctuated between -30 and 10 % with a mean inhibition of $-5.4 \pm 6.3 \%$ (Fig. 21).

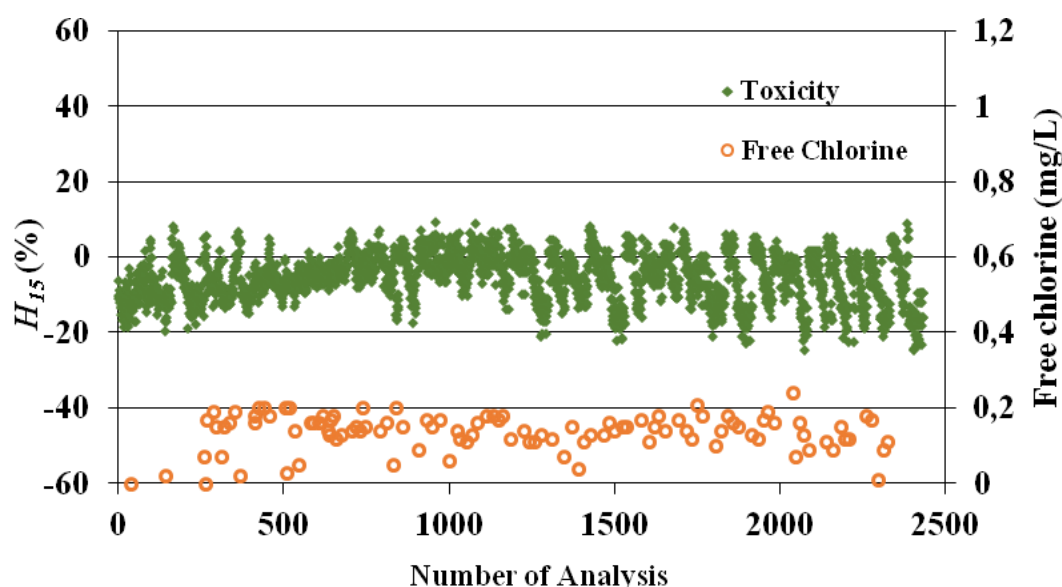


Figure 21: Trend of toxicity and free chlorine data in the EXPO water inlet from April to November 2015.

For every analysis, the luminescence kinetics of blanks and sample were shown in real time, whereas at the end of the analytical cycle, the final percentage inhibition was automatically calculated by the software. As required from EN ISO 11348, blank and sample deviations from their mean, percentage inhibition at time t , and f_{kt} value are listed in the “Table of results”. A typical trend of the non toxic sample analysed in EXPO is shown in Fig. 22. Mean *Blank* and *Sample* deviation from their mean were calculated to be 1.9 ± 1.4 and $1.6 \pm 1.2 \%$, respectively, in complete accordance with validity criteria required by the normative. Most importantly, the critical threshold of 20 % was never crossed during the period of the installation and all toxicity inhibition measurements were found non-toxic.

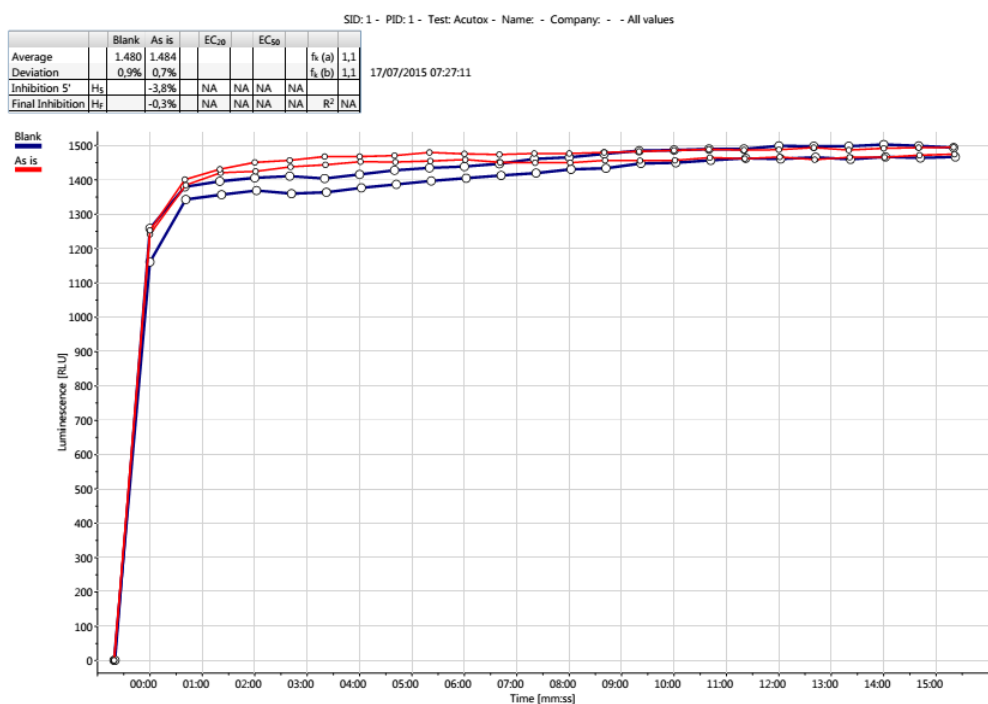


Figure 22: Software view of a non toxic sample analysis performed during EXPO.

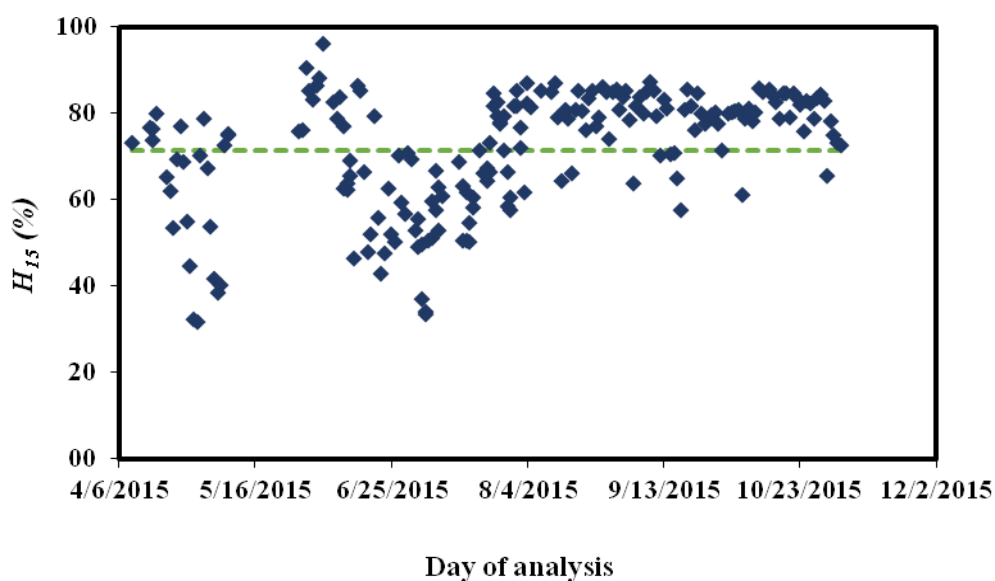


Figure 23: Daily inhibition to zinc measured as control at 12 am in the EXPO water inlet from April to November 2015.

Mean daily inhibition to zinc after 15-minute exposure at 25°C was calculated to be 71.4 ± 13.7 % with an improved percentage of deviation from their mean of blanks and positive controls of 1.8 ± 1.7 and 0.6 ± 0.6 %, respectively (Fig. 23).

A typical trend of a quality control run, as visualized by the software, was shown in Fig. 24.

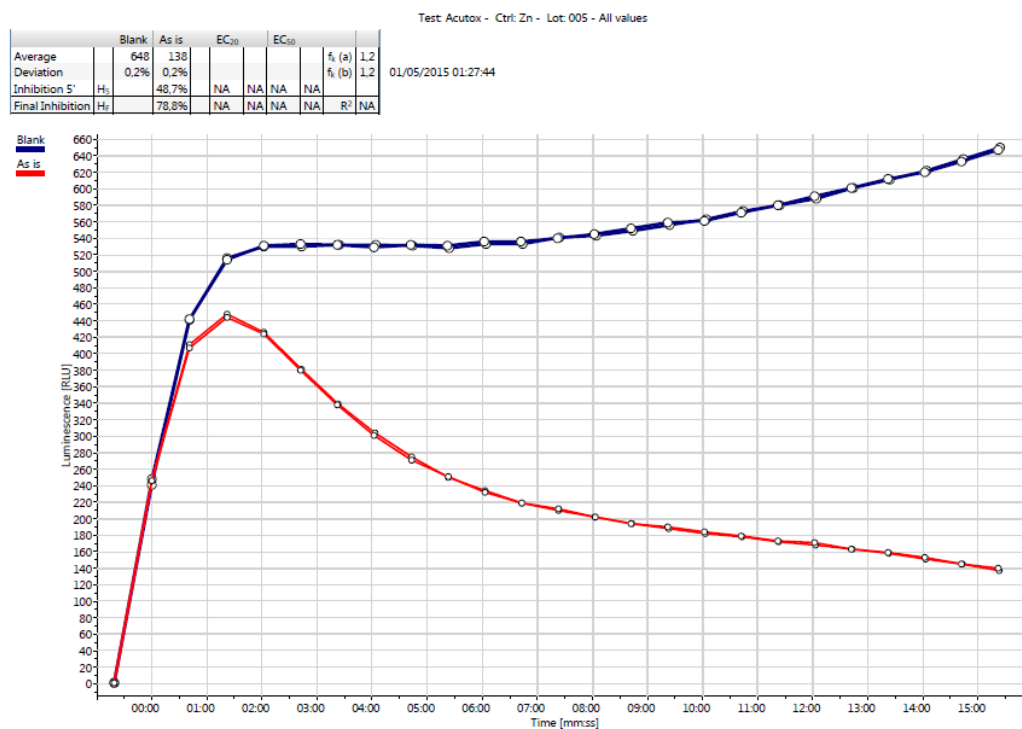


Figure 24: Software view of a quality control test performed during EXPO 2015 with 2.2 mg / L zinc ion solution.

2.3.2 Fluorescent Algae Toxicity Test

When algae were employed for performing toxicity tests within the analyser, the system sequentially measured Chlorophyll a fluorescence values versus time between 10^{-2} and 10^4 milliseconds. For each analysis the raw data graph is shown (Fig. 25), representing fluorescence kinetic curves of *Blanks* and *Samples*. Each of the curves consists of 2,000 points.

After data normalization (Fig. 26), the photosynthetic efficiency loss due to algal exposition to samples was automatically processed by the software according to an ad-hoc algorithm and toxicity was calculated as inhibition percentage of the relative fluorescence variation.

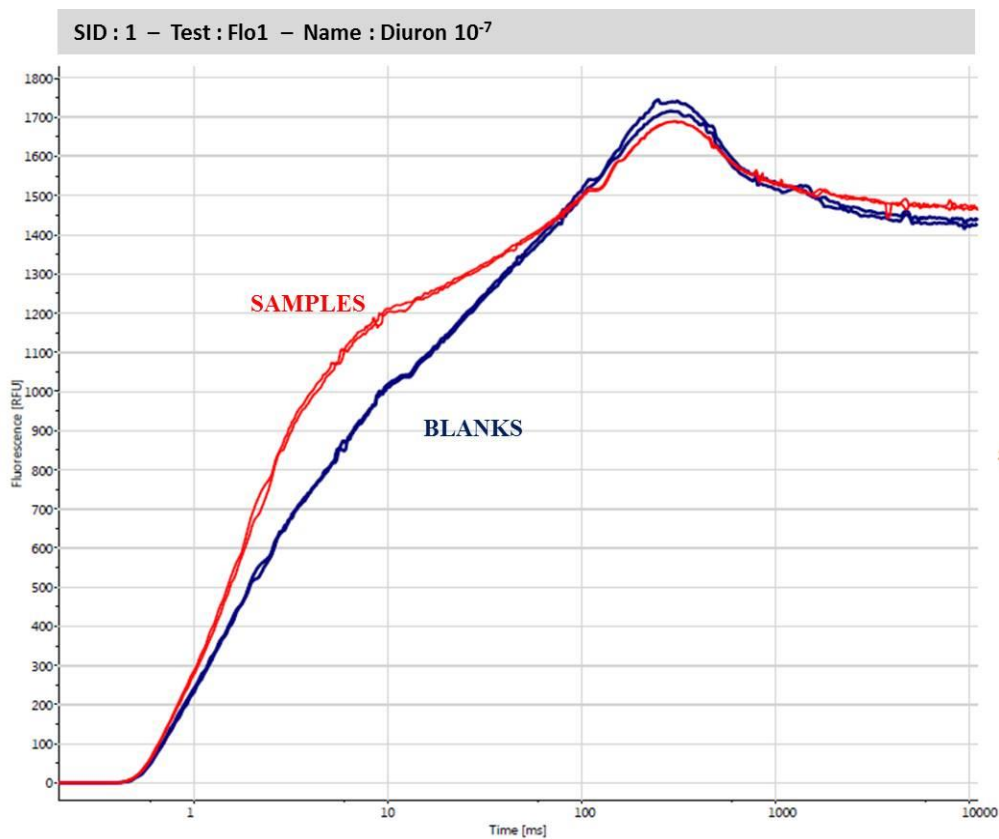


Figure 25: Chlorophyll a fluorescence induction transient of dark adapted algal suspension, excited with red (650 nm) LED, presented on a logarithmic time scale: raw data.

In particular, the herbicide toxicity was evaluated as the inhibition of the electron transport efficiency between PSII primary (Q_A) and secondary (Q_B) quinone acceptors, represented by the decrease of parameter $1 - V_J$ also expressed as:

$$H(\%) = [1 - (F_J - F_0) / (F_M - F_0)] \times 100 \quad (7)$$

where:

F_J is the fluorescence level 15 ms after the onset of excitation, the so-called J step in the fluorescence induction transient, whereas F_0 and F_M have been defined above.

In the presence of herbicides, the relative variable fluorescence at J step (V_J) increases proportionally to the herbicide concentration, thus revealing the progressive inhibition of the $Q_A - Q_B$ electron transfer and the accumulation of quinone Q_A in the reduced form.

Taking into account that, according to European legislations, herbicide content in agrifood should not exceed 0.05 - 0.1 mg / kg, and in particular, for Linuron herbicide, the safe limit corresponds to $1 \cdot 10^{-7}$ M, this herbicide was selected as reference toxic. Therefore, concentrations of this compound, ranging between $1 \cdot 10^{-8}$ and $1 \cdot 10^{-4}$ M, were automatically analysed by Easychem Tox On-line. Toxicity tests for each concentration were conducted in duplicate; control cultures containing no herbicide were also included. Starting from the inhibition values automatically determined by the software (Fig. 27), the EC_{50} caused by Linuron was manually calculated (Fig. 28) and the result was compared to that reported in literature for the algal test automatically performed by BBE-Algae Toximeter.

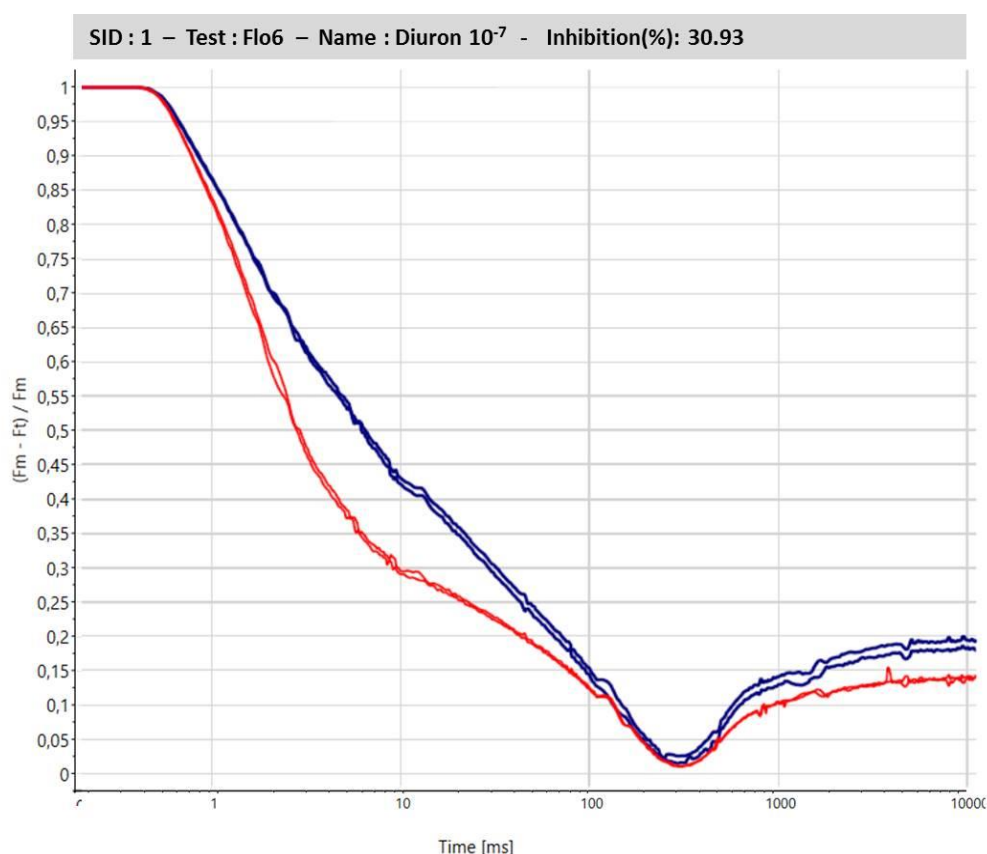


Figure 26: Chlorophyll a fluorescence induction transient of dark adapted algal suspension, excited with red (650 nm) LED, presented on a logarithmic time scale: Relative variable fluorescence after normalization.

When Diuron was employed as reference standard, small concentrations of the herbicide, corresponding to 10 $\mu\text{g} / \text{L}$, were found to cause an inhibition comprised between 10 and 15 %. When the algal toxicity test was performed within Easychem Tox On-line, the relative variable fluorescence caused by 6 ppb of Linuron was found to be 16%, perfectly in line with those reported in literature.

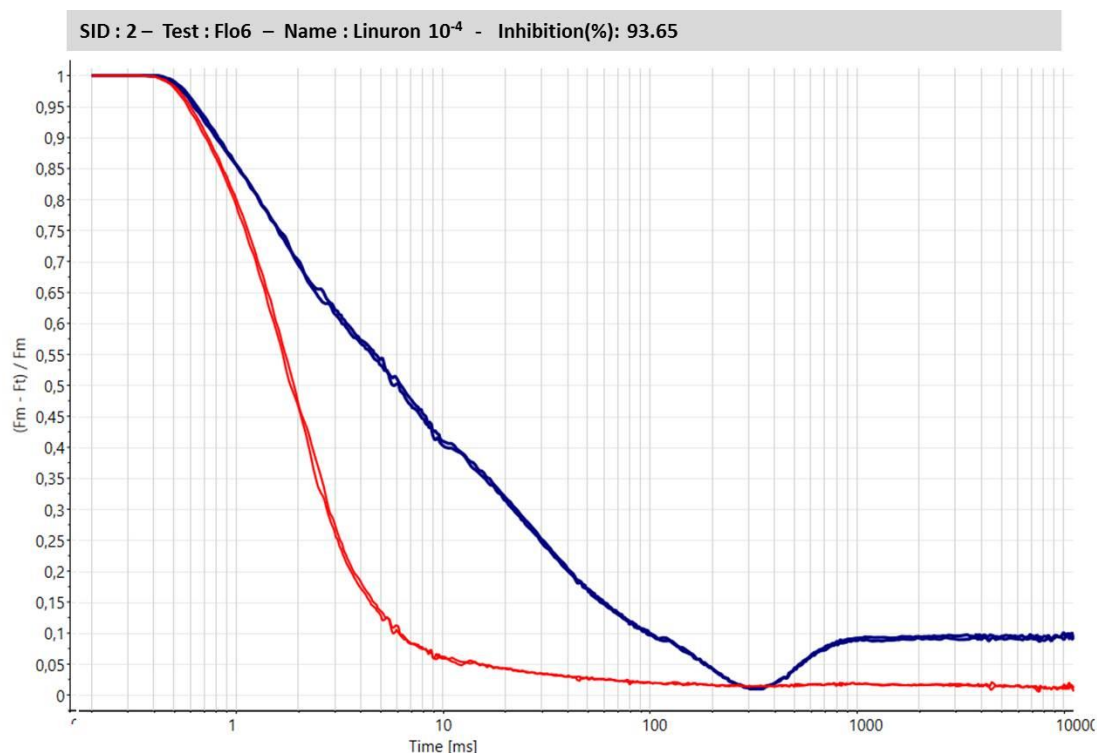


Figure 27: Inhibition percentage caused by a sample containing Linuron $1 \cdot 10^{-4}$ M.

[Linuron] (M)	[Linuron] (ppb)	RVF	$1 - V_j$ (%)
0	0	0.9997	0.003
$1 \cdot 10^{-8}$	0.6	0.9759	0.024
$1 \cdot 10^{-7}$	6	0.8342	0.165
$1 \cdot 10^{-6}$	62	0.3342	0.665
$1 \cdot 10^{-5}$	623	0.0485	0.951
$1 \cdot 10^{-4}$	6228	0.0635	0.936

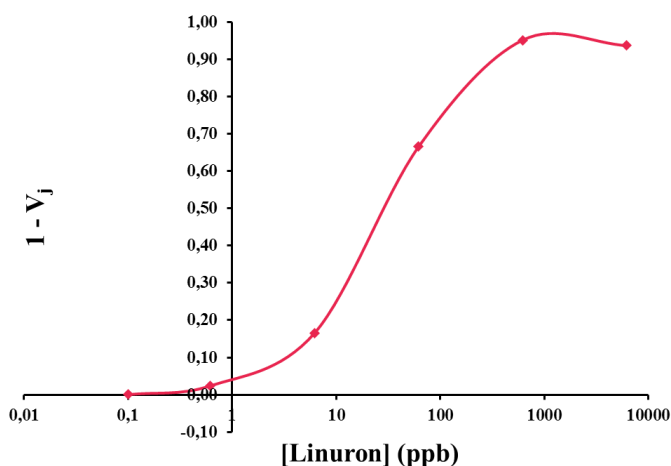
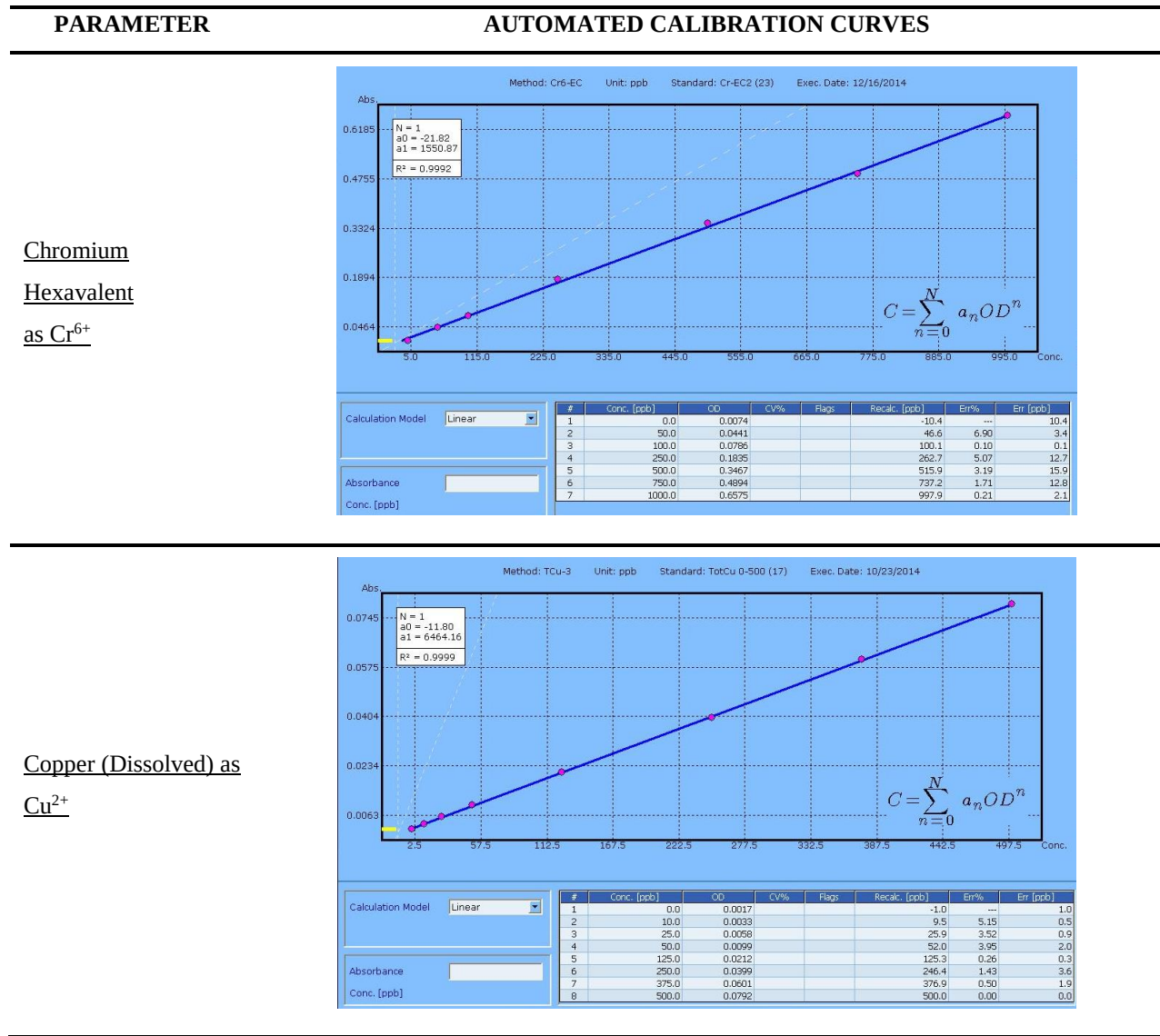


Figure 28: The toxic effect (EC_{50} value) of pure Linuron was determined in semi-automated mode by measuring the inhibition percentage caused by increasing concentrations of pesticide.

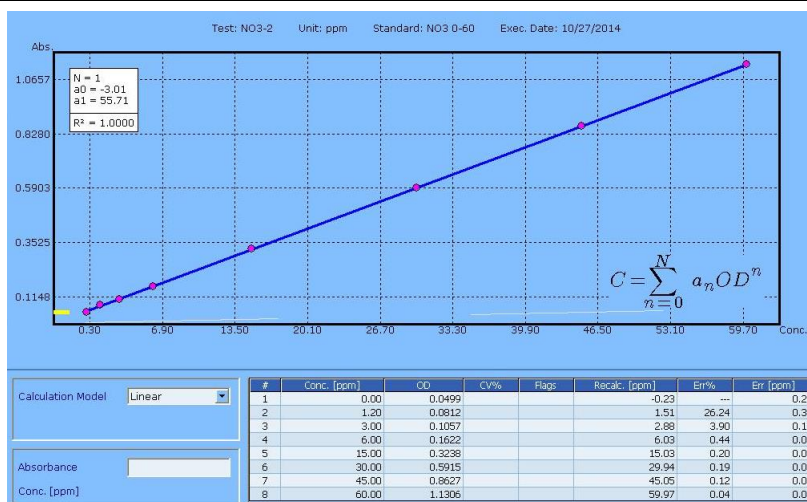
2.3.3 Chemical parameters

For each analysed parameter, the Optical Densities (O.D.) of a series of standard solutions, automatically prepared within the analyser, were plotted against the corresponding concentrations, in order to obtain the calibration curves. Data were automatically processed by the software on the panel PC.

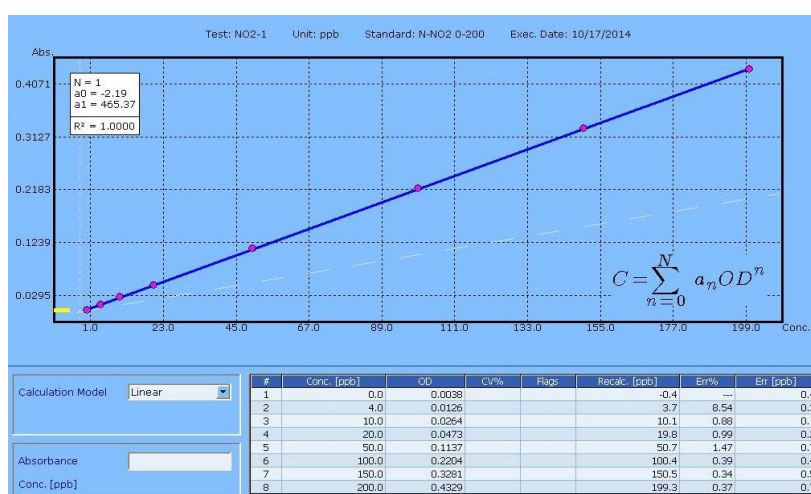
Table 9 – Calibration curves of chemical parameters.



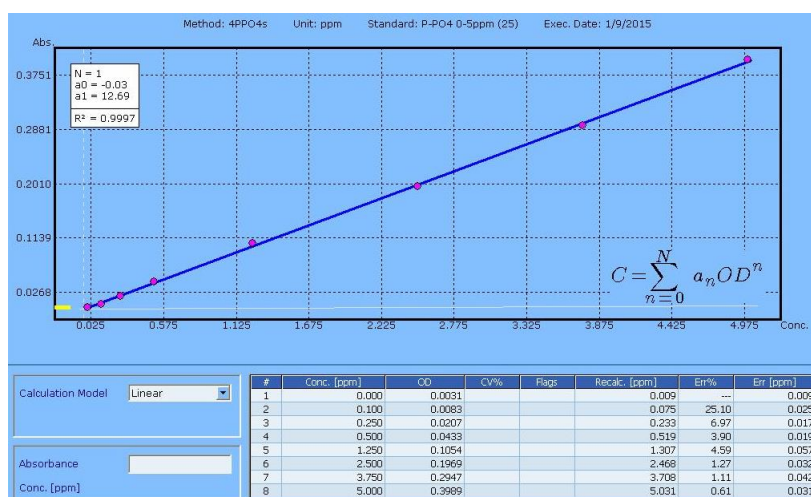
Nitrate as
N-NO₃



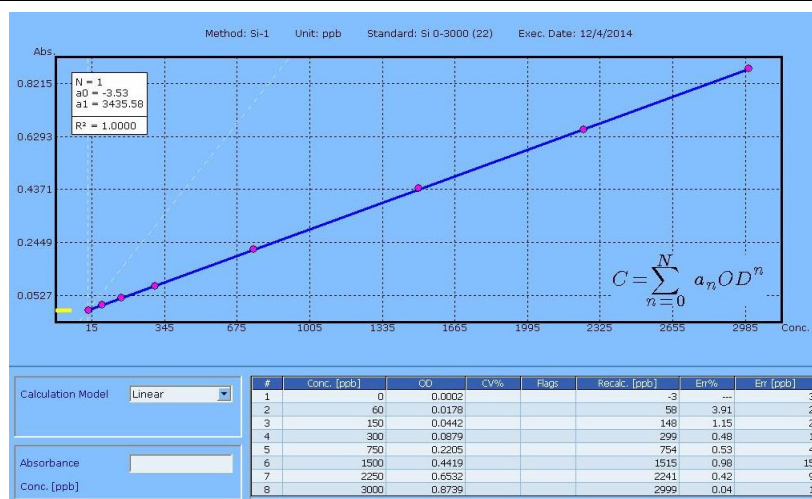
Nitrite as N-NO₂



Phosphate
as P-PO4



Silicon as Si



Each method was then tested by performing seven replicates of seven different known concentrations, in order to validate the automatic procedure. The coefficient of variation (CoV) was calculated according to ISO 8466–1 1990, as the ratio of the standard deviation of the results obtained for that method used to the mean of the working range of the method.

Table 10: Statistical analysis of data collected for the validation of the automatic analyses of nutrients and metals.

Target	Range (mg / L)	CoV (%)	Precision at 100 %	Accuracy at 100 %	Precision at 5 %	Accuracy at 5 %
Cr (VI)	0.003 – 0.200	± 1.45	± 1.1 %	± 0.3 %	± 3.6 %	± 1.0 %
Cu (I + II)	0.005 – 0.500	± 1.10	± 1.3 %	± 0.5 %	± 4.3 %	± 4.1 %
N-NH₄⁺	0.005 – 0.400	± 0.71	± 1.0%	± 0.2 %	± 5.0 %	± 4.6 %
N-NO₃⁻	0.200 – 15.00	± 5.07	± 3.2 %	± 2.0 %	± 3.4 %	± 2.0 %
N-NO₂⁻	0.001 – 0.200	± 2.03	± 1.8 %	± 0.9 %	± 4.9 %	± 4.3 %
P-PO₄³⁻	0.005 – 0.500	± 1.45	± 1.3 %	± 2.4 %	± 4.2 %	± 4.0 %
Si	0.005 – 0.500	± 0.83	± 1.0 %	± 1.8 %	± 1.7 %	± 4.5 %

Next, an on-line monitoring trial on non potable tap water contaminated with ppb levels of chromium (VI) and nitrite and ppm levels of nitrate was carried out for nine days. These parameters were tested every 3 hours at 25 °C in combination with the toxicity tests. Daily calibrations were automatically performed after midnight. After 80 analytical cycles, the measured outcomes, respectively 45.2 ± 2.5 and $123.9 \pm 4.6 \mu\text{g} / \text{L}$ for Cr (VI) and nitrite, and $10.82 \pm 0.85 \text{ mg} / \text{L}$ for nitrate, were found to be highly repeatable despite the inevitable sample variations. 30-minute sample toxicity oscillated between 13 and 31 % with a mean $\pm$ SD of $26 \pm 7 \%$ and no major toxicity events were detected.

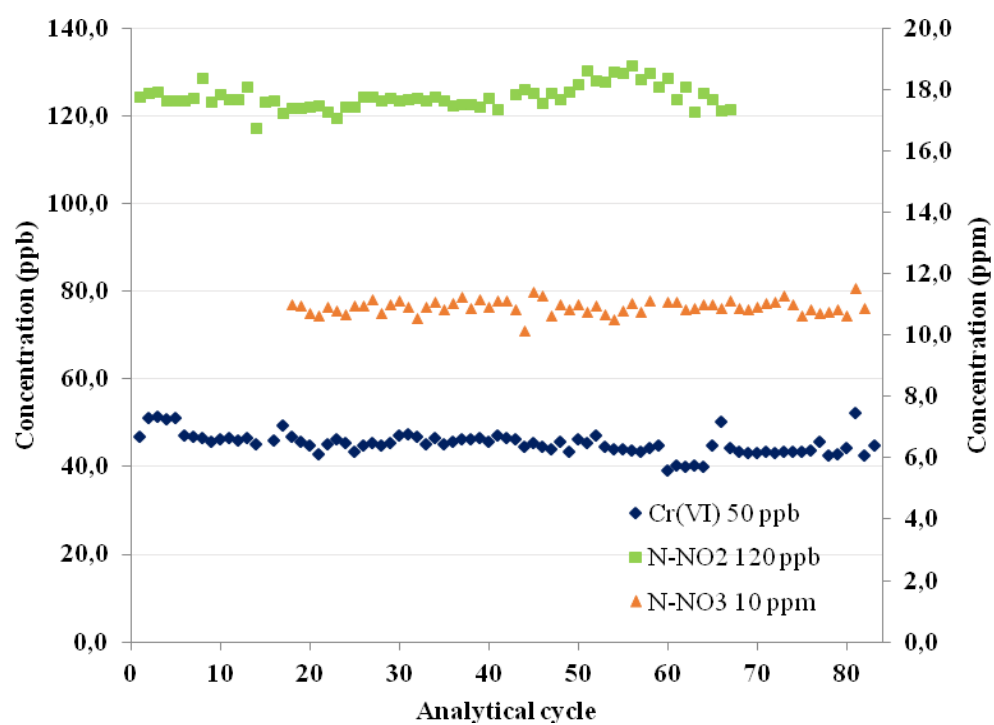


Figure 29: On-line monitoring of tap water spiked with 50 and 120 $\mu\text{g} / \text{L}$ of Cr and NO_2^- and 10 mg / L of NO_3^- .

2.4 Conclusions and future perspectives

The construction and development of an automated robotic platform, aimed for real-time monitoring of wastewater quality represented a significant improvement in environmental field.

First objective of this research was to establish an on-line biomonitor for toxicity evaluation in water samples, and, secondly, integrate it with chemical measurement. In contrast to chemical analysis, organism-based tests are able to detect all over toxic compounds in sample, and to assess their lethal effects. In order to provide an early warning system, biorganisms like bacteria and microalgae, sensitive enough to rapidly respond to changes in toxicity, were chosen. So, the full automation of both bacterial and algae standard toxicity tests within the innovative Easychem Tox On-line analyser, allowed to achieve significant improvements in environmental monitoring procedures.

The first objective was completely achieved, by developing an on-line luminescent bacteria based system to evaluate acute toxicity in water samples. The biomonitor was tested for long periods in order to demonstrate its ability to work in complete autonomy for up one month. At this scope, a wild type of *P. phosphoreum* was employed in the analyser, which preserved measurable bioluminescence and unaltered sensitivity for ten days; the option to automatically open two more vials extended the unattended time of operation to 30 days. Furthermore, the possibility to run at any time quality control cycles on any substance at known concentration in association with sample analysis allowed to check bacteria sensitivity and vitality. The main added value of Easychem Tox On-line was the ability to repeat sample analysis without waiting for the end of the current analytical cycle and to assess the extent of the danger by analysing different sample dilutions. These become indispensable features in case of toxicity alerts to exclude the occurrence of false positives and confirm the detected toxicity.

In order to extend the toxicity information to higher trophic levels, the application of algae based toxicity tests within Easychem Tox On-line analyser was investigated. The green algae *C. reinhardtii* was selected as biomonitor and the rapid measurement of Chlorophyll a fluorescence allowed to calculate the toxic effects of some common herbicides, by measuring the relative variable fluorescence. In future developments, the automated algal toxicity test needs to be improved to reach the ambitious target of 1-month of full autonomy. Aiming at this, three main aspects have to be investigated: (i) the prevention of bacterial contamination of the algal suspension; (ii) the continuous agitation of the algal suspension in order to avoid sedimentation and ensure homogenization; (iii) the algal culture shelf life, by routinely performing automated active Chlorophyll measurement.

Finally, since the most favourable solution for identification of the toxic substances might be the combination of different biological and chemical methods, the robotic system was improved for automatically analysing traditional chemical parameters in parallel with the toxicological tests. A laboratory trial demonstrated the possibility to incorporate toxicity tests with the required functionalities to measure some common chemical parameters. Because of that, Easychem Tox On-line has been proposed as an integrated water quality monitoring station, able to combine toxicological and chemical on-line control of water samples.

3. Easy coli:

Automated detection of *Escherichia coli* in wastewater

3.1. *Escherichia coli*: the best indicator of faecal contamination

3.1.1. Wastewater as source of faecal contamination

Public and ecological health protection requires the environmental safeguard of water bodies by regularly controlling the hygienic quality of the discharged wastewater effluents, which are considered the major source of faecal contamination of aquatic ecosystems. The establishment of sanitary practices in the late nineteenth century for the disposal of sewage and the increasing use of conventional chlorination, or, alternatively, ozonization, peracetic acid or UV-rays treatment of wastewater throughout the twentieth century resulted in a dramatic decrease in waterborne diseases such as cholera and typhoid fever. Despite these advances and several guidance designed to protect public water supplies from contamination, waterborne disease outbreaks still occur. In order to protect public health, it is important to have accurate, reliable, and scientifically defensible methods for determining whether source waters are contaminated by pathogens and to what extent.

The microorganisms associated with waterborne diseases (typhoid fever, cholera, shigellosis, hepatitis, jaundice fever, diarrhoea, amoebiasis, etc) found in polluted waters are members of bacteria, viruses, protozoa, and helminths (*Table 11*).

Table 11: Infectious agents potentially present in raw domestic wastewater (Hurst, 2002)

Agent	Disease	Clinical Symptom	Incubation Period	Source
Bacteria				
<i>E. coli</i> (enteropathogenic)	Gastroenteritis	Diarrhoea	2-6 days	Human faeces
<i>Salmonella typhi</i>	Typhoid fever	High fever, diarrhoea, ulceration of small intestine	7-28 days	Human faeces and urine
<i>Shigella</i>	Shigellosis	Bacillary dysentery	1-7 days	Human faeces
<i>Vibrio cholerae</i>	Cholera	Extremely heavy diarrhoea, dehydration	9-72 hours	Human faeces
Viruses				
Enteroviruses	Gastroenteritis, Heart anomalies	Paralytic diseases, respiratory illness	3-14 days	Human faeces
Hepatitis A	Infectious hepatitis	Fever, Anorexia	15-50 days	Human faeces
Rota virus	Acute Gastroenteritis	Gastroenteritis with nausea and vomiting	2-3 days	Human faeces
Protozoa				
<i>Giardia lamblia</i>	Giardiasis	Diarrhoea, nausea, indigestion	5-25 days	Human faeces
<i>Cryptosporidium parvum</i>	Cryptosporidiosis	Diarrhoea	1-2 weeks	Human faeces

The direct detection of pathogenic bacteria, viruses and protozoan parasites requires costly and time-consuming procedures and well-trained labour. The task would be enormous if one contemplates the monitoring of hundreds of pathogens on a routine basis in water and wastewater treatment plants, receiving waters, soils, and other environmental samples. Therefore, indicators of faecal pollution are much needed.

3.1.2. Indicator organisms of faecal contamination

For more than 100 years, public health personnel have relied extensively on an indicator organism approach to assess the microbiological quality of drinking water. More specifically, as early as 1914, the U.S. Public Health Service (U.S.P.H.S.) adopted the coliform group as an indicator of faecal contamination of drinking water. These enteric bacterial indicator microorganisms were typically used to detect the possible presence of microbial contamination of drinking water from human waste, and their use was later expanded and adopted for ambient, recreational, and shellfish waters and continues to focus on identification of faecal contamination.

Over the long history of their development and use, the current bacterial indicator approaches have become standardized, are relatively easy and inexpensive to use, and constitute a cornerstone of local, state, and federal monitoring and regulatory programs.

Microbial water quality indicators are used in a variety of ways within public health risk assessment frameworks, including evaluation of potential hazard, exposure assessment, contaminant source identification, and estimating effectiveness of risk reduction actions. However, no single indicator or analytical method is appropriate to all applications: a suite of indicators and indicator approaches is required for different applications and different geographies. For almost 50 years, Bonde's attributes of an ideal indicator (Bonde, 1966) have served as an effective model of how a faecal contamination index of public health risk and treatment efficiency should function.

Historic definitions of microbial indicators, such as coliforms, have been tied to the methods used to measure them. At the present, a variety of new methods are becoming increasingly available, providing several options for measuring each indicator group. Thus, separate criteria allow one to choose the indicator with the most desirable biological attribute for a given application and then match this with a measurement method that best meets the need of the application.

According to Bitton's definition an ideal indicator organism should respect the following criteria (Bitton, 2005):

- It should be a member of the intestinal microflora of warm-blooded animals.
- It should be present when pathogens are present, and absent in uncontaminated samples.
- It should be present in greater numbers than the pathogen.

- It should be at least equally resistant as the pathogen to environmental factors and to disinfection in water and wastewater treatment plants.
- It should not multiply in the environment.
- It should be detectable by means of easy, rapid, and inexpensive methods.
- It should be non pathogenic.

In the following, the commonly used microbial indicators are discussed and their main features are resumed in *Table 12*.

Total coliform (TC) is the broadest category of indicator bacteria and was originally believed to indicate the presence of faecal pollution. TCs belong to the family enterobacteriaceae and include the aerobic and facultative anaerobic, gram-negative, non-spore-forming, rod-shaped bacteria that ferment lactose with gas production within 48 hours at 35°C (APHA, 1998). These coliforms are discharged in relatively high numbers (2×10^9 coliform/day/capita) in human and animal faeces, but not all of them are of faecal origin, so the numerous non-faecal sources make this indicator too generic.

Faecal coliform (FC), a subgroup of total coliform, originates from the intestinal tract of warm-blooded animals. This subgroup is the most commonly used indicator of bacterial pollution in watersheds. Faecal coliforms are thermotolerant bacteria including all coliforms that can ferment lactose at 44.5 °C. The faecal coliform group comprises bacteria such as *Escherichia coli* or *Klebsiella pneumonia*. The presence of faecal coliforms indicates the occurrence of faecal material from warm-blooded animals.

Escherichia coli (*E. coli*) is a member of the faecal coliform subgroup, widely used because it correlates well with illness from faecal contaminated water. Some strains can cause gastrointestinal problems. Investigators have suggested the sole use of *E. coli* as an indicator of faecal pollution as it can be easily distinguished from the other members of the faecal coliform group (e.g., absence of urease and presence of β -glucuronidase).

Faecal streptococci (FS), also called faecal strep, is another group of bacteria associated with faeces from warm-blooded animals. The ratio of faecal coliform to faecal strep may indicate something about the source of the bacteria.

Enterococci are a subgroup of faecal strep bacteria. WHO recommended the use of Enterococci as an additional indicator, together with *E. coli*, because they generally do not grow in the environment and the majority of them were found to be true faecal species (Pinto, 1999).

As hinted above, Faecal coliform, *E. coli*, and Enterococci are the three groups of microorganisms that best comply with the requirements for useful microbial indicators for source and recreational water protection, even if a direct comparison with other candidate faecal indicators allows to

demonstrate that *E. coli* is far superior overall. For this reason, EPA targeted 2003 for all states to start using *E. coli* as sole indicator (USEPA 1999d), being this more specific for the presence of faecal contamination and so better for predicting health risk in primary contact water recreation.

Table 12: Main properties of the commonly used microbial indicators

Microbial indicator	Properties
Total coliforms (TC)	- Originally believed to indicate the presence of faecal pollution - Widely distributed in nature soils, water, flora, fauna - Contains member of <i>Escherichia</i>, <i>Citrobacter</i>, <i>Klebsiella</i> and <i>Enterobacter</i>, identified by incubation at 35 °C
Faecal coliforms (FC)	- Subgroup of TC - Coliforms that originate specifically from intestinal tracts of warm-blood animals - Cultured by increasing the incubation temperature to 44.5 °C - Remains the predominant indicator used to assess bacterial pollution in watersheds
<i>Escherichia coli</i>	- Member of FC group - Presence correlates with illness from swimming in both fresh and marine water - Has been shown epidemiologically to cause gastrointestinal symptoms - O157:H7 is a toxin-producing strain of this common bacterium
Faecal streptococci (FS)	- Differ from FC: (1) Less dominant in faeces (2) Not known to multiply in the environment (3) Die off more rapidly - Found in intestinal tracts of warm-blooded animals and may be found on vegetables and insects - FC/FS ratio used to determine faecal source - Includes <i>Streptococcus faecalis</i>, <i>S. faecium</i>, <i>S. bovis</i>, <i>S. equinus</i> and <i>S. avium</i>
Enterococci	- Subgroup of FS including <i>Streptococcus faecalis</i>, <i>S. faecium</i>, and <i>S. avium</i> - Commonly found in intestinal tract of humans and warm-blooded animals - Presence correlates well with illness from both fresh and marine water

3.1.3. *Escherichia coli*: index and indicator organism

Escherichia coli, originally known as *Bacterium coli commune*, was discovered in 1885 by German bacteriologist Theodor Escherich, who also showed that certain strains of the bacterium were responsible for infant diarrhoea and gastroenteritis.

This Gram-negative, facultative anaerobic, rod-shaped bacterium, belonging to the genus *Escherichia*, is commonly found in the lower intestine of warm-blooded organisms. *E. coli* is a thermotolerant bacterium with same fermentation properties of TCs (ferment lactose with gas and acid production within 48 h at 44.5 °C), produces indole from tryptophan at a temperature of 44.5 °C and gives a positive methyl red test result.

E. coli is the most common coliform among the intestinal flora of warm-blooded animals and its presence may be principally associated with faecal contamination. Most strains are harmless, even if some serotypes can cause serious food poisoning in their hosts, and are occasionally responsible for product recalls due to food contamination. The harmless strains are part of the normal flora of the gut, and can benefit their hosts by producing vitamin K2, and preventing colonization of the intestine with pathogenic bacteria. It is the only member of the coliform group that unquestionably inhabits the intestinal tract, and it is expelled into the environment within faecal matter: every human excretes in the faeces between 100 billion and 10 trillion individual *E. coli* per day.

E. coli has become the definitive biological marker of faecal contamination of water and food, being suitable both as index organism for warning of a serious health risk, and as indicator organism, according to WHO (WHO, 2002, Table 13).

Table 13: Definitions for indicator and index micro-organisms for public health concern and *E.coli* suitability.

	WHO definition	<i>E. coli</i>
INDEX ORGANISM	A group or species indicative of pathogen presence and behaviour respectively.	It is associated with the presence of ecologically similar pathogens EHEC, Salmonellae and Shigellae.
FAECAL INDICATOR	A group of organisms that indicates the presence of faecal contamination.	It is useful for assessing the efficacy of the treatments in removing or reducing faecal pollution.

3.1.4. Detection and enumeration of coliforms and *Escherichia coli*: traditional methods and emerging approaches

The US EPA has approved several methods for coliforms and *E. coli* detection, including the widely recognized multiple-tube fermentation technique, membrane filter technique, the presence/absence test and enzymatic technologies.

3.1.4.1. Multiple tube fermentation

The technique of enumerating coliforms by means of multiple-tube fermentation (MTF) has been used for over 80 years as a water quality monitoring method. The method consists of inoculating a

series of tubes with appropriate decimal dilutions of the water sample. Production of gas, acid formation or abundant growth in the test tubes after 48 h of incubation at 35 °C constitutes a positive presumptive reaction. Both lactose and lauryl tryptose broths can be used as presumptive media. The faecal coliform test performed by using an EC medium can be applied to determine TC that are FC (APHA, 1998): the production of gas after 24 h of incubation at 44.5°C in an EC broth medium is considered as a positive result.

The results of the MTF technique are expressed in terms of the most probable number (MPN) of microorganisms present. This number is a statistical estimate of the mean number of coliforms in the sample. As a consequence, this technique offers a semi-quantitative enumeration of coliforms. Nevertheless, the precision of the estimation is low and depends on the number of tubes used for the analysis. Many factors may significantly affect coliform bacteria detection, especially during the presumptive phase of the test. Interference by high numbers of non-coliform bacteria, as well as the inhibitory nature of the media, has been identified as factors contributing to overestimate or underestimate the coliform abundance. The time required to obtain results is higher than with the membrane filter technique that has replaced the MTF technique in many instances for the systematic examination of drinking water. Nevertheless, this method is extremely time-consuming, requiring 48 h for presumptive results, and necessitates a subculture stage for confirmation which could take up to a further 48 h.

3.1.4.2 Membrane filter technique

The membrane filter (MF) technique is fully accepted and approved as a procedure for monitoring water microbial quality in many countries. This method consists of filtering a water sample on a sterile filter with a 0.45-mm pore size which retains bacteria, incubating this filter on a selective medium and enumerating typical colonies on the filter. Many media and incubation conditions for the MF method have been tested for optimal recovery of coliforms from water samples. Among these, the most widely used are the m-Endo-type media in North America (APHA, 1998) and the Tergitol-TTC medium in Europe (AFNOR, 1990). Coliform bacteria form red colonies with a metallic sheen on an Endo-type medium containing lactose (incubation 24 h at 35 °C for TC) or yellow-orange colonies on Tergitol-TTC media (incubation 24 and 48 h at 37 and 44 °C for TC and FC, respectively). The predominant concern about MF is its inability to recover stressed or injured coliforms: a number of chemical and physical factors involved in wastewater treatment, including disinfection, can cause sublethal injury to coliform bacteria, resulting in a damaged cell unable to form a colony on a selective medium. The high number of modified media in use is a reflection of the fact that no universal medium currently exists which allows optimal enumeration of various coliform species originating from different environments and so present in a wide variety of

physiological states. MF is a useful technique for the majority of water quality laboratories as it is a relatively simple method to use. Many samples can be processed in a day, both with limited laboratory equipment and by a technician with basic microbiological training. Nevertheless, since this method is not sufficiently specific, a confirmation stage is needed, which could take a further 24 h after the first incubation period on selective media. This is why improvements to MF methods based on the enzymatic properties of coliforms have been proposed.

3.1.4.3 Presence/absence test by means of defined substrates.

Based on the enzymatic properties of coliforms, a defined substrate technology (DST) has been developed to overcome some limitations of the MTF and MF technique, such as the necessity of confirmatory tests. Unlike the previous methods, which eliminate the growth of non-coliform bacteria with inhibitory chemicals, the DST is based on the principle that only the target microbes are fed, and no substrate are provided for other bacteria, being the employed defined substrate a vital nutrient source for the target microbes (TC and *E. coli*). During the process, a chromogen or a fluorochrome is released from the substrate, indicating the presence of the target microorganisms. Edberg proposed a combined substrate consisting of the substrate ONPG for the constitutive enzyme β -galactosidase present in all coliforms and MUGlu for the specific detection of *E. coli* (Edberg, 1988). The method was basically constituted as a presence/absence test: the tubes, colourless after samples addition, were incubated at 35 °C. Any yellow colour in the test tube (indicating the hydrolysis of ONPG) was taken as positive for TC, and then it was exposed to long-wave UV light, for assessing the presence of *E. coli*, when a blue-white fluorescence took place.

3.1.4.4. Rapid enzymatic methods

The biochemical tests used for bacterial identification and enumeration in classical culture methods are generally based on metabolic reactions. For this reason, they are not fully specific, and many additional tests are sometimes required to obtain precise confirmation. The use of microbial enzyme profiles to detect indicator bacteria is an attractive alternative to classical methods.

Enzymatic reactions can be group-, genus- or species-specific, depending on the enzyme targeted, and always they are rapid and sensitive. Thus, the possibility of detecting and enumerating coliforms through specific enzymatic activities has been under investigation for many years now. Chromogenic and fluorogenic substrates produce, respectively, colour and fluorescence, upon cleavage by a specific enzyme and they have been used to detect the presence or the activity of specific enzymes in aquatic systems. Several authors have reviewed fluorogenic and chromogenic substrates used for bacterial diagnostics: by using these substrates, accuracy and detection improved, since methods for detection or enumeration may be performed in a single medium, thus bypassing the need for a time-consuming isolation procedure prior to identification.

In a large number of tests, the detection of total coliforms is based on the β -galactosidase activity. The enzyme substrates used are chromogenic substances, such as o-nitrophenyl- β -D-galactopyranoside (ONPG) or 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal, Lee, 1991). Fluorogenic substrates were also used and included 4-methylumbelliferone- β -D-galactoside (MUGal, Berg and Fiksdal, 1988) or fluorescein-di- β -galactopyranoside (FDG, Bitton *et al.*, 1995). On the other hand, the enzymatic method for detecting *E. coli* is based on the hydrolysing properties of β -D-glucuronidase, an enzyme which catalyses the hydrolysis of β -D-glucopyranosiduronic derivatives into their corresponding aglycons and D-glucuronic acid. Although this bacterial enzyme was discovered first in *E. coli*, its relative specificity for identifying this microorganism was not apparent until Kilian and Bulow surveyed the Enterobacteriaceae and reported that glucuronidase activity was mostly limited to *E. coli* (Kilian and Bulow, 1976). β -D-glucuronidase-positive reactions were observed in 94 – 96% of the *E. coli* isolates tested, whereas β -D-glucuronidase activity was found less common in other Enterobacteriaceae genus, such as Shigella, Salmonella and Yersinia strains.

To detect the presence of β -D-glucuronidase in *E. coli*, chromogenic substrates such as indoxyl- β -D-glucuronide (IBDG) (Brenner *et al.*, 1993) or 5-bromo-4-chloro-3-indolyl- β -D-glucuronide (X-Glu) (Watkins *et al.*, 1988) were used, even if the most frequent substrate is the fluorogenic 4-methylumbelliferyl- β -D-glucuronide (MUGlu). In the latter case, the end product is fluorescent and can be easily detected with a long-wave ultraviolet lamp. The assay generally consists of incubating the sample in lauryl-tryptose broth amended with 100 mg/L MUGlu, and observing the development of fluorescence within 24-hour incubation at 35°C. This assay can be adapted to membrane filters since β -glucuronidase-positive colonies are fluorescent or have a fluorescent halo when examined under a long-wave UV light.

3.1.4.5 MF technique conjugated to enzymatic detection

Different commercial agar media are now available, which include classical agar media for *E. coli* and coliform enumeration, modified with specific chromogenic and/or fluorogenic substrates for detection of β -D-glucuronidase and/or β -D-galactosidase. The use of these plate count methods allows far more rapid and accurate estimates of coliform and *E. coli* abundance in waters than the utilization of classical media. They can be used by any laboratory able to use conventional culture methods. However, they are more expensive and they do not satisfactorily solve the problems linked to the presence of non-culturable indicator bacteria.

3.1.4.6 Direct determination of enzymatic activity by fluorimetry

Contrarily to culture-based methods, procedures based on direct fluorimetry allow a rapid and direct estimate of the level of microbiological contamination of surface water, even if their detection

limits preclude their use for the monitoring of drinking water. These methods imply a direct determination of enzymatic activity for samples with high coliform abundance (surface water) and involve a growth phase for samples with low coliform abundance (drinking water). The detection system is based on fluorescence measurements, and particularly on the fluorescence emission of MU, molecule released by the enzyme reactions of either β -galactosidase with methylumbelliferyl- β -D-galactoside or β -glucuronidase with methylumbelliferyl- β -D-glucuronide.

3.1.5. Automated systems for *E. coli* detection in water

The most common methods for determining faecal contamination of municipal and industrial wastewaters rely on the time-consuming process of bacterial multiplication and require at least 24 hours from the sampling time to the possible determination that the water is unsafe for reuse: often it is feared that, by the time the contamination is detected, significant exposures have already occurred. New methods are therefore needed, permitting a rapid estimation of the sanitary quality of water to be carried out during raw water monitoring or emergencies involving water treatment failure or main breaks in a distribution network.

An ideal method for monitoring water quality has to be simple, sensitive, specific and able to perform analyses and to produce data in “real time”. Some physical-chemical parameters such as flow rate, turbidity, pH, temperature, conductivity and pressure are generally monitored on-line according to the above mentioned criteria; on the other hand, fully automated systems able to monitor hygienic water quality and to produce data in a timeframe, allowing corrective actions to be taken, do not already exist.

Automated methods for detection of *E. coli* have been developed and are useful for microbial source tracking, but they are still too complicated to be implemented in fully automated on-line instruments and, even more, they require special laboratory equipment and trained personnel. Being the measurement of enzymatic activity in water samples less complicated, easy-to-use field kits and automated instruments have been developed, according to this principle. Enzymatic activity was therefore suggested as a rapid “early warning” indicator of sewage contamination (Fiksdal; 2008).

In this context, instruments such as CA-100TM and ColiguardTM were developed with the aim to increase the number of samples processed and to reduce the analysis time. CA-100TM (Colifast Systems AS, Norway) is a semiautomated instrument able to perform presence / absence tests of FC by monitoring β -galactosidase activity (Angels d’Auriac, 2000). On the other hand, ColiguardTM (Mb Online GmbH, Austria) is a fully automatic analyser developed for the monitoring of *E. coli* both in drinking water supply, and in wastewater processing (Zibuschka, 2010). The quasi real-time

working system is based on the β -glucuronidase activity for rapidly calculating the *E. coli* equivalents, even if one sample at time could be analysed.

3.1.6. *E. coli* limits and detection methods in Italian Waste Water Treatment Plants.

According to the Italian law, various authorities are in charge of monitoring different types of water sample, and for each of them the *E. coli* limit is imposed, as shown in Table 14.

Table 14: *E.coli* limits imposed from Italian regulations

Type of water	Authority monitoring	<i>E. coli</i> limit
Water destined to human consumption such as: (i) mineral water, (ii) groundwater, (iii) swimming pools	D.Lgs.3/2001 enforcement of the European Directive 98/83/CE	Absence of <i>E. coli</i>
Bathing freshwaters	D.P.R. n.470/1982 National Sanitary System	<i>E. coli</i> < 100 CFU / 100 mL
Shellfish cultivation	D.Lgs.n.152/2006	<i>E. coli</i> concentration needs to be investigated directly in the body of the mussel
Wastewater reuse destined to (i) agricultural reuse and crop irrigation, (ii) urban reuse, (iii) industrial reuse.	D.M. n.185/2003	<i>E. coli</i> < 10 CFU/ 100 mL in 80 % of the samples (100 max value)

As specified by EU Directive, the reference methods for detecting *E. coli* in water samples are described in ISO 9308. The document is divided into three parts, each of them referring to a different procedure. ISO 9308-1 is based on membrane filtration technique which employs traditional media. The method has relatively low selectivity and is recommended for use with high water quality. Furthermore, two big limits of the method are in the readability of results and the time to get the definitive response to analyses. ISO 9308-2 describes a classic multiple tube fermentation based method, which results labour and time-consuming. On the other hand, part 3 of ISO 9308 refers to the enzyme-based most probable number procedure.

For monitoring the microbial contamination in effluents, Italian Waste Water Treatment Plants usually adopt two different enzyme-based methods, namely Colilert®-18 / Quanti-Tray® (approved by ISO 9308-3) and Chromogenic *E. coli* Agar (approved by APAT). Colilert®-18 / Quanti-Tray® is based on the MPN technique and it is a defined substrate technology, semi-automatic assay reduced to multi-wells. Positive results for *E. coli* are evidenced as yellow and fluorescent wells after incubation for 18 – 22 h at 36 ± 1 °C. Fluorescence is due to the production of 4-methyl-

umbelliferone, a fluorescent compound under the UV light (366 nm). Chromogenic *E. coli* Agar (C – EC Agar) is based on the membrane filtration method and prescribes the use of the EC X-Gluc Agar, a selective differential medium for the enumeration and immediate identification of *E. coli*. This medium contains bile salts for inhibition of gram-positive bacteria and the chromogenic substrate 5-bromo-4-chloro-3-indolyl- β -D-glucuronide for the detection of β -glucuronidase. Positive results are obtained after incubation at 36 ± 1 °C for 22 ± 2 h. In both procedures, the confirmation tests of the isolated colonies are not required.

3.1.7. Aim of the project

The demand for more rapid assays to be performed, in order to provide real-time results, has guided this research project towards the development of a new system able to rapidly detect *E. coli* in water samples, in complete and automated manner.

Part of this PhD thesis aimed to the formulation of a new analytical method, namely Easy Coli assay, for quantifying *E. coli* in water / wastewater samples within one hour. This rapid microbiological procedure was subsequently adapted to a complete automation within an innovative robotic system, the Easy Coli analyser. An automated, self-control prototype was therefore designed and developed with the final aim to provide the WWTPs with a rapid and simple *E. coli* detection system which ensures environmental protection of the receiving bodies, by both recording sudden variations of microbial contamination of the effluent and giving early warning in case of unexpected failures. Being able to almost instantaneously monitor the level of bacterial abatement, the system is also intended to provide a feedback tool to the disinfection process whose parameters, especially the amount of oxidizing agent, could be finely regulated according to the level of required disinfection.

3.2 Experimental

3.2.1. Apparatus: Easy coli analyser

Easy coli (*Fig. 30*) is a 65 (W) x 10 (H) x 50 (D) bench-top open analyser, managed by a dedicated Windows-based software. Two main sections compose the analyser: (i) on the left, a filtration unit, comprehensive of sampling and filtering modules; (ii) on the right, a detection unit, comprising a dosing station and a reaction carousel, which incorporates the fluorimetric station.



Figure 30: Final assembling of Easy coli analyser, consisting of the filtration unit in the left part and the detection unit in the right.

The filtration unit was designed and assembled to perform fully automated sampling and filtering operations (*Fig. 31*). Five different lodgings can be recognized: (i) filter and separator container on the very back, (ii) filtration site on the middle, (iii) disposal position for sample discharge and funnel rinsing on the front left, (iv) separator bin on the front right and (v) filter release hole on the reaction carousel on the very right. The device consisted of two automatic heads, controlling, respectively, sample and filter movements.

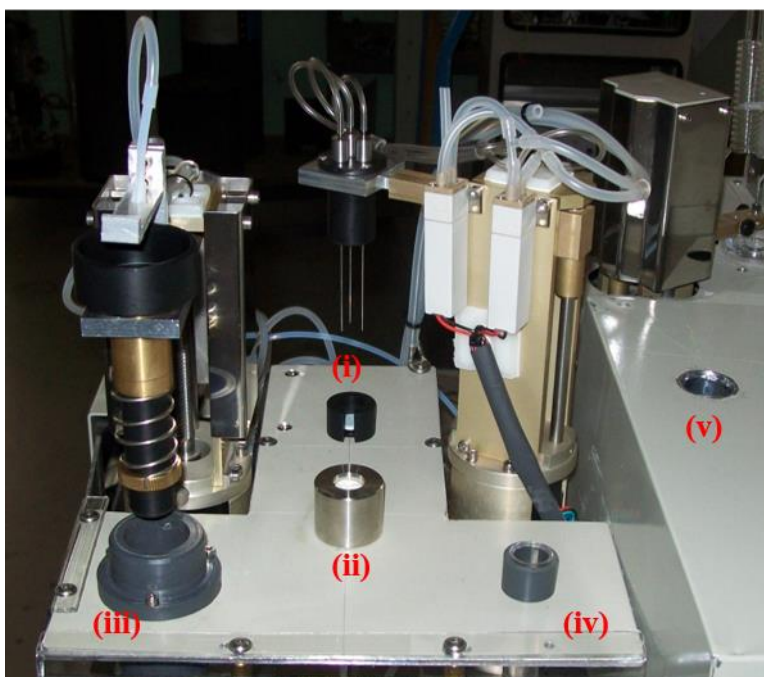


Figure 31: Filtration unit of Easy coli comprising: (i) filter and separator container, (ii) filtration site, (iii) sample discharge and funnel rinsing position, (iv) separator bin, (v) filter release hole, and two heads for filtering samples and moving filters.

The detection unit consists of a carousel provided with an upper opening, which encloses suitable containers for the addition of reagents by means of a dosing station, and an incorporated fluorimetric station for the detection.

The reagent dosing station (*Fig. 32a*) consists of an aspiration probe and a reagent dispenser syringe. By means of the positioning tower, probe's movements take place around two main axes: the Up / Down step allows to come in and out from the reagent tanks and the reaction vials, whereas the Probe Arm Rotation allows to move from the reagent tanks to the reaction vials. The dispenser syringe has the dual function to aspire the reagents in the probe when it is inside a reagent tank, and to dispense them in the reaction vials after the rotation.

The reaction carousel (*Fig. 32b*) consists of a 52-position rotating tray, that can hold 4-mL reaction tubes in order to run 52 sequential automatic measurements. The whole carousel is thermally insulated and the temperature can be maintained at fixed values between 30 and 50 °C, by using internal heaters and fast air recirculation.

The fluorimetric station, purchased from ESE GmbH, is based on the module FLUO SENS, a miniaturized fluorimeter characterized by confocal configuration. The compact module allows avoiding any sample transfer from the reaction tubes to the detection cell. The fluorimeter beam

passes through a 3 mm hole on the wall of the compartment and reaches the reaction vial positioned at a distance of about 10 mm.

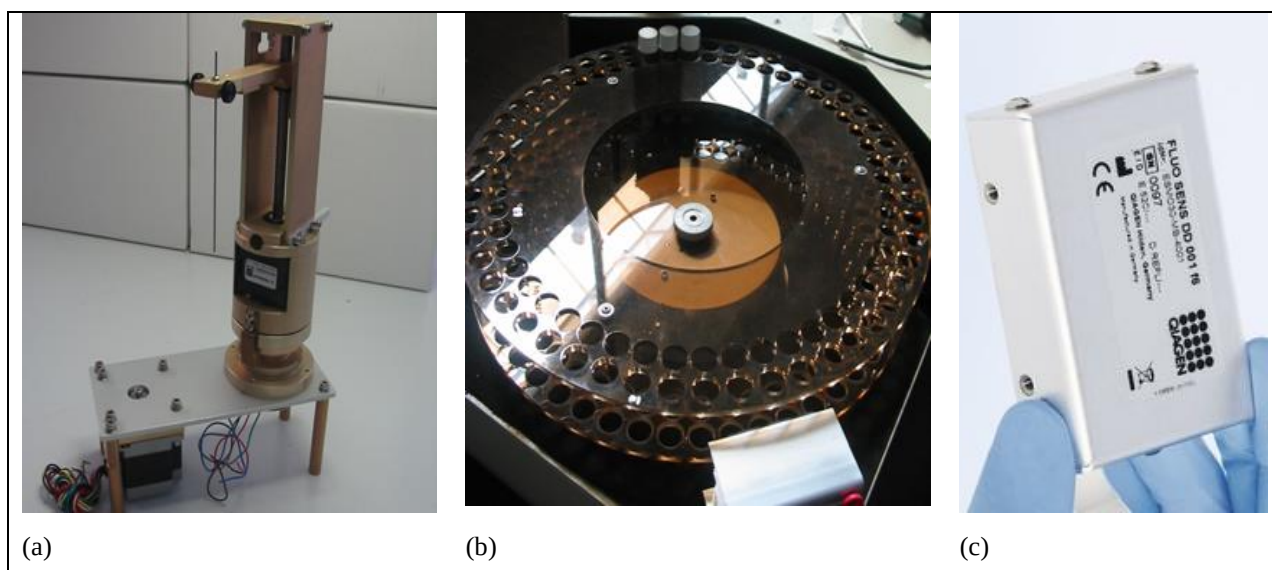


Figure 32: Main components of the detection unit: the aspiration probe, able to both move Up/Down or rotate (a), the rotating reaction carousel, holding 52 reaction tubes (b), and the fluorescence detector (c).

The user interacts with the analyser in laboratory or in the field, using Windows ® based software on the PC, which allows the complete remote control and management by a GSM/GPRS device.

3.2.2. Materials and Methods

3.2.2.1. Chemicals

Analytical-grade 4-methyl-umbelliferil- β -D-glucuronide, MW 352.30 g / mol, was purchased from Sigma-Aldrich (St. Louis, MO, USA). A nominal stock solution, containing a known weight of 5 g MUGlu / L was prepared in dimethyl sulfoxide, and 4 mL were added to 96 mL of a buffering solution, in order to prepare Reagent A.

Analytical-grade 4-methyl-umbelliferone, MW 176.20 g / mol, was purchased from Sigma-Aldrich. A nominal stock solution, containing a known weight of 0.018 g MU / L was prepared and then 1 mL Stock Solution were diluted in 1 L in order to prepare 1×10^{-7} M MU, used for performing a daily automated calibration.

Sodium phosphate dibasic, Sodium hydroxide, Potassium hydrogen phosphate anhydrous, and Magnesium sulfate heptahydrate were purchased from Sigma-Aldrich.

3.2.2.2. Reagents

The incubation medium was prepared by mixing buffering salts, surfactants and MUG, and it was employed in Easy coli analyser as *Reagent A*. A nominal 2 N solution NaOH (*Reagent B*) was prepared and used in the analyser as fluorescence enhancer solution.

3.2.2.3. Reference medium

C – EC Agar was purchased from Biolife (Milan, Italy) and was employed as reference medium for performing standard Membrane Filtration procedure.

3.2.2.4. Reference methods

ISO 9308-1

The ISO 9308-1 membrane filtration method was performed by using Tergitol-7 agar (*Fig.33*) supplemented with triphenyltetrazolium chloride (Oxoid, Basingstoke, UK).

According to the method, membrane filtration is performed by using 0.45- μ m-pore-size, 47-mm-diameter membrane filters. Filtered samples are then incubated on Lactose TTC agar with Tergitol 7 at 36 ± 2 °C for 21 ± 3 h. Orange-yellow colonies are recorded as the presumptive count (*Fig. 4*). In order to discriminate *E. coli* from coliforms, the standard procedure prescribes confirmation tests, such as oxidase activity and indole production from tryptophan, so that results are available within 72 h.

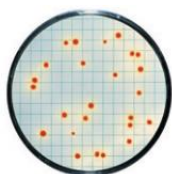


Figure 33: Enumeration of E.coli on Tergitol-7 Agar: positive bacteria give yellow colonies surrounded by a yellow halo

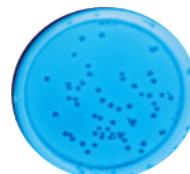


Figure 34 : Enumeration of E.coli on C-EC Agar: positive bacteria give blue-green colonies that fluoresce under u.v.-light (366 nm)

C-EC Agar

As recommended by the Italian National Authority for the Protection of Environment APAT, a membrane filtration method based on C-EC Agar (*Fig.34*) was performed (Metodi Analitici per le Acque, 2003). According to the reference procedure, membrane filtration is performed by using 0.45- μ m-pore-size, 47-mm-diameter membrane filters. An aliquot of the sample or its dilution is filtered and, after 18-24 hours of incubation at 44 ± 1 °C, results are read under ultra-violet lamp (366 nm). The compound 4-methylumbelliferyl- β -D-glucuronide (MUG), incorporated into C-EC Agar, is hydrolyzed by β -glucuronidase, releasing the compound 4-methylumbelliferone which then produces colonies of blue-green colour, fluorescent to ultraviolet light. The results are expressed as Colony Forming Units (CFU) per 100 mL of sample. This method allows counting the number of colonies of *E. coli* grown on a membrane placed on agar medium supplemented with chromogens. For high concentrations of *E. coli* in the sample, the reading of the results can be complicated by the spread and the confluence of the fluorescence produced by the typical colonies. Therefore, the

method is most suitable to the analysis of treated or little contaminated water.

3.2.2.5 Manual Easy coli assay

The scientific principle of the rapid Easy coli assay refers to an analytical solution for the detection of both coliforms and *E. coli*, based on the overexpression of the enzyme β -glucuronidase (GUS) in absence of cell growth (Bodini, 2003). Starting from this study, the improvement of Easy coli assay was obtained, by considering some important assumptions.

- The β -glucuronidase enzyme (GUS) is species-specific in *E. coli*, being GUS-negative *E. coli* strains less than 10 % of the total.
- The interferences caused by both free enzyme and non-target GUS producing organisms are negligible or weak.
- GUS concentration in environmental strains is 2 log units higher than in lab-induced *E. coli*, so that an enzyme induction step is not required.
- Disinfection may cause a decrease by 2-4 log units in culturability without influencing GUS activity.
- Non-culturable bacteria may retain enzyme activity.
- The hydrolysis rate of the enzyme is hyperbolically regulated by the starting concentration of the chemical fluorophore MUG.
- The analysis shall be performed within 4 - 6 hours from sampling, in order to avoid modifications in the bacterial content.

According to the well-defined Easy coli assay, 1 mL of environmental sample was filtered manually with a 13 mm-filter apparatus (Millipore) on PVDF filters, 1.3 cm diameter, 0.45 μ m cut-off. The filters were then placed into 10 mL glass tubes containing *Reagent A* and held in a thermostatic bath at 44.5 °C for a 60-min incubation time. By the end, the reaction was stopped by adding 0.1 mL of *Reagent B* up to pH 11-12. The resulting solution, containing the fluorescent product MU, was gently mixed and then transferred to a 10 $\times$ 10 mm quartz cuvette. The fluorescent measurement was performed by means of the PerkinElmer LS50B spectrofluorimeter, by programming the required parameter: heating block was set at 44.5 °C, the excitation wavelength at 362 nm, and the emission wavelength at 445 nm, with both slit widths at 2.5 nm.

3.2.2.6. Automated cycle of analysis

The full automation of the manual Easy coli assay for the quantitative analysis of *E. coli* was achieved within Easy coli analyser. The process for analysing and monitoring aqueous samples included two main steps, the former consisting in the automatic filtration within the filtration unit, and the latter comprising both the incubation and the measurement stages which took place within the detection unit.

Sampling and filtration cycle

The sampling and filtering procedure are performed by means of a filtration funnel, provided with a cone plug and a rotating head. During sampling and shifting, the plug is shut, whereas, during filtering, disposing and washing, it is open. The funnel is allowed to shift between the filtration and the disposal positions, whereas the moving head can rotate to move filter in different positions.

The process for automatically sampling, filtering sample and moving filters could be summarized as follows.

- Aqueous samples are directed into filtration funnel programmed in close-mode, by means of the peristaltic pump.
- The funnel shifts from the disposal to the filtration position, descends, seals with the filter holder and turns into open mode.
- The fluid crosses the filter membrane forced by vacuum.
- When the filtration ends, the funnel shut and go back to the disposal position. At the same time, the automatic head moves to the filtration position for loading the filter and moving it towards the reaction carousel.
- After descending through the release hole, the automatic head releases the filter within a reaction vial, in the rotating carousel.

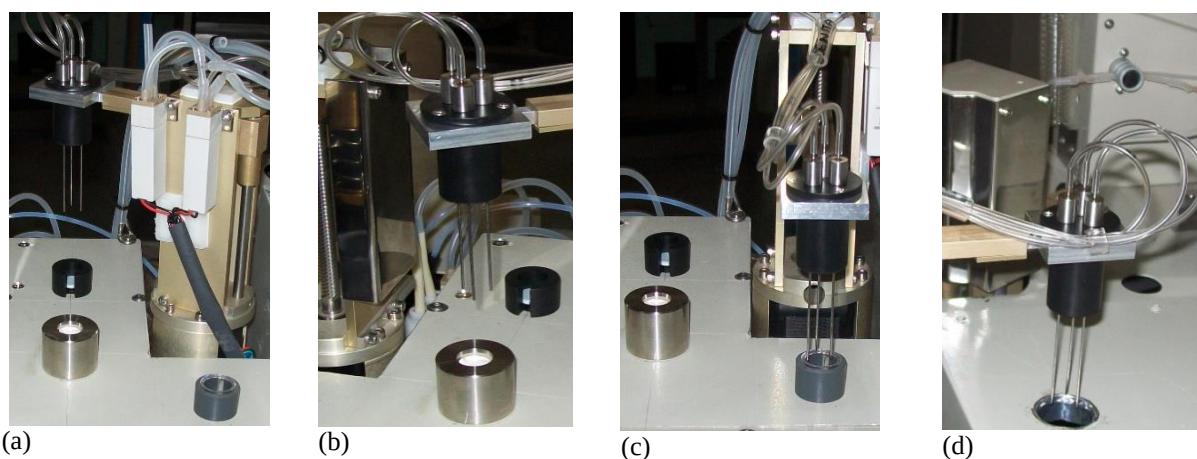


Figure 35 – Movements of the rotating head during the filter cycle.

Reaction cycle and detection

After automatic filter releasing in the reaction vial, the reaction and detection stages takes place. First, 2.1 mL of *Reagent A* are automatically poured on the filter and incubated for 60 minutes at 44.5 °C. When incubation time elapses, 0.2 mL of *Reagent B* are added, the vial rotates in front of the fluorimeter and the fluorimetric measurement is performed.

3.3 Results and Discussion

3.3.1. Validation of Easy coli assay

The use of rapid enzymatic assays for the quantification of *E. coli*, based on the hydrolysis of MUG by the specie-specific enzyme β -glucuronidase, has been the basis of numerous works, being George *et al.* (2000), Farnleitner *et al.* (2001) and Garcia-Arimesen *et al.* (2006) among the earliest. When results achieved by the Easy coli assay were compared with data in literature, good agreement were found. This allowed to validate the rapid procedure and encouraged the research toward its automation within Easy coli analyser.

Table 15: Validation of Easy coli assay by comparing results with data in literature

Author	Cells	Range	Slope	Intercept	Count
Easy coli	<i>E. coli</i>	10^2 - 10^5	0.78	-2.50	Plate
Garcia-Armisen	<i>E. coli</i>	10^2 - 10^8	0.73	-3.66	Plate
Farnleitner	Faecal coliforms	10^2 - 10^5	0.49	-2.70	Plate
George	Faecal coliforms	10^1 - 10^8	0.61	-3.07	Plate

3.3.2. Laboratory campaign: set up parameters for the improvement of the manual procedure within Easy coli analyser

The stability of Easy coli analyser was at first tested during a laboratory testing campaign. Four main goals characterized the experimental trials: (i) to improve the performances of the reaction medium, (ii) to evaluate and implement all components in the filtration unit (iii) to select the better reference method for testing the accuracy of the automatic process and (iv) to test the repeatability and reproducibility of results achieved with Easy coli analyser. In most of the experiments performed in this part of research, the quantification of *E. coli* required the use of pure culture cell suspensions, whose concentration was linearly correlated with the measured fluorescence.

3.3.2.1. Cell preparation, filtration and counts.

Fresh cultures of *Escherichia coli* ATCC 25922 were prepared by overnight growth in Luria-Bertani (LB) medium at 37 °C. Tests were performed on diluted cell samples after centrifugation

(1800 × *g*, 4 min) and pellet resuspension in 0.85 % NaCl physiological solution. The reference microbial cell count was made by plating the pure environmental samples on TTC Tergitol-7, at 44 °C for 24 h.

3.3.2.2. Improvement of the performances of the assay medium

The original medium, employed in the manual assay, was composed by different salts and surfactants (*Reagent A*), the enzyme substrate, and a fluorescence enhancer (*Reagent B*). The adaptation to the automated system required the examination of both the composition and the properties of the medium, with the aim to increase the analytical performances.

Finding optimum pH of Reagent A

According to literature (Fiksdal, 1994) the enzymatic activity and efficiency of GUS was strongly affected by pH of the reaction medium; in particular, a range between pH 6.9 and 7.2 was found as suitable for GUS hydrolysis.

Since both analyser components and materials may potentially alter pH, different starting pH values of Reagent A were investigated and compared, in order to find the optimum pH value for performing tests. Reagent A was adjusted with either HCl 1 M or NaOH 2 N in order to reach the following pH values: 6.0, 6.4, 6.7, 6.9, 7.2. Environmental samples were analysed within Easy coli, by employing the different Reagent A solutions and results were compared in terms of the measured fluorescence (*Table 5*). As shown in *Fig. 7*, was observed that little pH differences caused up to 30% decrease, and the better results were obtained in the range pH = 6.7 – 7.2. According to these observations, it was decided to use Reagent A at a pH value of 6.9, being this value exactly in the middle of the optimum range: in such a way, potential interferences on pH, taking place during the enzymatic assay, could be buffered.

pH	Fluorescence (rfu)
6.0	628.2
6.4	736.3
6.7	851.8
6.9	830.5
7.2	865.8

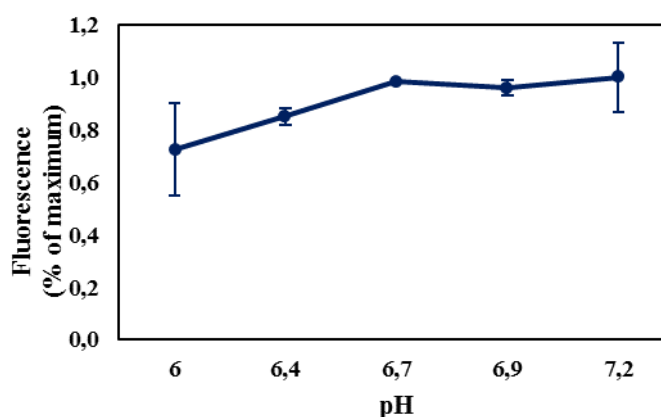


Figure 36: Assay performances at different Reagent A pH values

Selecting the final MUG concentration

Enzymatic reactions are significantly affected by the substrate concentration according to the Michaelis-Menten kinetics, which relate reaction rate v to the concentration of substrate $[S]$, according to Equation 8

$$v = d[P] / dt = V_{max}[S] / (K_M + [S]) \quad (8)$$

The suitable concentration of MUG was therefore investigated, by preparing MUG in six different concentrations ranging from 0.02 to 1.46 mg/mL. For each concentration, two replicates were tested, by adding them according to the automated analytical cycle.

MUG concentration and enzyme activity were related hyperbolically and saturation was reached at the concentration of about 0.6 - 0.7 mg/mL (Fig. 37). Keeping these results in mind, the originally selected MUG concentration of 0.21 mg / mL was considered already sufficiently close to the saturation point, and therefore suitable for the automatic analysis.

Studying the effect of Chloroform addition

Chloroform is known for increasing the cell-membrane permeability and for allowing the release of cellular components, the effect of its addition in the medium assay was studied in order to improve the release of the enzyme β -glucuronidase from the cells.

In particular, two experiments were performed, each of them consisting of five replicates with and five replicates without chloroform in the *Reagent A*. Despite previous results confirmed the efficiency of chloroform in improving the performances of the β -galactosidase, it was not possible to reproduce the same effect on β -glucuronidase, since a precipitate formed. The resulting solution was still turbid and was characterized by reduced fluorescence values of 80 ± 4 % than that measured in samples treated without chloroform (Fig. 38).

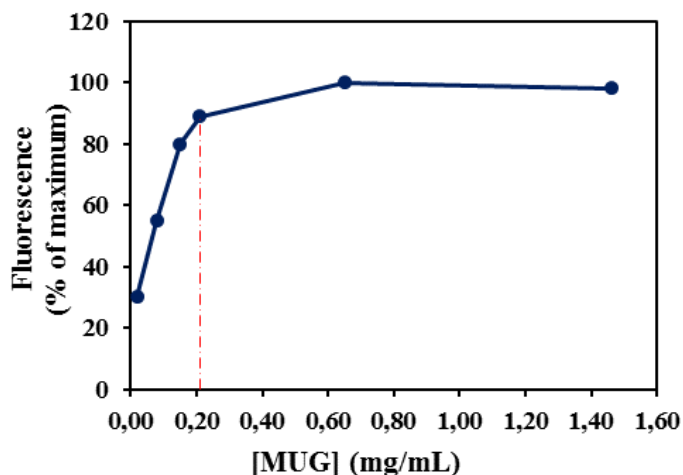


Figure 37: Saturation curve for MUG

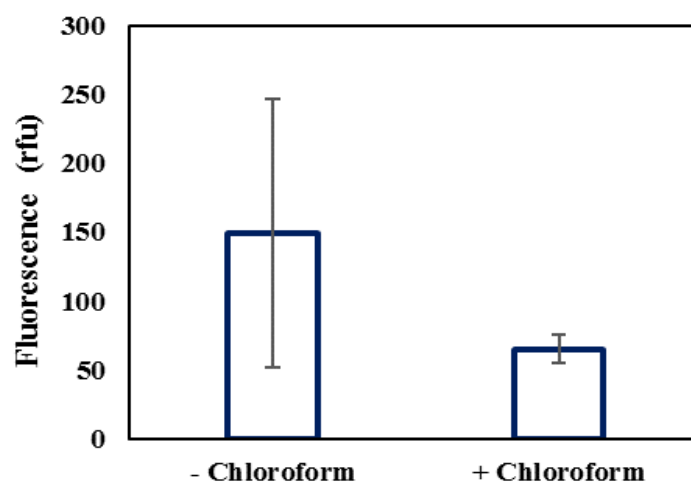


Figure 38: Chloroform influence on the fluorescence

Checking the optimum temperature for medium storage

Reagent A is composed by components easily consumable by bacteria, so that an improper storage could lead to bacterial growth and/or component deterioration. Therefore, the temperature to store reagents in the analyser represented a very important parameter to check. A series of experiments were carried out in order to compare media stored at $-80\text{ }^{\circ}\text{C}$ with identical media stored both at room temperature (RT) and $+4\text{ }^{\circ}\text{C}$. Every day, five samples in water MilliQ and three in Reagent A, were prepared with the addition of 0.5 mL of MU (10^{-6} M) and/or 0.2 mL of MUG (5 mg/mL). After 60-min incubation at $44.5\text{ }^{\circ}\text{C}$, 0.1 mL of Reagent B were added to 2.4 mL of medium and the fluorimetric measurement was performed, according to the automated analytical cycle.

Tests performed on media kept at RT showed that water and Reagent A give identical results and maintain a stable fluorescence, throughout the days. On the contrary, MUG at RT kept increasing the fluorescence signal, due to auto-hydrolysis, and after twenty days, the fluorescence values measurement exceeded the instrumental range, whereas MUG at $-80\text{ }^{\circ}\text{C}$ kept a stable signal (Fig. 39).

On the contrary, when the experiments were conducted by employing media stored at $+4\text{ }^{\circ}\text{C}$, all fluorescence values were always comparable with the corresponding data obtained at $-80\text{ }^{\circ}\text{C}$, and all different compositions of Reagent A preserved stable for two months (Fig. 40).

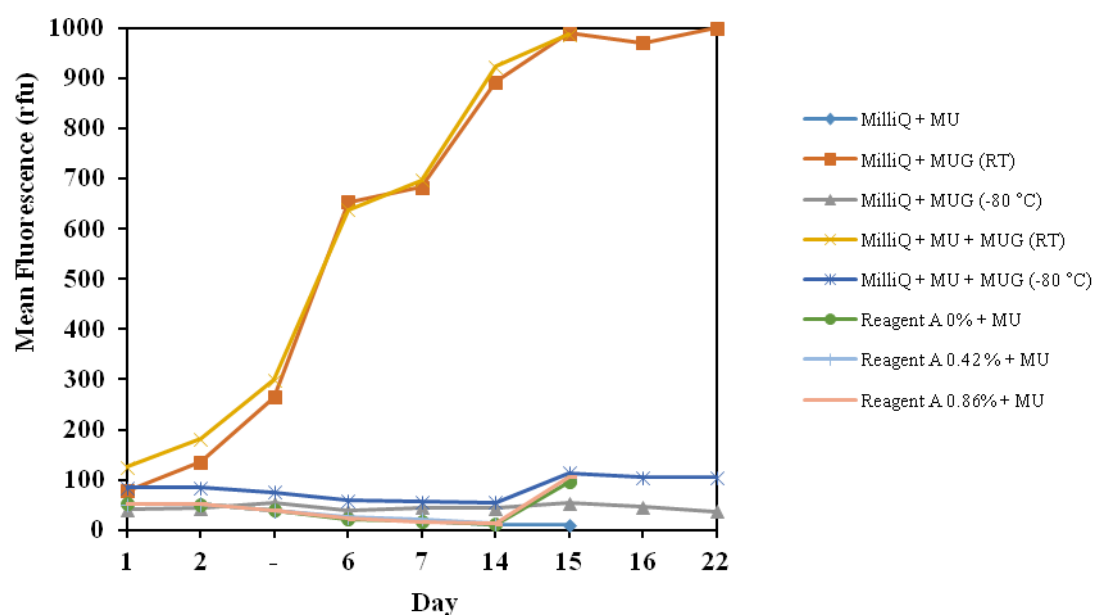


Figure 39 – Media stability at Room Temperature and at -80 °C

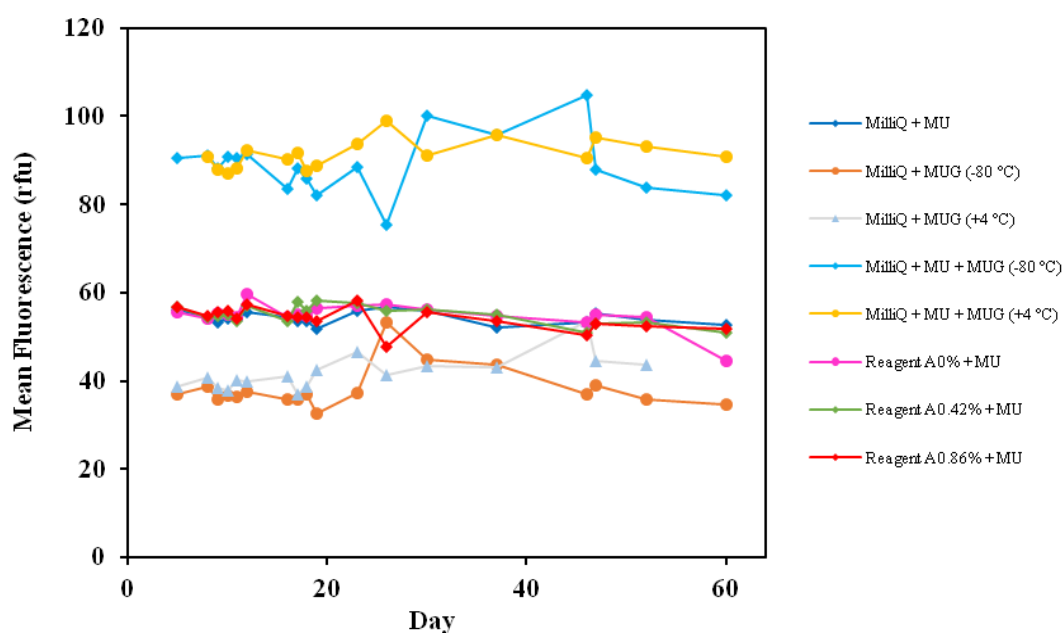


Figure 40 – Media stability at +4 °C and at -80 °C

Selecting the best combination of Reagent A and Reagent B

In order to determine the best combination of reagent volumes to be mixed with samples, some experiments were performed, both in automatic and in manual manner, by adding different volumes of both Reagent A or Reagent B, and measuring the resulting fluorescence (Table 16).

When the manual assay was performed, the best combination of reagents resulted 2.1 mL of *Reagent A* and 0.2 mL of *Reagent B*, which was also found among the best combinations tested in the automatic procedure. Due to smaller amounts required, if compared with equivalently suitable combinations, this mix was confirmed to be used in the automated analyses.

Table 16 Selection of best performing combinations of Reagent A and Reagent B volumes.

		<i>Manual Easy coli assay</i>	<i>Automated Easy coli procedure</i>
<i>Reagent A (μl)</i>	<i>Reagent B (μl)</i>	<i>Fluorescence (rfu)</i>	<i>Fluorescence (rfu)</i>
2000	200	1038	1109
2000	300	1042	977
2100	200	1168	1141
2200	200	1004	1134
2100	300	959	1065
2300	300	1016	1124
2400	300	1132	1189

Sample-reagent mix

One of the basic principle behind automatic analysers is the need to homogenize the sample - reagent zone, and Easy coli analyser made no exception because:

- mixing allowed better contact between microbes and reagents, thus quicker enzyme activation;
- Reagent B needed to be homogeneously distributed over the volume to avoid the creation of a pH gradient affecting the final measurement;
- the product of the enzyme reaction MU was required to emit the same amount of fluorescence at any tube height to avoid artefacts.

For these reasons, when the analytical cycle was carried out simply via addition of sample and reagents, followed by fluorescent measurement at the end of the incubation time, fluctuating results were obtained. In order to increase reproducibility of the process, a turbulent flow created by air bubbles was introduced and inserted every time a reagent was added within the reaction environment, namely, after the addition of *Reagent A* and after the addition of *Reagent B*. Furthermore, after having pumped air in the reaction tube, the needle automatically was forced to enter a glass tube filled with routinely changed water, in order to be subjected to a washing cycle and to avoid cross-contamination events.

3.3.2.3 Improvement of the filtering process

Being the automated filtration an innovative feature in Easy coli analyser, the filtration unit was deeply studied, by analysing some of the most important aspects which could affect the filtration process.

Checking the filter material

Within the automatic device, the physical properties of the filters, such as electrostatic adherence or resistance to deformation, could change respect to the manual procedure. Checks on both the most suitable filter material and cut-off were, therefore, necessary. Four different types of filters, namely Polyvinylidene fluoride (PVDF), Polyethersulphone (PES), Polytetrafluoroethylene (PTFE) and Polycarbonate (PC), having the two standard cut-off (0.22 μm and 0.45 μm), were tested for performing automated analyses on environmental samples (*Table 17*).

Table 17: The tested filters differ for material and cut-off.

Material	Diameter (cm)	Cut-off	
		0.22	0.45
PC	1.3	0.22	0.45
PVDF	1.3	0.22	0.45
MCE	1.3	0.22	0.45
PES	1.3	0.22	0.45

The recovery performances of the different materials were compared in terms of measured fluorescence, whereas the run of Blank samples confirmed that different filter materials induced no interferences on the fluorescence measurements. As for the filter material, the only materials allowing reproducible recoveries higher than 90 % were PVDF and PES, whereas PC and MCE filters produced recoveries comprised between 20 and 70 % (*Fig. 41*), and were excluded due to their physical properties which caused their suction into the disposal vessel by the pump. PVDF was found the most effective due to better handling, i.e. less electrostatic and better recoveries of *E. coli*, therefore it was selected as suitable filter material. On the other hand, results achieved by using 0.20 μm or 0.45 μm filters were similar and, after a statistical evaluation, they were evaluated not significantly different. Considering that filtration through 0.45 μm was quicker and, if necessary, higher volumes can be filtered, this cut-off was chosen as the most suitable one.

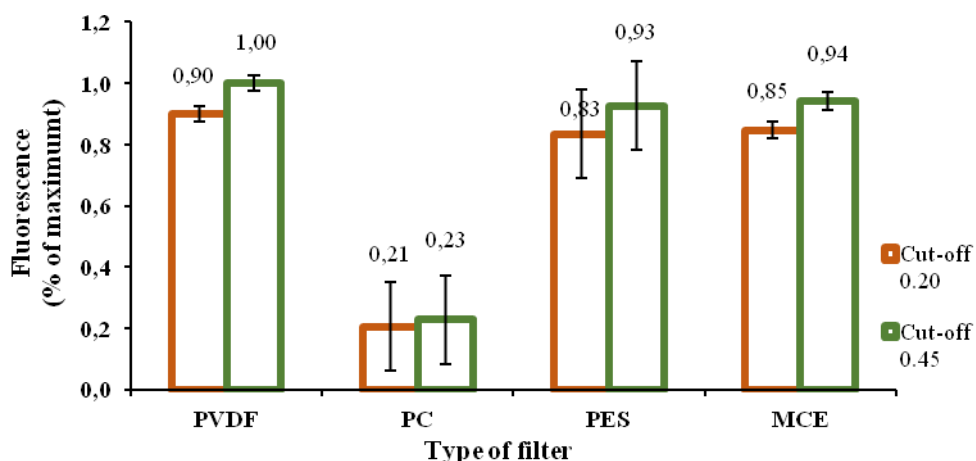


Figure 41: Recovery performances of different type of filters.

Selecting sample volume and dilution

Bacterial contamination and the presence of suspended solids in water are strictly related, because of two main reasons: (i) coming from domestic sewage, the organic contaminants in wastewater effluents have inevitably a faecal derivation and (ii) like other bacteria, *E. coli* has the tendency to be adsorbed to solids in suspension, which are most probably faecal matter. These considerations resulted central when deciding the volume of sample to be automatic filtered and the possibility to enclose a pre-filtration stage.

Some basic consideration about sample volumes guided the experimental design.

- Since the official procedures leave the operator free to choose the sample volume to be filtered, also in the automatic analysis the user will have the possibility to manually set the sample volume.
- If the sample volume is changed, it will be always possible to relate fluorescence and bacterial count, since this value is expressed as concentration, and takes into account the volume originally filtered.
- As the environmental samples tested were rich in *E. coli* and suspended solids, the filtration of 100 mL of sample may cause filter obstruction. A maximum volume of 10 mL was selected as and it was observed to be always possible without causing filter block, being wastewater commonly subjected to primary and secondary treatment before discharge.

In order to establish the effect of dilution on the final results, several experiments were performed, to compare the performances of diluted and non-diluted samples in terms of fluorescence. Three replicates of 10 mL of non-diluted environmental samples and three replicates of 0.5 mL of samples diluted in 9.5 mL physiological solution were filtered on PVDF, Ø 1.3 cm, cut-off of 0.45 µm.

Times required for filtration were carefully measured. The analytical cycle following the filtration was automatically performed within Easy coli analyser; the relative fluorescence automatically measured was related to the microbial count made by plating 20 µl of pure environmental sample on TTC Tergitol-7, at 44 °C for 24 h. As shown in *Fig. 42*, performances of diluted samples were 34 ± 3 % higher than non-diluted.

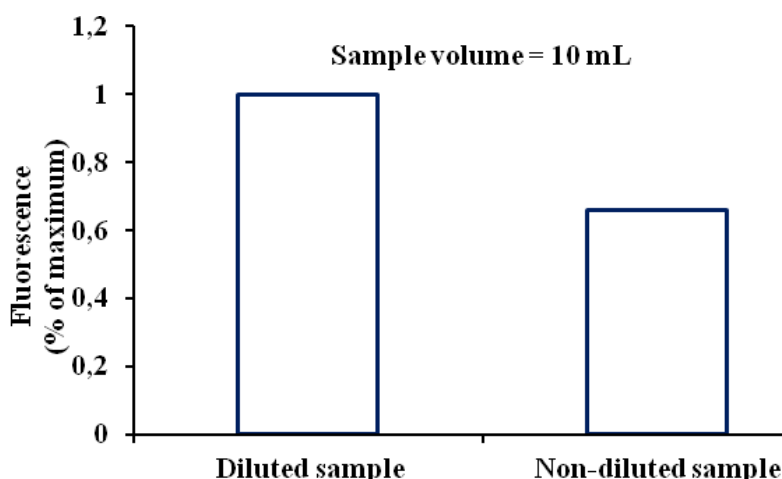


Figure 42 – Influence of dilution on the final results

3.3.2.4 Selection of the reference method and evaluation of process accuracy

In order to evaluate the process accuracy, the automated Easy coli procedure was correlated to official methods. The environmental samples were initially plated on TTC Tergitol-7 by MF, according to the method ISO 9308-1, but poor results were afforded. For these reason, C-EC Agar from Biolife was employed as reference medium. Data coming from a large amount of experiments were used for building the regression line, and, in particular, the correlation between automated process and reference method was achieved by relating the logarithm of the relative fluorescence measured within Easy coli analyser with the logarithm of cell count.

Four replicates of six different environmental samples effluents were analysed according to the automatic cycle in order to measure the corresponding fluorescence values; calibration with 10^{-7} M MU was performed before each analysis. In order to compare the performance of Easy coli analyser with C-EC Agar, different sample dilutions were filtered, plated and incubated at 44 °C for 24 h. The linear regression produced a satisfactory value of $R^2 > 0.8$. Since the selection of C - EC Agar as reference medium allowed to considerably improving the process accuracy, it was used in all the following tests.

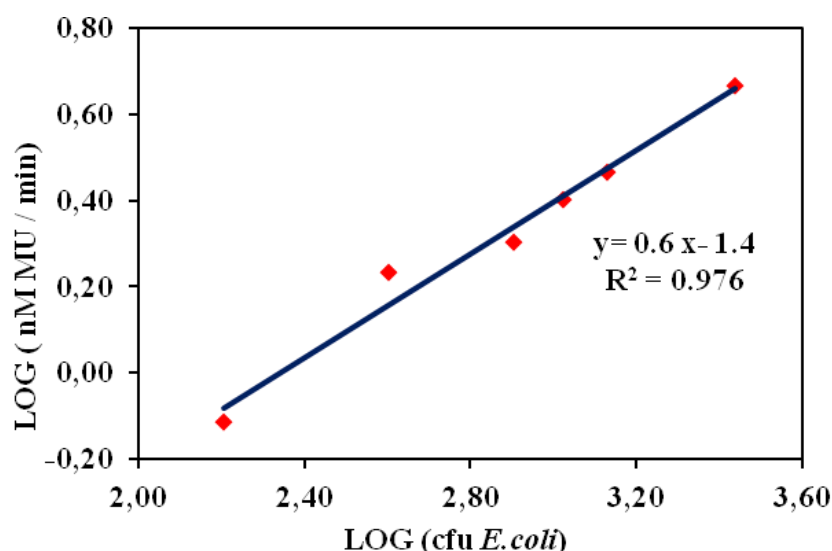


Figure 43: – Log-Log linear regression between *E.coli* quantification performed by Easi coli analyser and cell counts on C-EC Agar.

3.3.2.5. Process repeatability and reproducibility

Repeatability and reproducibility of blank samples

Whit the aim to verify the proper functioning of every stage in the automated process, some experiments were performed on blank sample, for checking the repeatability and reproducibility. In particular, eight experiments, consisting of 2 - 4 replicates of blank samples were performed, by filtering 50 mL of ultrapure water on PVDF filters and by analysing them according to the automated analytical cycle. Blank daily repeatability was expressed in percentage error, whereas the reproducibility of the blank samples was determined to be the mean of daily repeatability. Processing all data obtained in these tests, the maximum and minimum percentage errors were calculated respectively equal to 6.64 % and 0.01 %, while the reproducibility resulted to be 2.23 ± 4.41 %.

As shown in *Fig. 44*, the calculated percentage error lower than 20 % indicated that the final measurement was not affected by all the automated operations which took place in Easy coli analyser, such as sampling, filtration, fluid transfer, addition of reagents and fluorimetric measurement.

Daily repeatability and day-to-day reproducibility

In order to calculate the daily process repeatability, data coming from all the experiments done during the laboratory trial were used, whereas day-to-day reproducibility could not be calculated on the same samples as the bacterial concentration in environmental samples is typically subject to remarkable variations after six hours.

Fluorescence data of replicates performed in identical conditions and achieved by 12-day experiments were firstly averaged, and for each sample the percentage standard error was calculated for determining the repeatability of the process. The process repeatability was comprised between minimum percentage error E%, 1.08 % and maximum E%, 6.87 %. Then, the average value of all the daily standard errors, namely the mean percentage error E% was used as an estimate of day-to-day reproducibility on different samples and was calculated to be 3.52 ± 2.44 % (Fig. 45).

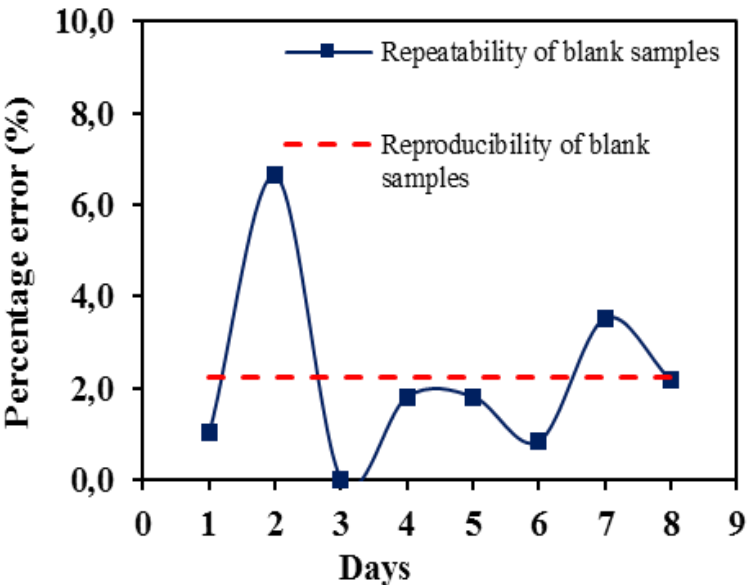


Figure 44: Repeatability and reproducibility of blank samples

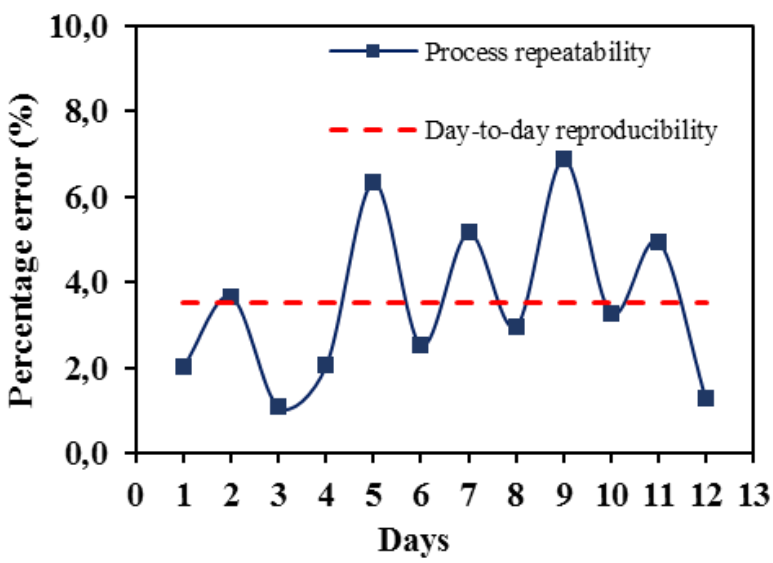


Figure 45: Process repeatability and reproducibility

Calibration stage

In order to avoid any failure of either reagents or equipment and to escape possible weather - dependent fluctuations of the fluorimeter, a calibration stage was inserted at the beginning of each analytical cycle, in order to keep the analysis under control. Furthermore, the calibration curve let to express experimental data in measurable units. Calibration was performed automatically by the analyser and it could be programmed at will.

A linear relationship between 4-methyl-umbelliferone concentration and fluorescence values was observed for [MU] ranging 10^{-8} to 10^{-6} M (Fig. 46), therefore $[MU]=1 \cdot 10^{-7}$ M was selected as reference concentration for performing the calibration.

In this way, all results were reported as concentration of released MU following the enzymatic reaction, and both process failure or potential interferences due to seasonal fluctuation could be notified if calibration values suddenly increased or decreased.

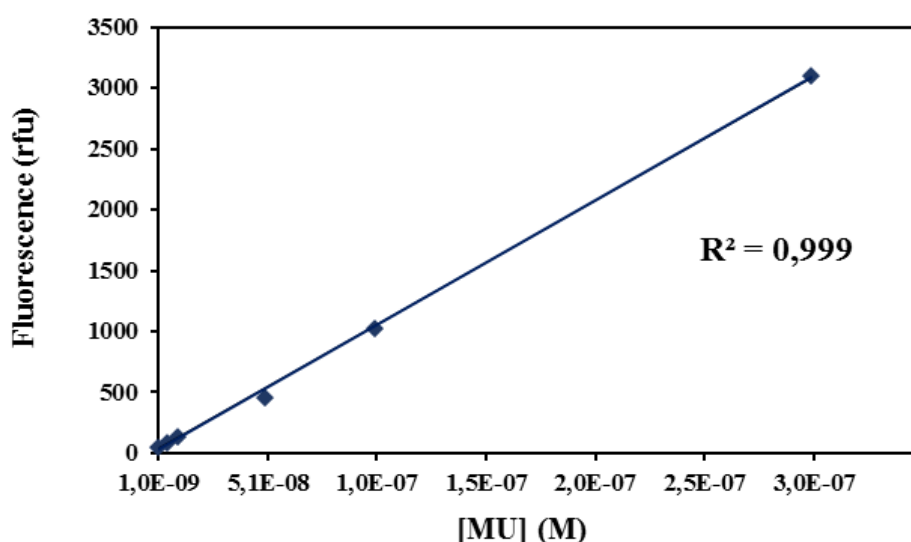


Figure 46 – Linearity between [MU] and fluorescence

3.3.3. Field campaign in a wastewater treatment plant

3.3.3.1. Set up optimal parameters

Finally, a test campaign was conducted in field with the installation of Easy coli analyser in an urban wastewater treatment plant. The system was employed for quantifying *E. coli* in waste water effluents and its efficiency was assessed.

In order to validate the detection performances of the analyser, results coming from the automatic analyses were constantly compared with the official procedure. The optimal parameters were setup

by taking into account all the considerations and results achieved during the previous laboratory campaign (Table 18).

Table 18: Parameters set up for performing the automatic analyses in the field campaign.

Sample	Volume	10 mL
	Dilution	0.5 mL sample in 9.5 mL physiological solution
Reagent A	Composition	Phosphate buffer
	Volume	2.1 mL
	pH	6.9
	Storage Temperature	4 °C
	[MUG]	0.21 mg / mL
	Incubation time	60 min
Reagent B	Composition	NaOH 2 N
	Volume	0.2 mL
	Storage Temperature	4 °C
Filter	Material	PVDF
	Diameter	1.3 cm
	Cut-off	0.45 µm
Calibration	Composition	MU $1 \cdot 10^{-7}$ M
Detection	Fluorimetric kinetic MU-determination	
	Measurement time	60 sec
Reference method	Membrane Filter on C-EC Agar	

Installed at the end of the treatment plant line, Easy coli analyser was programmed to perform *E. coli* quantification in one-hour, at 44.5 °C: next to the automatic sampling and filtration, samples were incubated within the detection unit in presence of Reagent A. The enzyme reaction was stopped by adding 200 µl of Reagent B and fluorescence was measured after few seconds, by positioning the reaction vial in front of the fluorimeter. A solution $1 \cdot 10^{-7}$ M MU was employed for automatically calibrating the system.

3.3.3.2 Analytical cycle

The principles behind the routine analytical procedure adopted during the field trial had the objective to minimize the occurrence of random and systematic errors, such to create a regression line faithfully reflecting the relationship between the automatic procedure and the official method. By means of the dedicated software, a working list was programmed in order to automatically perform the fluorescence measurements of 14 test tubes, including blanks, calibrants and filtered

samples (Fig. 47). Contemporarily, 4 Petri plates, containing C-EC Agar and membrane filters, were employed for filtering known volumes of real sample according to the reference APAT procedure.

Pos	Name	FU Start	FU End	Conc	Status	R1 Start time	R1 Incub. Time	R2 Start time	R2 Incub. Time
1	H2O	240,762	254,522	1011,544		14.27.12	1 h 00 m 09 s	15.27.19	0 h 00 m 59 s
2	H2O	283,081	309,213	1840,434		14.28.09	1 h 00 m 06 s	15.28.16	0 h 01 m 00 s
3	H2O	248,905	295,735	3227,14		14.28.58	1 h 00 m 07 s	15.29.05	0 h 00 m 59 s
4	BcampRind05	231,166	299,28	4653,109		14.30.28	1 h 00 m 05 s	15.30.35	0 h 01 m 00 s
5	BcampRind05	315,27	401,673	5878,421		14.31.27	1 h 00 m 05 s	15.31.32	0 h 01 m 15 s
6	B RA	588,583	774,739	12561,59		14.32.39	1 h 00 m 05 s	15.32.45	0 h 01 m 00 s
7	B RA	228,789	783,794	37273,44		14.33.32	1 h 00 m 05 s	15.33.37	0 h 01 m 00 s
8	B RA	277,069	846,121	38214,55		14.34.23	1 h 00 m 07 s	15.34.29	0 h 01 m 21 s
9	cal [Vol=0]	248,792	1449,651	80543,84		14.35.41	1 h 00 m 06 s	15.35.47	0 h 01 m 00 s
10	cal [Vol=0]	260,464	1648,817	93105,41		14.36.34	1 h 00 m 05 s	15.36.39	0 h 01 m 00 s
11	cal [Vol=0]	205,804	1454,036	83717,7		14.37.24	1 h 00 m 07 s	15.37.30	0 h 01 m 09 s
12	Sample Rind05	193,102	1110,4	61546,05		14.38.28	1 h 00 m 08 s	15.38.35	0 h 01 m 03 s
13	Sample Rind05	252,392	1062,33	54353,23		14.39.27	1 h 00 m 07 s	15.39.34	0 h 01 m 00 s
14	Sample Rind05	237,914	1129,667	59834,61		14.40.22	1 h 00 m 06 s	15.40.28	0 h 01 m 22 s

Figure 47: Worklist of a typical automated routine performed during the field trial

The different run programmed in the worklist aimed to achieve different type of information.

Table 19: Different information achieved from a typical automatic routine

NAME	MEANING	TYPE OF ANALYSIS
H ₂ O	Water Blank	Contains ultrapure water in place of Reagent A and a filter on which ultrapure water was filtered. This measure allows to monitor the system hygiene and calculate background interference due to water.
B Sample	Sample Blank	Contains ultrapure water in place of Reagent A and a filter where a volume equal to that of real sample used for the analysis, was filtered. In this way, chemical interferences of suspended / adsorbed solids are evaluated.
B Reagent.	Reagent Blank	Contains Reagent A, with no filter. This measure allows to calculate the background value of Reagent A, due mainly to MUG self-hydrolysis.
Cal.	Calibration	Contains Reagent A and MU $1 \cdot 10^{-7}$ M, in order to calculate the daily relative fluorescence unit value of MU.
Sample	Filtered sample	Contains Reagent A and a filter where a known volume of real sample was filtered.

The data visualised on the software window were then processed by means of appropriate calculator programs. Specifically, the dataset was organized in a well-defined Excel worksheet, as shown in Table 20.

Table 20:– Excel worksheet for processing analytical data

Automated analytical cycle							
Name	FU Start	FU End	Start –End	Type of Data	Value		
H ₂ O	169.755	209.695	40	Mean (H ₂ O) ►	31		
H ₂ O	250.62	271.809	21				
B Sample	286.512	329.353	43	Mean (B Sample) ►	35	M (B Sample) – M (H ₂ O) ►	4
B Sample	255.923	283.189	27				
B Reagent	217.665	609.302	392	Median (B Reagent) ►	392		
B Reagent	188.251	566.543	378				
B Reagent	153.454	581.102	428				
Cal	185.054	1416.083	1231	Median (Cal) ►	1231	Cal - B Reag / 100 (1nM MU) ►	8.394
Cal	187.602	1397.291	1210				
Cal	177.7	1556.385	1379				
Sample	288.774	1114.495	826	Median (Sample) ►	715	Sample (MU nM) ►	38.04
Sample	275.121	865.316	590				
Sample	288.75	994.369	706				
Sample	296.311	1021.601	725				
						Volume filtered (mL) ►	1.0
						Time►	60 s
Standard method							
	cfu (on Petri)	cfu (100 mL)					
MF 1	12	12000		Median MF ►	18000		
MF 2	16	16000					
MF 3	22	22000					
MF 4	20	20000					
Correlation							
Sample MU (nM / min)		0.634		LOG MU (nM / min)		-0.198	
cfu		180		LOG (cfu)		2.26	

The meaning of data calculated in the Excel worksheet are explained in the following.

- *Start – End*. Final values were obtained by subtracting Fluorescence start values (FU Start) from fluorescence end values (FU End) for each blank, calibrant and sample. In this way, every background interference caused by intrinsic fluorescence of the reaction tube was removed.
- *Mean (H₂O)*. Mean value of Water Blanks.
- *Mean (B Sample)*. Mean value of Sample Blanks.
- *Median (B Reagent)*. Median value of Reagent Blanks.
- *Median (Cal)*. Median value of Calibrants.
- *Median (Sample)*. Median value of Samples.
- *Median MF*. Median value of colony forming units counted on the Petri plates, multiplied by the suitable dilution factor such to estimate real *E. coli* concentration in 100 mL.
- *Volume filtered*. The volume of effluent sample analysed within the automated system.
- *Time*. The incubation time of the analysed blanks, calibrants and samples.

Finally, data processing was performed as follows:

- *Sample Background* = $M(B\ Sample) - M(H_2O)$.

This value represented the background fluorescence value caused by physical / chemical interference of particles adsorbed to the membrane filter.

- *MU Value (1 nM MU)* = $Median(Cal) - Median(B\ Reagent) / 100$.

This value represented the daily fluorescence of 1 nM MU.

- *Sample (MU nM)* = $[Median(Sample) - Median(B\ Reagent) - Sample\ Background] / MU\ Value(1\ nM\ MU)$.

This value represented the sample measurement achieved in the automatic analysis, expressed in terms of nM concentration of MU produced in the enzymatic reaction. The potential interferences coming from both sample particles and MUG autohydrolysis, were subtracted to the *Median(sample)* value, and the result was normalised respect to the daily fluorescence value of 1 nM MU.

- *Sample MU (nM / min)* = $Sample(MU\ nM) / Time$.

This value represents the sample value achieved in the automated analysis expressed in nM MU produced per unit time.

- *cfu* = $Median\ MF \times Volume / 100$.

This value represents the estimate of *E. coli* concentration in the real sample analysed.

- *Sample MU (nM / min)* and *cfu* were finally converted in logarithmic units and introduced in the standard regression line respectively as LOG MU (nM / min) and LOG cfu.

3.3.3.3 Treatment of samples in disinfection process

Four main types of disinfection agents are commonly used in order to drastically decrease the microbiological contamination in effluents: chlorine, peracetic acid, ozone and UV rays.

Chlorine-based compounds are the most commonly used disinfectants; their efficacy requires the accomplishment of two main principles: concentration, which has to be in the range 2 - 8 ppm and time of exposure, less than one minute. Other factors such as temperature, pH and presence of organic particulate (which reacts with halogens and reduce the disinfection capacity), considerably influence the process.

Peracetic acid comes from the reaction between hydrogen peroxide and acetic acid and represents a powerful antimicrobial agent since causes the breakage of sulphur-sulphur bonds in proteins and inhibits the chemiosmotic role of lipoproteins of the cytoplasmatic membrane. By oxidizing essential enzymes within the cells, the action of peracetic acid affects the metabolic cycle, the active transport and the level of intracellular solute. In twenty minutes, 1.5 - 2 ppm of peracetic acid are able to inactivate 97% of *E. coli* in wastewater.

Ozone disinfection is generally used at medium to large sized plants after at least secondary treatment. Ozone disinfection has the ability to achieve higher levels of disinfection than either chlorine or UV; being an extremely reactive gas, O_3 rapidly reacts, forming O_2 and reducing the microbial load.

An UV ray disinfection system transfers electromagnetic energy from a mercury arc lamp to an organism's genetic material, retarding its ability to reproduce. The effectiveness of UV ray system depends on the characteristics of the wastewater, the intensity of UV radiation, the amount of time the microorganisms are exposed to the radiation and the reactor configuration.

With reference to the experimental field trial, wastewater was mainly disinfected by ozonisation or treated within secondary settlers.

3.3.3.4 Data collecting and data processing

During the field trial, samples from ozone-treated effluents and from secondarily treated but not disinfected effluents were analysed with Easy coli analyser. Effluents sampling was performed in order to obtain measurable results, circumventing the occurrence of over-range values. Since most of the analysed effluents were highly contaminated, small sample volumes, ranging between 0.5 and 5 mL were sufficient to fulfil these requirements.

All samples were tested in quadruplicate, higher and lower values were discarded, and the means were calibrated with daily MU values as described in the above. Reference counts were performed by C-EC agar plating.

Building regression line

Among the great number of data achieved in the field campaign, 35 ozone-treated or non-disinfected-samples were considered for relating Easy coli performances with official procedures. The processed results, coming from the selected experiments, were listed in *Table 21* and were used for building the standard regression line.

Table 21: Mean data coming from ozone-treated and non-disinfected samples analysed by both Easi coli analyser and C-EC Agar Membran Filtration method. For each type of samples Standard Deviation was determined, too.

Type of Treatment	Automated Easy coli			MF		
	MU (nM/ min)	LOG (nM MU/min)	SD (nM MU/min)	E. coli (cfu)	LOG(cfu E. coli)	SD (cfu E. coli)
Ozone	2.525	0.402	0.767	1068	3.03	305
Ozone	2.927	0.466	0.202	1350	3.13	96
Secondary	0.770	-0.114	0.296	160	2.20	32
Ozone	4.627	0.665	0.180	2750	3.44	480
Ozone	1.523	0.183	0.786	400	2.60	191
Ozone	2.007	0.303	0.089	800	2.90	299
Ozone	6.203	0.793	1.636	1450	3.16	411
Secondary	0.718	-0.144	0.020	22.5	1.35	4
Ozone	0.271	-0.567	0.244	10	1.00	17
Ozone	3.329	0.522	0.199	680	2.83	136
Secondary	0.712	-0.148	0.135	100	2.00	173
Ozone	1.168	0.067	0.596	120	2.08	30
Secondary	4.301	0.634	0.539	1275	3.11	1025
Ozone	0.221	-0.655	0.089	15	1.18	8
Secondary	0.992	-0.003	0.567	100	2.00	34
Secondary	1.897	0.278	0.256	420	2.62	61
Ozone	3.052	0.485	1.255	450	2.65	104
Secondary	1.914	0.282	1.885	350	2.54	61
None	7.147	0.854	0.710	2350	3.37	0
Secondary	0.718	-0.144	0.433	90	1.95	57
Ozone	1.304	0.115	0.317	195	2.29	74
Secondary	0.883	-0.054	0.029	210	2.32	38
Secondary	4.184	0.622	0.428	1020	3.01	0
Secondary	0.660	-0.180	0.448	70	1.85	13

Ozone	3.128	0.495	0.290	405	2.61	94
Secondary	1.496	0.175	0.102	130	2.11	50
Ozone	1.526	0.183	0.357	30	1.48	0
Secondary	1.057	0.024	0.032	50	1.70	0
Secondary	1.462	0.165	0.458	120	2.08	49
Secondary	0.600	-0.222	0.076	172.5	2.24	53
Ozone	1.189	0.075	0.151	302	2.48	8
Ozone	1.585	0.200	0.245	240	2.38	37
Secondary	6.457	0.810	1.382	475	2.68	106
Secondary	6.019	0.780	0.354	1480	3.17	594
Ozone	0.634	-0.198	0.192	160	2.20	50

Despite few samples were poorly contaminated, a significant 76 percent of the variance of enzyme activity detected by Easy coli was explained by linear relationship with official bacterial counts (Fig. 48).

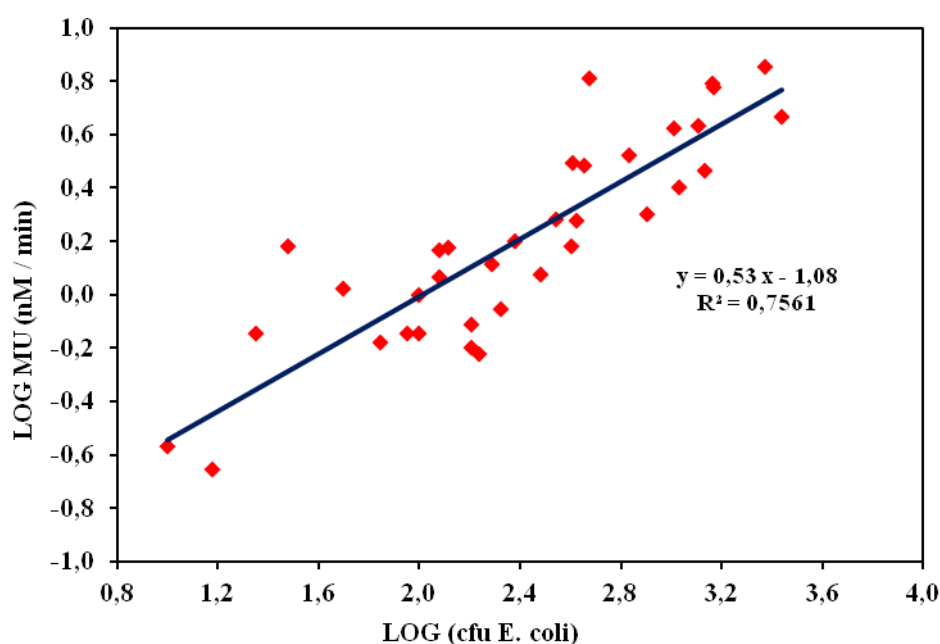


Figure 48: Non-treated and ozone-treated effluents, regression line

Statistical analysis of results

All data, obtained by both the automatic procedure and the reference method were statistically analysed, by calculating the standard error for each sample. As shown in Fig. 49, the standard errors of colony counts (horizontal purple bars) achieved via MF procedure resulted larger than those achieved when using the automatic Easy coli procedure (vertical green bars).

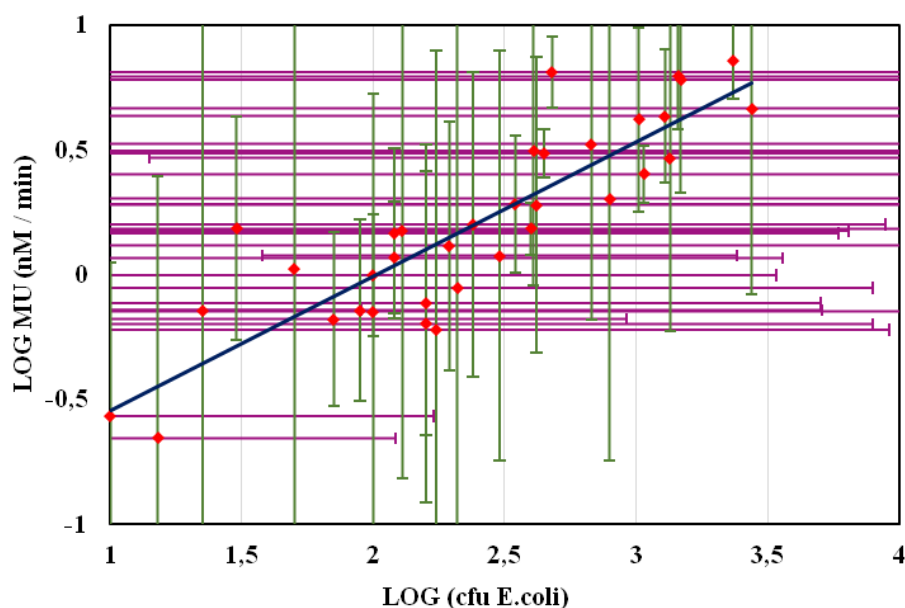


Figure 49: Non-treated and ozone-treated effluents, results with standard deviation

Calibration accuracy

The fluorescence measurement of calibrant was introduced in order to improve the accuracy of Easy coli results. Despite in the vast majority of the experiments, 1 nM MU ranged from 6.9 to 8.7 rfu, this value was sporadically observed to oscillate between 3 and 10. It is likely that these oscillations may be attributed to random errors rather than to a natural variation in the relationship between MU concentration and fluorescence emitted. The mean value, directly calculated from the experimental values, was found to be equal to 7.555 ± 1.371 rfu.

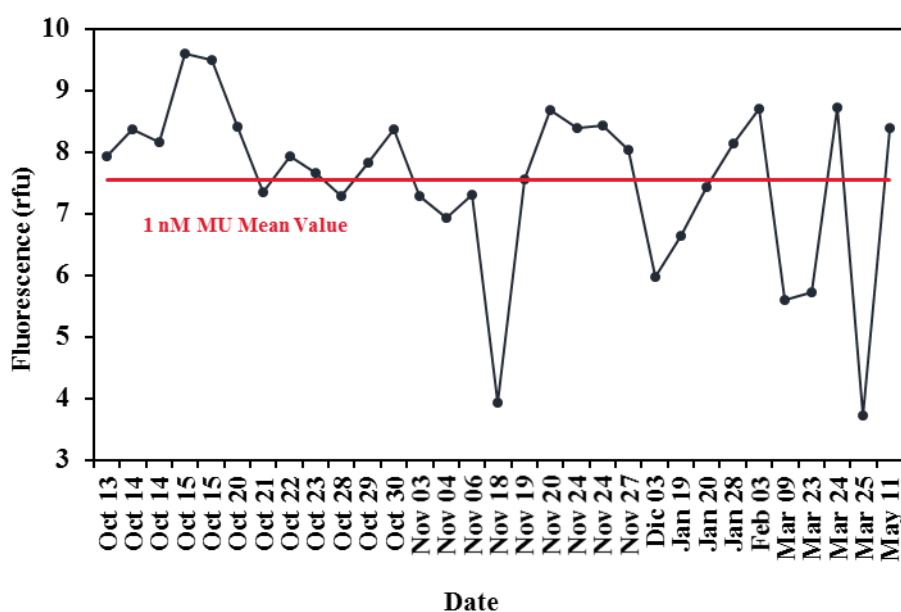


Figure 50: Fluorescence of 1 nM MU, daily measured for calibrating the analyser.

3.4 Conclusion

One of the main purposes of this PhD Thesis was the development of Easy coli analyser, a fully automated on-line self control system for detecting and quantifying *E. coli* in wastewater effluents and surface waters, before and after disinfection.

Firstly, a rapid one-hour procedure was set-up, optimized, carried out and compared with official Membrane Filtration methods. Secondly, the assay was improved within the robotic device in order to provide analytical automation in each step of analysis, from sampling to detection and data treatment. All units were found suitable for microbiological procedures. The software was able to monitor and control the whole process. The reagent delivery was tested and times and volumes were profitably automated. The detection unit provided encouragingly good results in terms of low detection limits, high sensitivity and significant reproducibility.

A field campaign was successfully performed, by quantifying *E.coli* on real samples coming from a wastewater treatment plant. A significant linearity was obtained, when the results collected from the automated Easy Coli were correlated with those determined using the official *MF* method.

In conclusion, by monitoring the efficacy of disinfection treatments, this novel technology provides feedback to this process whose parameters, especially the amount of oxidizing agent, could be finely regulated according to the level of disinfection required.

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Acknowledgments

I would like to express my deepest gratitude to Pompeo Moscetta and Systea S.p.A. that funded this research and made available an astonishingly unique combination of facilities, staff and expertise.

A very special thanks goes out to my supervisor, Sergio Bodini, whose expertise, understanding, and patience, added considerably to my graduate experience. I appreciate his vast knowledge and skill in many areas, and his assistance in guiding me through these years of studying, learning and growing.

I must also acknowledge Prof. Maria Teresa Giardi and the staff at the Institute of Crystallography, National Research Council of Italy (CNR), Area of Research of Rome for their valuable cooperation.

I would also like to thank my family for the support they provided me through my entire life and in particular, I'm very grateful to Antonio, Mary and Max for being my "little family", without whose love, encouragement and editing assistance, I would not have finished this thesis.

In conclusion, I recognize that this research would not have been possible without the support and assistance of Prof. Stefania Masci, DAFNE, University of Tuscia, Viterbo, Italy.