CZECH UNIVERSITY OF LIFE SCIENCES PRAGUE
FACULTY OF ENVIRONMENTAL SCIENCES
DEPARTMENT OF APPLIED ECOLOGY

Assessment of the ATP assay for monitoring microbial water quality

BACHELOR THESIS

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2024

CZECH UNIVERSITY OF LIFE SCIENCES PRAGUE

Faculty of Environmental Sciences

BACHELOR THESIS ASSIGNMENT

Kateřina Khollová

Environmental Engineering

Thesis title

Assessment of the ATP assay for monitoring microbial water quality

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Objectives of thesis

The overall aim of the bachelor thesis is to assess the usability of the ATP measurement for observing microbial quality of water. The theoretical part of the work will focus on the current state of measuring microbial contamination in the aquatic environment, available standardized protocols, and related Czech and EU legislations. The practical part of the work will be performed on process water samples by using selected ATP methods and microbial cultivation techniques.

Methodology

The bachelor work is experimental. Methodologically, it will be structured as the comprehensive literature review followed by the practical part conducted in the laboratory conditions in compliance with instruments' guidelines and related ISO/EN standards.

The proposed extent of the thesis

50-60 pages incl. appendixes

Keywords

adenosine triphosphate, total, free, ME, CFU, Escherichia coli, microorganisms, aquatic

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Recommended information sources

Affek, K., Muszyński, A., et al., 2020: Evaluation of ecotoxicity and inactivation of bacteria during ozonation of treated wastewater. Desalin. Water Treat. 192:176–184. DOI: 10.5004/dwt.2020.25775

ISO 6222:1999-ed.2.0: Water quality – Enumeration of culturable micro-organisms – Colony count by inoculation in a nutrient agar culture medium. Geneva: International Organization for Standardization, 4 p.

ISO 9308-1:2014-ed.3.0: Water quality – Enumeration of Escherichia coli and coliform bacteria – Part 1: Membrane filtration method for waters with low bacterial background flora. Geneva: International Organization for Standardization, 10 p.

Pan, R., Zhang, K., et al., 2021: Characteristics of biostability of drinking water in aged pipes after water source switching: ATP evaluation, biofilms niches and microbial community transition. Environ. Pollut. 271:116293. DOI: 10.1016/j.envpol.2020.116293

Schmidt, T.M. (ed.), 2019: Encyclopaedia of Microbiology. Fourth Edition. Amsterdam: Elsevier, Inc. ISBN 978-0-12-811737-8

Vang, Ó.K., 2013: ATP measurements for monitoring microbial drinking water quality. PhD Thesis. DTU Environment, Department of Environmental Engineering, Technical University of Denmark, Lyngby, Denmark, 169 p.

Expected date of thesis defence

2023/24 SS – FES

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Electronic approval: 19. 12. 2023

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Electronic approval: 6. 2. 2024

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Dean

Prague on 25. 03. 2024

Bachelor thesis author's statement

I hereby declare that I have independently elaborated the bachelor/final thesis with the topic of: Assessment of the ATP assay for monitoring microbial water quality, and that I have cited all of the information sources that I used in the thesis as listed at the end of the thesis in the list of used information sources.

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Acknowledgements

I would like to thank to my supervisor doc. Ing. Lenka Wimmerová, MSc., Ph.D., for letting me be a part of this work and providing me with guidance during this research. Then I would like to thank to Anshu Shaw, M.Sc., who helped me to learn new things during this research. My experience and knowledge have significantly grown during this research.

Abstract

The overall aim of the bachelor thesis was to assess the usability of the ATP measurement for observing microbial quality of water. The theoretical part of the work focused on the current state of measuring microbial contamination in the aquatic environment, available standardized protocols, and related Czech and EU legislations. The practical part of the work was performed on process water samples by using selected ATP methods and microbial cultivation techniques. The secondary objective was a comparison of the achieved results recalculated to the ME's and CFU.

According to the results that were obtained in the experimental part completed in laboratory, the DeltaTox, FLAA ATP Bioluminescent Assay and the SystemSURE Plus standards were all accurate and showed a high precision of the best fit. The achieved R2 values were higher than 0.98 for all used standard's kits and the measuring devices. The measured water samples showed the similar ATP results using the SystemSURE Plus kit and the FLAA ATP Bioluminescent Assay kit. The results achieved for the DeltaTox kit was a significantly higher. The *E. coli* cultivation results revealed that one colony (CFU) isolated from the same water samples have the same but much higher content of ATP than it is used for the microbial equivalent (ME) recalculation. The correlation between CFU/mL and Number of cells/mL had showed to be high.

Key words

Adenosine triphosphate, total, free, ME, CFU, *Escherichia coli*, microorganisms, aquatic

List of abbreviations

AMP – Adenosine Monophosphate

ATP – Adenosine Triphosphate

CFU – Colony Forming Units

fATP – Free ATP

GDWQ – Guidance for Drinking-water Quality

IME - Individual Microbial Equivalent

ISO – International Organization for Standardization

LRI – Lower Respiratory Infection

ME – Microbial Equivalent

PPi – Pyrophosphate

RLU – Relative Light Units

STEC – Shiga toxin-producing *Escherichia coli*

tATP – Total ATP

WFD – Water Framework Directive

WHO – World Health Organization

YLLs – Years of Life Lost

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1. Introduction

Water quality is essential aspect of daily life, and it has impact on personal well-being. Among various parameters affecting water quality, the main concern for people is microbiological content of water, particularly drinking water. The Water Framework Directive serves as the main law for Europe. It gives the standards and legislation according to which practices the water should be treated. The main part of ensuring water quality is to ongoing monitoring and testing the content of water to preserve public health and environmental sustainability.

Water contains a variety of microorganisms derived from various sources, such as soil, vegetation, domestic and industrial wastes. Estimation of the microbial load provides useful information for the assessment and observation of water quality. Separate counts are usually made of the microorganisms which can grow and form colonies on nutrient agar media at 36 °C and 22 °C. Colony counts are useful for assessing the integrity of ground water sources and the efficiency of water treatment processes such as coagulation, filtration and disinfection and provide an indication of the cleanliness and integrity of the distribution system. They can also be used to assess the sustainability of water supply required for the preparation of food and drink, where the water should contain no microorganisms that could contaminate the product. The main value of colony counts lies in the detection of changes from those expected, based on frequent, long-term monitoring. Any sudden increase in the count can be an early warning of serious contamination and calls for immediate investigation (EN ISO 6222, 1999). For these reasons the development of testing methods is important for the right and accurate results.

2. Objectives of thesis

The overall aim of the bachelor thesis is to assess the usability of the ATP measurement for observing microbial quality of water.

The theoretical part of the work was focused on the current state of measuring microbial contamination in the aquatic environment, available standardized protocols, and related Czech and EU legislations.

The practical part of the work was performed on process water samples by using selected ATP methods and microbial cultivation techniques. The secondary objective was a comparison of the achieved results recalculated to the ME's and CFU.

3. Literary research

3.1.Microbiology

Microbiology is the study of microscopically small organisms, which are a group of simple life forms that include bacteria, archaea, algae, fungi, protozoa, and viruses. It is a vast and multidisciplinary field that overlaps with other sciences such as genetics, biochemistry, molecular biology, and engineering. Microbiology aims to understand and expand our knowledge of microorganisms by studying their metabolism, morphology, reproduction, and genetics. The research has some key roles in understanding some past as well as present challenges. It has been of great help in discovering problems related to health, food, and water. Research in microbiology has led to knowledge and development in concerning areas such as control of infectious diseases, medicine, veterinary medicine, the food industry, and environment related issues.

Prior to the 17th century, people only knew about some entities responsible for the decay and diseases of animals and humans. When the microscope was invented, at the beginning of 17^{th} century, the study of microbes came into existence and microbiology became more studied and developed. Hence, the invention of microscope, led to microbiology becoming its own specialization and helped in discovering the large and diverse groups of microorganisms.

The categories of microbiology studied are as follows:

- Morphology;
- Nutrition;
- Physiology;
- Reproduction and growth;
- Metabolism;
- Pathogenesis;
- Antigenicity;
- Genetic properties;
- Inter-microbial interactions.

3.1.1. Pathogenic microorganisms

Microorganisms inhabit every environment on the planet and can be found in the human body, mostly in the gut. Most microorganisms are harmless to humans and play an important role in the health of plants, humans, and animals. However, some pathogenic microorganisms can cause diseases in the host. There are four main types of pathogens: bacteria, viruses, fungi, and parasites. Throughout history, there were significant events caused by pathogens, which mostly comprised of viruses and bacteria, and have a huge impact on the human population.
There are three main categories of pathogens, from which only a minority of species can cause diseases.

- Facultative pathogens can colonize their hosts without causing infections. These are primarily environmental bacteria and fungi. Examples include *Escherichia coli*, *Streptococcus pneumoniae*, *Neisseria meningitidis*, and many others.
- Obligated pathogens require a host to complete their life cycle. The pathogen is dependent on the cell wall of the host for reproduction. Some require more than one host to fulfil their life cycle. Examples include *Plasmodium* species, *Halipegus occidualis*, human immunodeficiency virus, and many others.
- Opportunistic pathogens are benign but have a latent ability to cause diseases. This group consists mostly of bacteria.

Pathogens can cause various illnesses through many ways. Pathogenesis involves the ability of a pathogen to enter the host, evade the host's immune defences, multiply, and cause tissue damage. The immune system can respond to the pathogen by killing, or it can overreact and start to kill infected and uninfected cell tissues, which can lead to self-harming of the organism by its own immune system. Some pathogens benefit from host immune reactions. They can be spread by air, touch, bite, or transmitted during pregnancy; as a result, the pathogen can easily infect other hosts.

Bacterial infections have a large impact on public health. Generally, bacterial infections are easier to treat than viral infections since the armamentarium of antimicrobial agents with activity against bacteria is more extensive. Moreover, due to the ability of viruses to change their genetic makeup which in turn changes the receptors on their outer surface, it is challenging to target them directly. Therefore, it is easier to attack bacteria by the help of antibiotics. However, bacterial resistance to antimicrobials is a rapidly growing problem with

potentially devastating consequences (Doron, Gorbach, 2008). For the year 2019, there were 7,7 million deaths caused by pathogenic bacteria (Antimicrobial Resistance Collaborators, 2022).

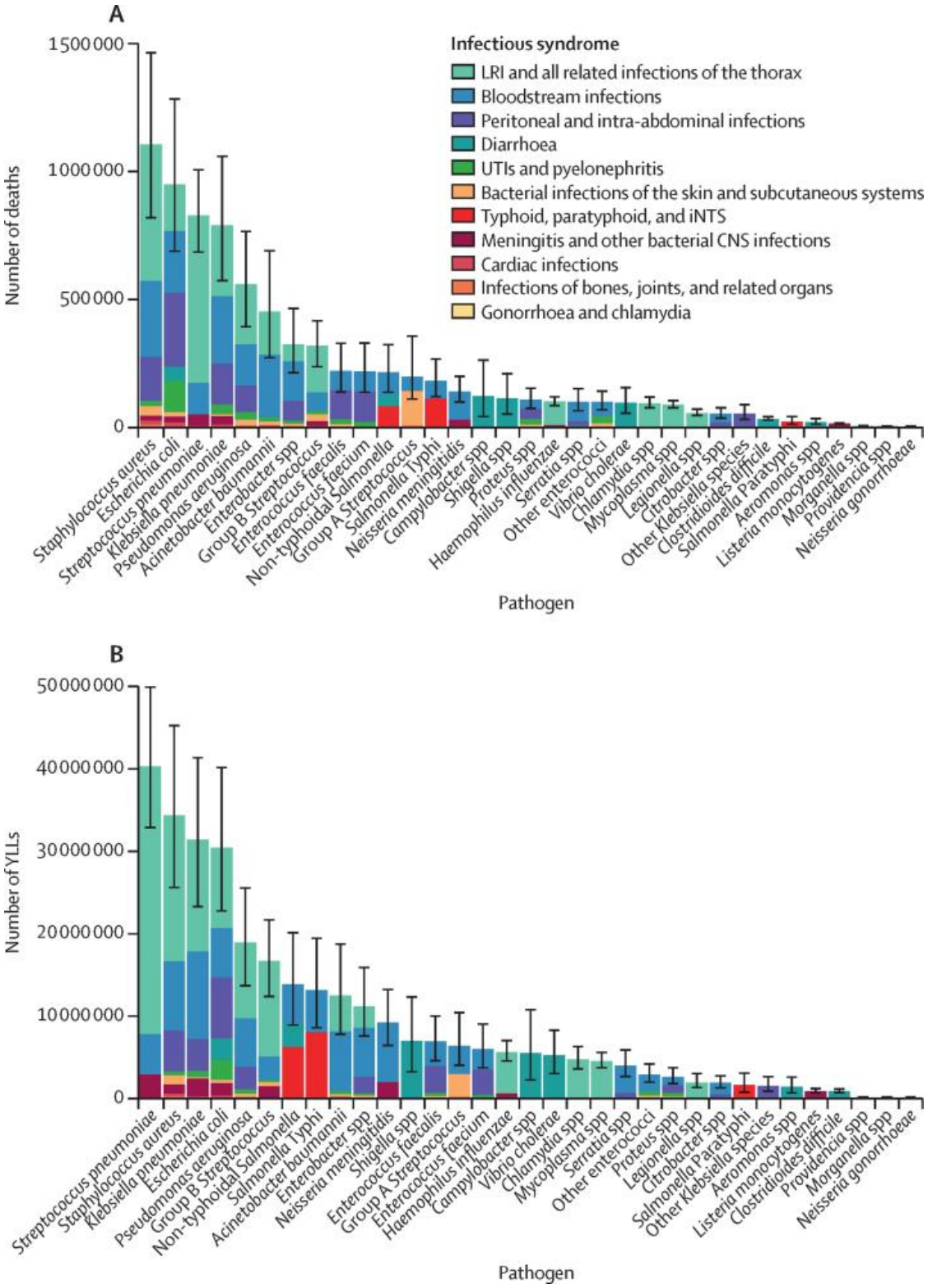

Figure 1: Global number of deaths (A) and YLLs (B), by pathogen and infectious syndrome, 2019 Columns show total number of deaths for each pathogen, with error bars showing 95% uncertainty intervals, with the bars split into infectious syndromes. LRI=lower respiratory infection (Antimicrobial Resistance Collaborator, 2022)

Throughout history, various diseases caused by pathogenic viruses have had a significant impact on the population. The main argument for the occurrence of these pathogens is linked to agriculture, which is based on the closeness between traditional farming with livestock and the rise in human population density. However, this neglects the fact that pathogens can evolve fast. The events caused a high number of deaths, but they helped in the development of new medicine and a better understanding of microbiology. For most diseases, there are vaccinations, and for that reason, their occurrence is low to non-existent.

3.1.2. Aquatic microorganisms

Many microorganisms play fundamental roles in aquatic ecosystems, capturing energy from the sun through photosynthesis, decomposition, and releasing nutrients stored in organic tissue, which play important roles in the cycling of nutrients such as nitrogen, phosphorus, and carbon. They are found naturally in fresh water and saltwater, where they need to adapt to extreme conditions, such as low and extremely high salinity. Others must deal with extremely cold or high temperatures. These include bacteria, cyanobacteria, protozoa, algae, and other small animals. These microorganisms also play important roles in the food chain.

Drinking water is typically most tested and protected against microbial contamination. In many countries there are regulations to specify, how often are water resources tested, how the sampling is done, how the analysis is done, and the accepted limits for different types of microorganisms. There are also methods using ATP testing for water quality, where the total ATP in drinking water is the sum of microbial ATP and free ATP. The concentrations in drinking water are very similar across countries, water supply systems with different sources, and different treatment trains in waterworks (Vang, 2013). Testing of microbes, that cause diseases, is sometimes expensive, and the bacteria may escape the detection, because they can be in low numbers. A highly standardized and routine laboratory procedure to determine water quality is based on the detection of *E. coli*. Historically, for example, drinking water was stored in jugs that were made from metal, which is currently known for its antibacterial effects. Another type of water sterilization is boiling, which kills bacteria. The use of chemicals, such as chlorine, has become popular in the early decades of the twentieth century. Currently, there are operations for cleaning water at sewage treatment plants, where bacteria are used to break down human waste. Filtration and chlorination are very common in drinking water.

3.1.3. Escherichia coli

Escherichia coli is a Gram-negative bacterium that is commonly found in the intestines of human and warm-blooded animals. *E. coli* is a large and diverse group of bacteria. Most strains of *E. coli* are harmless, but some of them can cause severe foodborne diseases. STEC is kind of *E. coli*, that produce toxins, known as Shiga-toxins, that are similar to the toxins produced by *Shigella dysenteriae*. The symptoms of STEC include abdominal pain, fever, vomiting and diarrhea, which, in some cases, can progress into bloody diarrhea. The incubation period is in the range from 3 to 8 days. Most patients recover within 10 days, but around 5-10% of infected people can also be diagnosed by the hemolytic uremic syndrome, which can be life threatening. For the STEC, there are several tests that diagnose the disease, and then the next step is treatment with antibiotics. There is no special treatment for patients having *E. coli* infections. It subsides by natural immune defences. The virulence lends the ability to evade host defences and develop resistance to common antibiotics.

E. coli is transmitted to humans mostly through food, that was contaminated. Most of the time it is raw or undercooked meat products and raw milk. Faecal contamination of water and other foods, as well as cross-contamination, will lead to infection. The prevention of infection requires control measures at all stages of the food chain, from agricultural production to the household. For the industry it mostly depends on good hygienic conditions of slaughtering practices, that can reduce the contamination. Prevention in household involves keeping everything clean, separating cooked and raw food, cooking on high temperature and for enough time, storage of food at right temperature, and using safe water and raw materials. The temperature during cooking should reach at least 70 °C. Washing of fruits and vegetables thoroughly is also a very important step.

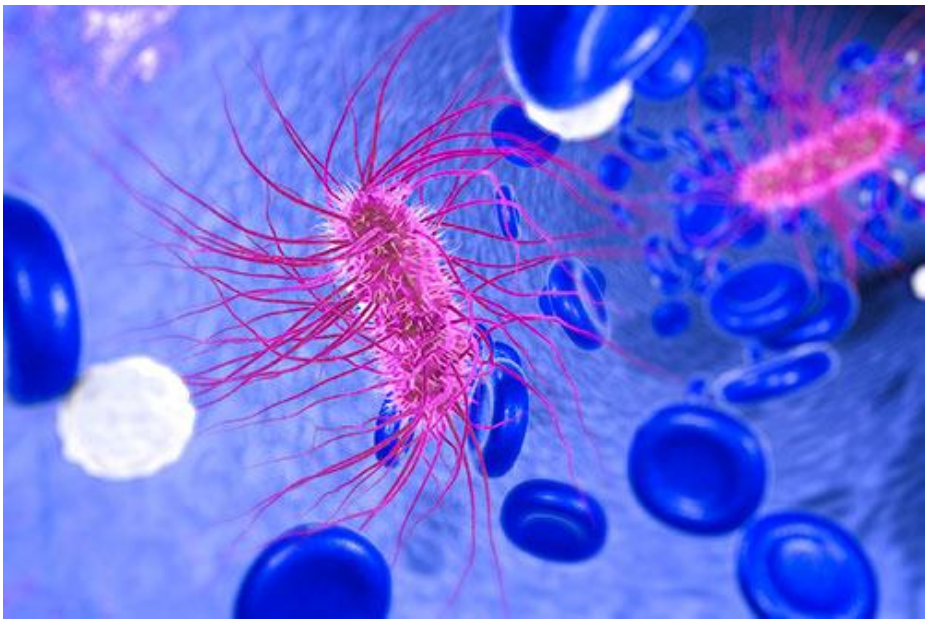

Figure 2: *Escherichia coli* (FDA, 2019)

One significant application of *E. coli* lies in the utilization as a reference organism for converting ATP to microbial equivalents, which shows the quantification of microbial contamination in various samples. Thanks to *E. coli* living in gastrointestinal tract of warm-blooded organisms, including human, it is one of the most studied genomes. These attributes position *E. coli* as a perfect candidate for calibrating ATP measurements to microbial levels. The availability of standardized methods for detecting and quantifying *E. coli* furthermore solidifies its role and simplifies the use of it in laboratory analysis. This supports the monitoring of levels of *E. coli* in food, water, and clinical samples, which lead to safeguarding of public health.

3.2. Environmental legislation

The aim of the environmental legislation is to prohibit, restrict, and regulate environmentally harmful practices. Environmental regulations can have a profound effect on economic activities, and these effects should be included in the assessment of the implementation of these regulations. Succes of environmental legislation mainly depends on the way it is enforced, where public can be big help. The forming of environmental legislation in 1960s lead to awareness of these problems to public.

The main concerns of these legislations are:

- Air quality;

- Water quality;
- Waste management;
- Contamination by chemicals.

There are specific standardized instructions, how to proceed during specific experiments, which play significant role in science. This gives the scientists the opportunity to get accurate results, which can be compared to each other. These standards provide step by step guide during the testing, collecting, and determination of the results. The standards are updated if there are any specific changes. The International Organization for Standardization (ISO) is an international non-government organization with a membership of 169 national standards bodies, that ensure the products and services, which are used daily, are safe, reliable, and of high quality. It also includes guide business in adopting sustainability and ethnical practices. ISO was founded in 1974 and it published over 24,500 international standards covering almost all aspects of technology and manufacturing.

3.2.1. Water legislation

Water is essential for the existence of life on this planet, serving not only as a vital resource for drinking but also for sustaining various essential activities such as farming, fishing, energy production, manufacturing, and transportation. However, to safeguard water resources and ensure their sustainable use, there exist rules and regulations for the protection of water quality. The Water Framework Directive (WFD) stands as a pivotal piece of legislation, focusing on maintaining good ecological and chemical status in water bodies to safeguard human health, secure water supply, preserve natural ecosystems, and protect biodiversity. Central to its objectives is the reduction and elimination of pollutants that pose risks to human health and ecosystems alike. Originally issued in 2000, the WFD has since emerged as the cornerstone of water protection in Europe. Among its primary goals is the dissemination of knowledge regarding water-related challenges and fostering public interest in addressing them. The directive's scope encompasses inland, transitional, and coastal surface waters, as well as groundwaters, ensuring an integrated approach to water management that respects the integrity of entire ecosystems. This holistic approach includes the regulation of individual pollutants and the establishment of corresponding regulatory standards. EU action is imperative due to the transboundary nature of river basins and pollution. The adoption of a river basin approach, whereby Member States divide river basins and associated coastal areas

into 110 river basin districts, represents the most effective strategy for managing water resources. Implemented through six-year recurring cycles, the WFD has yielded satisfactory results in surface waterbodies during its initial cycle from 2009 to 2015. From a scientific perspective, the implementation of the WFD has significantly advanced understanding of the ecology of European surface waters, particularly in regions that have received limited investigation. This has led to the development of numerous methods for sampling and studying aquatic ecosystems, resulting in the generation of substantial amounts of data (Hering et al., 2010). As a result, ongoing efforts under the WFD continue to enhance scientific knowledge and inform effective water management strategies across Europe.

The European Parliament and the Council have fulfilled a complete water policy aimed at establishing limits and parameters for water quality across various water bodies, including rivers, lakes, transitional waters, and coastal waters. Under this policy framework, the classification of ecological status for these water bodies is based on several key quality elements. These include biological elements, which assess the health and diversity of aquatic ecosystems; hydromorphological elements, which evaluate the physical characteristics and habitat structure supporting biological elements; and chemical and physico-chemical elements, which analyse the composition and properties of water supporting biological elements. Additionally, the policy framework encompasses general parameters for assessing overall water quality and specific pollutants that may pose risks to human health and the environment. In the context of groundwater, the determination of chemical status relies on specific parameters, including conductivity and concentrations of pollutants. These parameters serve as indicators of groundwater quality and help assess the potential risks associated with contamination and pollution. By establishing clear guidelines and standards for water quality assessment, the European Parliament and the Council aim to ensure the protection and preservation of water resources, promoting sustainable water management practices across Europe. Through ongoing monitoring and evaluation efforts, policymakers get to address emerging challenges and maintain the ecological integrity and health of aquatic ecosystems for present and future generations.

Table 1: General definition of ecological quality for rivers, lakes, transitional waters, and coastal waters (European Union, 2000)

Element	High status	Good status	Moderate status
General	There are no, or only very minor, anthropogenic alterations to the values of the physico-chemical and hydromorphological quality elements for the surface water body type from those normally associated with that type under undisturbed conditions. The values of the biological quality elements for the surface water body reflect those normally associated with that type under undisturbed conditions, and show no, or only very minor, evidence of distortion. These are the type-specific conditions and communities.	The values of the biological quality elements for the surface water body type show low levels of distortion resulting from human activity but deviate only slightly from those normally associated with the surface water body type under undisturbed conditions.	The values of the biological quality elements for the surface water body type deviate moderately from those normally associated with the surface water body type under undisturbed conditions. The values show moderate signs of distortion resulting from human activity and are significantly more disturbed than under conditions of good status.

3.2.2. ISO standards

This International Standard 6222:1999 specifies a method for the enumeration of culturable micro-organisms in water by counting the colonies formed in a nutrient agar culture medium after aerobic incubation at 36 °C and 22 °C. The method is intended to measure the operational efficiency of the treatment process of public drinking water supplies and for general application to all types of water. It is particularly applicable to the examination of water intended for human consumption, including water in closed containers and to natural mineral waters (ISO, 1999). The results for this test norm are counted as a number of colony forming units in 1 ml of sample (CFU/ml) for each cultivating temperature. If there are no colonies in the culture medium with a certain volume of the undiluted sample, it is reported as a “not determined in 1 ml”. If there are more than 300 colonies on the plate with highest degree of dilution, then the results are expressed as >300 or only as approximately.

The ISO 9308-1:2014 specifies a method for the enumeration of *Escherichia coli* and coliform bacteria. The method is based on membrane filtration, subsequent culture on a chromogenic coliform agar medium, and calculation of the number of target organisms in the sample. Due to the low selectivity of the differential agar medium, background growth can interfere with the reliable enumeration of *E. coli* and coliform bacteria, for example, in surface waters or shallow well waters. This method is not suitable for these types of water. ISO 9308-1:2014 is especially suitable for waters with low bacterial numbers that will cause less than 100 total colonies on chromogenic coliform agar (CCA). These may be drinking water, disinfected pool water, or finished water from drinking water treatment plants. Some strains of *E. coli* which are β-D-glucuronidase negative, such as *Escherichia coli* O157, will not be detected as *E. coli*. As they are β-D-galactosidase positive, they will appear as coliform bacteria on this chromogenic agar (ISO, 2014).

There are different acceptable values of *E. coli* for different types of waters:

Table 2: The set limit's values for *E. coli* (European Union, 2006, 2020)

Parameter	Excellent quality	Good quality	Sufficient
Marine water	250 CFU/100mL	500 CFU/100mL	500 CFU/100mL
Fresh water	500 CFU/100mL	1 000 CFU/100mL	900 CFU/100mL
Drinking water	0 CFU/100mL		

3.3. Water testing

The quality of drinking water is very important for overall well-being of human population, where number of bacteria or pathogens can be risk to the public health. The evaluation of drinking water distribution system is a great indicator for measuring the safety and quality of drinking water, nonetheless continuous sampling is needed for improvement of water quality. This can in future be a good indicator in early stages, when some accident that can contaminate the water supply system. However, methods for this testing mostly focus on bacterial regrowth, or on the nutrients that support that regrowth. Unfortunately, these methods can be very time consuming and are known to have many limitations. Because of this, there is the ATP assay, which measures all active microorganisms. It was also firstly used in 1960s to analyse the microbial activity in aquatic sample (Somasundaram, 1966). For this, it is a very common for testing drinking water and all variety of industrial water. ATP assay

can rapidly detect either total ATP if an extraction reagent is use, or free ATP only without reagent. Therefore, the microbiological ATP content can be calculated as a difference between total and free ATP. These methods can bring some disadvantages to the process. ATP assay bring the measurement of all living cells within minutes from the test, but on the other hand, there are no specific results, what exact species are in the water. In contrary, the culture-based method is very consuming in time, and the results take long time to show, but we can have indicators especially for faecal coliforms and *E. coli*, where is the sensitivity and specificity of the detection.

3.3.1. Drinking water

Safety of drinking water is one of the major concerns of society. The contamination of drinking water can lead to public health problems. Due to this, testing and checking water quality is one of the main concerns in water management. To provide safety for consumers, there are specific standards and regulations, which provide limit values for possible contamination of water.

The Guidelines for drinking-water quality identify 43 microbial parameters, which include bacterial, viral, protozoan, and helminth pathogens, as well as toxic cyanobacteria. The verification of microbial water safety is normally based on testing of indicator organisms, and the GDWQ include a guidance value for *E. coli* or thermotolerant coliforms. Countries and territories in the survey designated numerical standards for 24 microbiological parameters. However, nine of these parameters were designated by only one country and a further nine by less than ten countries and territories (WHO, 2018).

Table 3: Microbiological parameters on drinking water (European Union, 1998)

Parameter	Parametric value
Escherichia coli	0/250 mL
Enterococci	0/250 mL
Pseudomonas aeruginosa	0/250 mL
Colony counts 22 °C	100/mL
Colony counts 37 °C	20/mL

3.3.2. Industrial water

Industrial water is type of water that is sourced from natural or engineered sources, with various treatments or processes. Industrial water could be treated to meet specific quality standards, such as purity, pH, conductivity, and microbial content, depending on its intended use and regulatory requirements. The composition of industrial water can be different, depending on factors such as the type of industry, the specific processes involved, and the quality of the source water. It also may contain dissolved minerals, organic compounds, suspended solids, and trace elements, which can influence its physical, chemical, and biological characteristics. Industrial water can be contaminated from pollutants, chemicals, and microbial organisms from industrial activities, which then has to be treated and have management measures to ensure water quality and environmental protection.

Table 4: Reclaimed water quality requirements for agricultural irrigation (European Union, 2020)

Minimum reclaimed water quality class	Crop category	Quality requirements			
		E. coli (number/100 mL)	BOD5 (mg/L)	TSS (mg/L)	Turbidity (NTU)
A	All food crops consumed raw where the edible part is in direct contact with reclaimed water and root crops consumed raw	≤ 10	≤ 10	≤ 10	≤ 5
D	Industrial, energy and seeded crops	≤ 10,000	25	35	-

Note: *BOD5 = Biochemical Oxygen Demand measured during 5 days of incubation, TSS = Total Suspended Solids, NTU = Nephelometric Turbidity Unit

3.3.3. Wastewater

Wastewater is discharged from households, businesses, and institutions. This water includes water from the toilet, sinks, showers, and any other processes done in household. This water is very easily contaminated by pollutants, organic material, suspended solids, or microbes. When the water is heavily polluted, there are significant problems related to it. This can lead

to environmental contamination, the degradation of water quality, and big impact on aquatic and public health. Due to this, there are certain legislations that should not be acceded to and if these numbers are higher, it can lead to many problems.

The treatment of wastewater differs depending on environmental condition and the standards. These treatments involve series of processes, that aim to remove contaminants and pollutants from the water, which is then safely discharged into the environment or reused. There are stages of treatment of wastewater. A purification process generally consists of five successive steps:

1. preliminary treatment or pre-treatment (physical and mechanical);
2. primary treatment (physicochemical and chemical);
3. secondary treatment or purification (chemical & biological);
4. tertiary or final treatment (physical and chemical);
5. treatment of the sludge formed (supervised tipping, recycling, or incineration).

In general, the first two steps are gathered under the notion of pre-treatment or preliminary step, depending on the situation (grégorio, Lichtfouse, 2018).

Water supply systems can be contaminated by, for example, wastewater or surface water with increased amounts of microorganisms and/or readily available substrates which may then lead to aftergrowth. It is unclear to what degree the ATP assay can detect microbial contaminations or how sensitive it is to minor changes in the microorganism amount. This has so far been limited to investigations of drinking water spiked with *E. coli* combined with incubation of water samples (Vang, 2014).

3.4. Adenosine triphosphate

Adenosine triphosphate (ATP) is the source of energy used and stored at the cellular level due to the phosphate groups that link through phosphodiester bonds. First mentions of ATP began in 1920s during study of muscle contractions. Organisms use molecules held in the fats, proteins, and carbohydrates, which are eaten, as a source of energy to make ATP. Organisms require this energy for their growth and other metabolic processes, which include intracellular signalling, DNA and RNA synthesis, Purinergic signalling, synaptic signalling, active transport, and muscle contraction. This process is called hydrolysis. Mitochondria make ATP by the process of cellular respiration, which can be achieved through aerobic respiration, that

requires oxygen, or anaerobic respiration, which does not require oxygen. Aerobic respiration produces ATP from glucose and oxygen, during three-step process, that involves glycolysis, the Krebs cycle, and electron transport chain. Anaerobic respiration uses chemicals other than oxygen, and the process is primarily used by archaea and bacteria that live in anaerobic environments, where they convert the energy from sun into energy that can be used by cell by means of photosynthesis.

The structure of ATP consists of the ribonucleoside adenosine to which three molecules of phosphate are bonded in series. Two of the phosphate bonds are phosphoanhydrides and have high free energies of hydrolysis greater than 30 kJ (Vang, 2013). ATP is formed through catabolic reactions of cells, where energy is generated, and is used in anabolic reactions, where energy is consumed. ATP is transformed back into ADP and P_{i-} ATP is generated during certain exergonic reactions and consumed in certain endergonic reactions (Brock and Madigan, 1991).

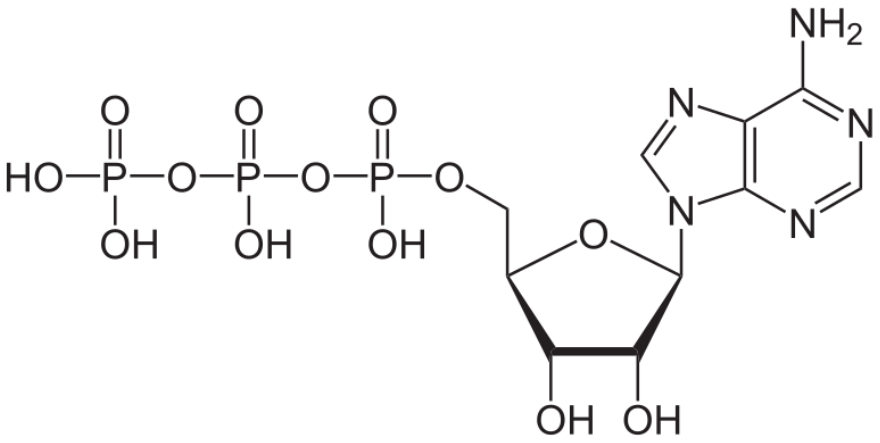

Figure 3: Chemical structure of adenosine triphosphate (Vang, 2013)

Originating in the food and beverage industry, measurement of ATP is nowadays frequently used to measure cleanliness of surfaces in hospitals. By measuring ATP, the presence of all kinds of organic material is measured, e.g. microbial contamination and organic contamination (skin flakes, bodily fluids, food scraps, etc.). As the amount of ATP is quantified, ATP measurements give insight into the level of environmental contamination within the healthcare setting. The ATP results are available within seconds which enable immediate feedback (van Arkel, 2021).

For the threshold of ATP testing, it depends on the device and on the environment, where it is tested. Usually, the benchmark in hospitals is set to 100 RLU (Pontes, 2023), when if it is exceeded, the test is evaluated as a hygiene failure.

Recent research has shown that even viruses can produce ATP by glycolytic and fermentation processes to support their replication. They achieve this by engaging enzymes by the viral replication proteins and produce ATP locally, within the replication structure (Nagy, 2020).

3.4.1. Free, total, and intracellular ATP

Free ATP is the freed ATP by dying or dead cells. When the cell dies, it loses its membrane integrity. As it is unstable molecule in unbuffered water, it is rapidly destroyed. Its stability depends on many parameters like pH, temperature, presence of stabilators, type of biocide use and presence of other microorganisms. And therefore, it is difficult to assess. If the environment provides good stability, then free ATP will increase in number. On the other hand, if the environment is not favourable, the free ATP disappears very quickly.

Intracellular ATP is found in living cells, and it plays a vital role in intracellular energy transfer, serving as the primary energy currency of the cell. It is constantly renewed and recycled within the cell through metabolic processes. The presence of intracellular ATP is indicative of cellular viability, as it ceases upon cell death. Moreover, total ATP, comprising both free ATP and intracellular ATP, serves as a comprehensive indicator of cellular activity and health. Furthermore, in microbial analysis, ATP can be recalculated to estimate microbial equivalents and even correlate with colony-forming units, providing valuable insights into microbial biomass and activity within a given sample.

3.4.2. Colony forming units

Colony-forming units are a measure of viable colonogenic cell numbers in CFU/mL. This is an indication of the number of cells that remain viable enough to proliferate and form small colonies (Sankaranarayanan et al., 2014). CFU is used to quantify microbial load, quality control, and research. Colonies appear under specific conditions such as the medium, time, and temperature. For this method, a Petri dish with agar is required. The sample, mostly diluted by some margin, was then spread, or streaked onto the agar, and placed in an incubator

for cultivation. After cultivation, colonies on agar were counted using a colony counter. The formula for calculating the CFU/mL:

$$\text{CFUs/mL} = \frac{\text{Number of Colonies Counted}}{\text{Dilution Factor} \times \text{Volume Plated}}$$

Where: CFU – Colony Forming Units

There are studies, which are concern in correlation of ATP and CFU. The studies describe a low correlation between the amount of ATP measured and the amount of microbial contamination. An explanation for this low correlation could be the interference of biological parameters that are measured along with ATP measurement. The amount of bacterial/fungal contamination on a surface could be low, while contamination with other biological material (e.g. food scraps, skin flakes, etc.) is higher (van Akel, 2021).

3.4.3. Microbial Equivalents

In microbial analysis, estimating microbial equivalents based on ATP measurement is a valuable technique for assessing living biomass within a sample. To achieve this, the tATP content is initially determined, which includes both free ATP and intracellular ATP within the sample. To isolate the living biomass represented by intracellular ATP, free ATP is subtracted from the total ATP. The resulting value represents the amount of ATP associated with living cells. Afterward, to ease comparison with traditional culture-based methods, the total ATP results are converted into Microbial Equivalents per mL (ME/mL). This conversion is based on the established relationship that 1 *E. coli*-sized bacterium contains 0.001 picograms (pg) of tATP, which is equivalent to 1 femtogram (fg). The formula for calculating tATP in ME/mL is:

$$tATP(ME/mL) = tATP\ (pg\ \text{ATP}\ /\text{mL}) x \frac{IME}{0.001\ pg\ ATP}$$

Where: ATP – Adenosine Triphosphate

IME – Individual Microbial Equivalent

ME – Microbial Equivalent

tATP – Total Adenosine Triphosphate

Given the convenience and familiarity of reporting results in similar formats, total ATP results in ME/mL are often expressed using Scientific Notation (i.e., #. # x 10#) or in a Log10 format for ease of comparison across different analytical techniques and studies (MODERN WATER DeltaTox ATP User manual, 2012).

3.5. ATP measurement

ATP testing is used in many facilities in different industries, where is checked the cleaning procedure. This is much needed in healthcare industry, where if the test failed, there can be a lot of consequences concerning health of people in the facilities. The ATP test show mostly two values. One is the effectiveness of the cleaning procedure. The monitoring of this is very important and can show correct way to clean the environment. The other value is for checking if the values are below the given standards. When they are, there is a significant risk for public health (Pontes, 2023).

For checking the ATP values in non-laboratory facilities is mostly used industrial measuring device, which is light, small and can be easily used in terrain. For the test that are done in labs, the devices are on base of bioluminescence testing.

3.5.1. ATP assay principles

There are many criteria for ATP assay, which can significantly influence the results. The factors, which can be cause of bad results, are potential inhibitors, temperature, pH, and stability in catalytic activity over time. From the inhibitors, there can be decrease of light production due to increased salt, where calcium is the main inhibitor. One of the important conditions, when ATP assay is used, to maintain as constant conditions as possible through the whole testing. If this is not kept in mind, the results can differ. The emitted light is quantified in the relative light units (RLU) with the use of luminometer.

Many methods can calculate intracellular ATP levels. They can be divided into these groups of techniques:

- Fluorescence methods;
- Bioluminescence methods;
- Chromatographic methods.

The chromatographic methods have that include ion exchange, high-performance liquid chromatography (HPLC), or thin-layer chromatography, can be used simultaneously for detection of nucleotides in a mixture. Ion exchange is a technique for separating compounds based on their net charge. The disadvantage of this method is the low stability of ion-exchange columns, because of this, the separation is not always adequate (Khlyntseva, 2009). With use of HPLC method it is possible to perform structural, and functional analysis, and purification of many molecules within a short time. This technique yields perfect results in the separation, and identification of amino acids, carbohydrates, lipids, nucleic acids, proteins, steroids, and other biologically active molecules (Coskun, 2016).

The fluorescence method can be used as a detection technique in chromatographic determination of nucleotides, but also as an independent analytical technique. It is an analysis, in which the molecules of the analyte are excited by irradiation at a certain wavelength and emitted radiation at a longer wavelength is measured. Fluorescence emission involves the measurement of light against a dark background and is not based on different measurements like most absorption techniques. ATP determination with fluorescence is subdivided into two group: fluorescence enhancement and fluorescence quenching (Vang, 2013).

3.5.2. ATP bioluminescence

Bioluminescence methods includes the use of firefly luciferase enzyme, which was shown since 1940s, and are the most used enzyme for determination of adenine nucleotides. The advantage of this methods are their high sensitivity, selectivity, and relative ease of application (Khlyntseva et al., 2009). The bioluminescence is based on correlation between a bioluminescent reaction and the effect of tested compound. Therefore, the more bacteria or cells presented in the sample, the more ATP is presented and the stronger the bioluminescence produced (Y. Tang et al., 2023). The bioluminescence reaction occurs very rapidly and begins right after the reagent is added, the test sample and the reagent are incompletely mixed.

The overall reaction that proceeds in the luciferin-luciferase system of fireflies is described by the following scheme:

$$\text{D-luciferin} + O_2 + \text{ATP} + Mg^{+2} \xrightarrow{\text{Firefly luciferin}} \text{oxyluciferin} + \text{AMP} + \text{PPi} + \text{light quantum } (\sim 560\text{nm})$$

Where: AMP – Adenosine Monophosphate

ATP – Adenosine Triphosphate

PPi – Pyrophosphate

The scheme shows that an organic substance (luciferin) is rapidly oxidized in the presence of ATP and magnesium ions by air oxygen to oxyluciferin with simultaneous formation of pyrophosphate (PPi) and adenosine monophosphate (AMP). Initially the oxyluciferin is formed in an electronically excited state, and a light quantum is emitted during the transfer of the product to the ground state. The advantages of firefly luciferase include its absolute specificity towards ATP and high emission quantum yields (Lomakina, et al., 2015). Cellular ATP is extracted through cell lysis and subsequently reacts with luciferase and luciferin. The emitted light is quantified in the relative light units (RLU) using a luminometer and can be converted into ATP concentration due to a proportional relationship between the measurements (Khlyntseva, et al., 2009).

The knowledge of the biochemistry of the beetle bioluminescence has been based on the firefly luciferase from the *Photinus pyralis*. The bioluminescence system is dependent on four components: oxygen, the Luc enzyme, the substrate luciferin, and the complex ATP-Mg^{2+}. In 1947 there was a study concerning the energy source of bioluminescence by McElroy. In this study was determined, that the energy source of bioluminescence system was the molecule ATP, and the strength of the light produced during bioluminescence is dependent on the mount of ATP of the sample. Unfortunately, despite the progress in past decades, the knowledge about this system is not yet fully understood (Fraga, 2008).

But RLU/ATP is not a good indication of the level of contamination of spore forming bacteria likely due to the low metabolic activity in the endospore state. When evaluating the presence/absence or concentration of spore forming bacteria, using standard microbiological culture methods are best practices (Gibbs, 2014).

3.5.3. ATP result interpretation

Interpreting ATP results is crucial for evaluating microbial control in various processes. ATP-based measurements are highly sensitive to changes in total microbial quantity, making them effective tools for assessing microbial activity and contamination levels. In general, processes

exhibit optimal microbial control when tATP levels are minimized. Modern Water's ATP test kits offer a practical solution for checking microbial quantity, allowing for the identification of area within a process that may require immediate attention. For process control purposes, daily monitoring using ATP test kits provides accurate total microbial quantity parameters that can be trended over time against process characteristics and performance. However, it's essential to recognize that every process is unique, and during audits, relative comparisons from point to point serve as reliable indicators of process performance. For daily monitoring, establishing a baseline trend before making control decisions is crucial. To facilitate this process, Modern Water offers guidelines in units of picograms of tATP per milliliter (pg tATP/mL). These guidelines help ensure effective utilization of ATP test kits for microbial control and process optimization.

Table 5: DeltaTox ATP Interpretation Guideline (Modern Water Manual DeltaTox, 2012)

Application	Good control (pg tATP/mL)	Preventative action (pg tATP/mL)	Corrective action (pg tATP/mL)
Cooling & Process Water Oxidised Biocides	<10	10 to 100	>100
Cooling & Process Water Non-Oxidising Biocides or Non-Chemical Treatment	<100	100 to 1,000	>1,000
Papermaking Product Quality (Newsprint, Fine Papers)	<1,000	1,000 to 10,000	>10,000
Papermaking Odour Control (Paperboard, Recycle Water	<10,000	10,000 to 100,000	>100,000

3.5.4. ATP assay limitations

In the interpretation of ATP assay results, it is essential to consider both fATP and tATP measurements. When fATP is measured, it is advisable to subtract this value from the tATP prior to interpreting the results. This adjustment helps provide a more accurate assessment of microbial activity and contamination levels within a given sample. It's important to note that different types of biocides, such as oxidizing biocides (e.g., chlorine, bromine, ozone) and non-oxidizing biocides, as well as non-chemical treatments, may impact ATP measurements differently. Oxidizing biocides tend to reduce both fATP and tATP levels due to their

mechanism of action, whereas non-oxidizing biocides and non-chemical treatments may result in higher residual tATP levels, particularly when processes are well controlled.

However, it's crucial to acknowledge the limitations of ATP assay interpretation guidelines. These guidelines are designed to provide generic risk management guidance and may not fully capture the complexities of every situation. Therefore, users are strongly encouraged to establish their own control ranges based on their specific processes and requirements.

4. Methodology

The methodology, that was used in the experimental part of this thesis for measuring *E. coli*, was done in accordance with the ČSN EN ISO 9308-1:2014/Adm. 1:2016 Water quality – Enumeration of *Escherichia coli* and coliform bacteria – Part 1: Membrane filtration method for waters with low bacterial background flora.

ATP was measured by three different kits. These kits came with step-by-step manuals for their testing and that was followed through the testing procedures.

4.1. Materials and equipment

4.1.1. Chemicals

- FLAA ATP Bioluminescent Assay Mix - lyophilized powder containing luciferase, luciferin, MgSO4, DTT, EDTA, BSA, and tricine buffer salts (Sigma-Aldrich, Czech Republic)
- FLAA ATP Standard (Sigma-Aldrich, Czech Republic)
- HY-LiTE ATP Standard (Sigma-Aldrich, Czech Republic)
- DeltaTox ATP reagent (Modern Water, U.S.A.)
- E C BROTH (OXOID, UK)
- Ethanol 96% (Penta, Czech Republic)
- ECC agar (OXOID, Germany)

4.1.2. Equipment

- Beakers 25, 50 and 100 mL (P-Lab, Czech Republic)
- Automatic micropipettes 10-100 μL and 100-1000 μL (RAININ – METTLER TOLEDO, U.S.A.)
- Glass cuvette (Modern Water, U.S.A.)
- Metal strainer (ACO Industries, Czech Republic)
- Weighing scales (KERN, Germany)
- MS Excel program (Microsoft, U.S.A.)
- RStudio (Posit, Austria)

4.1.3. Apparatus

- Microtox® FX DeltaTox analyser (Modern water, U.S.A.)
- Luminometer system SURE Plus (SKATEC, Czech Republic)
- AquaSnap TOTAL (Hygiena, Czech Republic)
- Vacuum filtration system (Startorius stedim, Germany)
- Incubator (POL-LAB, Czech Republic)
- Chamber (MERCI, Czech Republic)
- Electric heater (Heidolph, Germany)

Figure 4: System SUPER Plus (Hygiena, 2020)

Figure 5: Microtox® FX (photo: K. Khollová)

4.2. Methodology

All laboratory work was done in labs at the Department of Applied Ecology, on Faculty of Environmental Sciences, Czech University of Life Sciences Prague.

4.2.1. Preparation of samples

In total, six samples of water were collected for analysis. Among these samples, three originated from biotope, which is located in front of the Faculty of Environmental Sciences. Once, the samples of biotope were collected in front of the Faculty of Forestry and Wood Science, due to freezing of the original biotope. There was nothing done with the first sample from the biotope, the second biotope sample was strained through metal strainer, and the third biotope sample was filtrated using vacuum filtration system. Another type of sample was sourced from tap water in school. One sample was obtained from tap water from home in Dobřejovice. The final sample consists of distilled water.

4.2.2. Preparation of FLAA ATP Standard (stock solution)

To prepare the ATP Standard stock solution, the contents of one vial of ATP Standard were dissolved in sterile water. This solution has stability for at least 24 hours when stored at

temperatures between 2 to 8 °C or for over two weeks if stored frozen at -20 °C. The ATP Standard solution was prepared by series of dilutions with sterile water (Table 6). These dilutions maintain stability for up to 8 hours when stored in ice (Sigma-Aldrich, 2015).

4.2.3. Preparation of DeltaTox ATP test Reagent

The ATP reagent was prepared through a process known as freeze-drying, which optimizes product stability prior to use. Before incorporating this reagent into experimental procedures, it is necessary to rehydrate it appropriately. This involves mixing the freeze-dried powder with a liquid buffer solution and allowing the mixture to incubate for a minimum of 5 minutes. During this incubation period, the rehydrated reagent becomes fully activated. However, it is crucial to exercise caution to prevent contamination during the rehydration process (Modern Water, 2012).

4.2.4. Preparation of FLAA ATP Assay mix (stock solution)

For the preparation of the ATP Assay Mix, the content of one vial was dissolved in 5 ml of sterile water to create a stock solution with a pH of 7.8. Then mixed by gently inverting or swirling the solution until complete dissolution. It is recommended to allow the solution to stand in ice for at least one hour to ensure thorough dissolution. The resulting stock solution remains stable for at least two weeks when stored at temperatures between 0 to 5 °C and protected from light. If there is slight decrease in light production, new standard curve needs to be prepared for each measuring. To prolong shelf life, the stock solution can be frozen for future use, remaining stable through 2 to 3 freeze-thaw cycles (Sigma-Aldrich, 2015).

4.2.5. Preparation of dilution series

Each standard underwent dilution process, calculated to achieve a range of concentrations covering the range of the assay. These calculations of the concentrations allowed for the generation of a comprehensive dataset.

The dilution series was used for calibration curves, which served as valuable tools for quantifying and interpreting the results obtained from the experimental samples. These

calibration curves provided a reference framework with which the experimental samples could be compared and evaluated.

Table 6: Dilution of ATP Standards with distilled water

ATP standard (µL)	H_2O (µL)	Final concentration (ng/mL)
200	0	1
100	100	0,.5
50	150	0,.25
25	175	0,.125
12,.5	187,.5	0,.0625
6,.2	193,.8	0,.0313
3,.1	196,.9	0,.0156
1,.5	198,.5	0,.0078
0,.75	199,.25	0,.0039
0,.38	199,.62	0,.0020

4.3. Procedure of tests

Accompanying the devices and kits, manuals with detailing procedures for preparation of the samples and performance of the tests. These manuals served as a resource, offering detailed, step-by-step instructions for ensuring the optimal outcome of the experiments. They also provided valuable insights and recommendations to anticipate and address any questions that may happen during the testing process.

4.3.1. *E. coli* cultivation and testing

The cultivation of *E. coli* followed the guidelines outline according to the ISO 9308-1:2014/Adm.1:2016 standards. Additionally, the EC broth was added for selective detection of coliforms in the chosen water samples. The preparation of this broth was done by adding 3,7 g into 100 mL of distilled water, followed by through boiling to ensure complete dissolution of the powder. Afterwards, the prescribed steps in the standard were followed for the cultivation and detection of *E. coli*.

After the preparation procedure, where one colony of *E. coli* was added into distilled water, the samples were ready for testing. Each sample underwent testing procedure using the

SystemSURE Plus kit, following the same procedures employed for testing of water samples. Each *E. coli* preparation and measurement was performed three times.

4.4. Data assessment and calculations

All experimental results were obtained from the instrument display, where they were presented in RLU units. Afterwards, these numbers were then insert into Excel, where they were then used for further calculations.

Following the data obtained from the device, the next step was to plot graph in Excel. This graph shows data, where values on Y-axis give the RLU against concentration of the standard on the X-axis. For visualization of this relationship the trendlines was added into the graph. Additionally, the R-squared values were added, which serve as an indicator of the goodness of fit for each trendline. The most appropriate trendline was determined with linear regression. It is important to state that the closer the R-squared value is to 1, the more accurate the experiment was performed.

For the experimental samples, the data was obtained also from the device display. These numbers were put into Excel tables, where they were calculated by using these equations:
The equation for Total ATP:

$$tATP(pg\ ATP/mL) = \frac{RLU_{tATP}}{RLU_{ATPI}}\ x\ 2{,}000(pg\ ATP/mL)$$

The equation for Free ATP:

$$fATP(pg\ ATP/mL)\ = \frac{RLU_{fATP}}{RLU_{ATPI}}\ x\ 1{,}000(pg\ ATP/mL)$$

Where: ATP – Adenosine Triphosphate

ATPI - median of calibration

tATP – Total Adenosine Triphosphate

RLU – Relative Light Units

fATP – Free Adenosine Triphosphate

Subsequently, the values gained from these equations were divided by 1,000, converting the units to nanograms per millilitre (ng/mL).

The calculation of *E. coli* was done in Excel tables. The dilution was done by 10^4, and the volume plate was 1 mL of distilled water.

The CFU are given by:

$$CFU/mL = \frac{(number\ of\ colonies \times dilution)}{volume\ plated}$$

Where: CFU – Colony Forming Units

The Number of cell/mL is given by:

$$Number\ of\ cell/mL = \frac{ATP\ concentration}{0.001}$$

Where: ATP – Adenosine Triphosphate

5. Results

These results were obtained by methods mentioned in the Methodology chapter.

5.1. Results achieved by DeltaTox kit

For obtaining these results, the DeltaTox kit was used with the Microtox® FX device.

5.1.1. Calibration

Following the measurement and calculations of collected data, analysis was conducted in the creation of graph. This plot visualises the good fit for this specific kit, which shows high accuracy of the performed measurement.

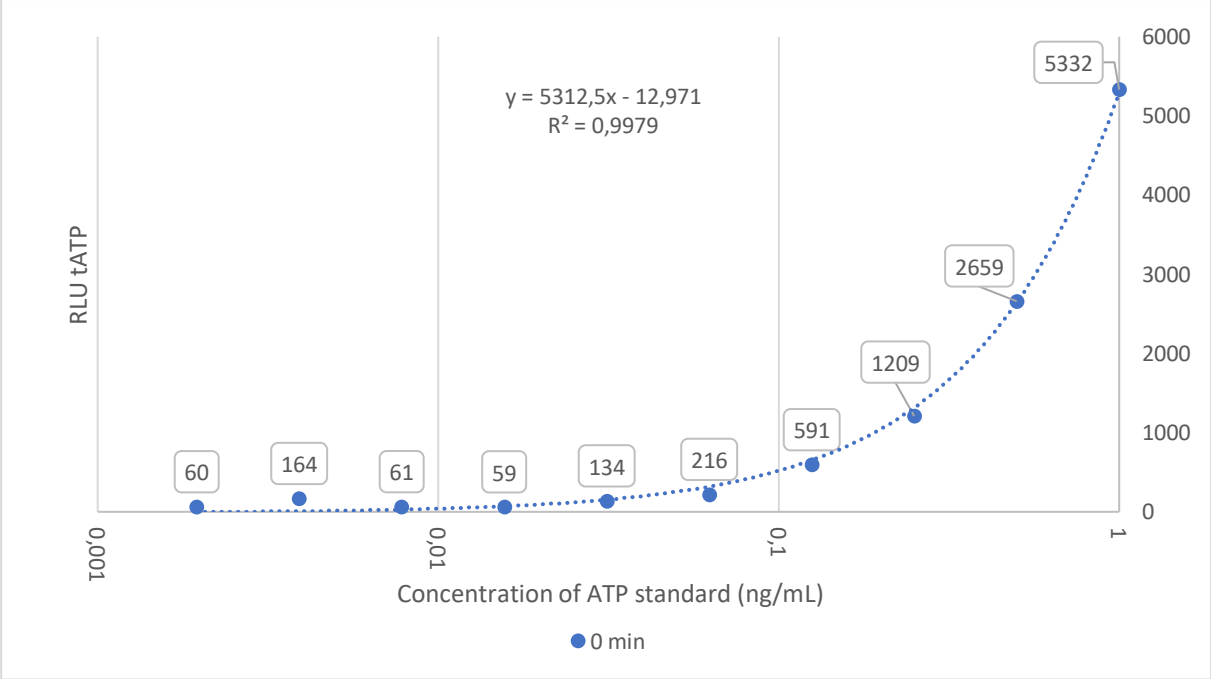

Figure 6: Standardisation curve for the DeltaTox kit

5.1.2. Water samples

These results were obtained and calculated by the procedures mentioned in the Methodology. For these specific results was obtained tATP, as well as fATP.

Table 7: Results of water samples using the DeltaTox kit

Samples	tATP RLU	ng/mL	fATP RLU	ng/mL
Non-filtered biotope	2393	0,.9069	141	0,.0267
Strained biotope	1392	0,.5275	228	0,.0432
Filtered biotope	579	0,.2194	397	0,.0752
Tap water home	2385	0,.9038	1830	0,.3467
Tap water school	158	0,.0598	123	0,.0233
Distilled water	72	0,.0272	51	0,.0096

These results were close to what was expected and measured in past for these sources of water samples. The only surprise were the results from tap water home, where the numbers were higher than it was anticipated.

5.2. Results achieved by FLAA ATP Bioluminescent Assay kit

For obtaining these results, one standard from the FLAA ATP Bioluminescent Assay kit was used. The second one was from the DeltaTox kit. All test were done on the Microtox® FX device.

5.2.1. Calibration

This plot visualises the equation for good fit for standard from FLAA ATP Bioluminescent Assay kit. In this case, the number is high which shows high accuracy.

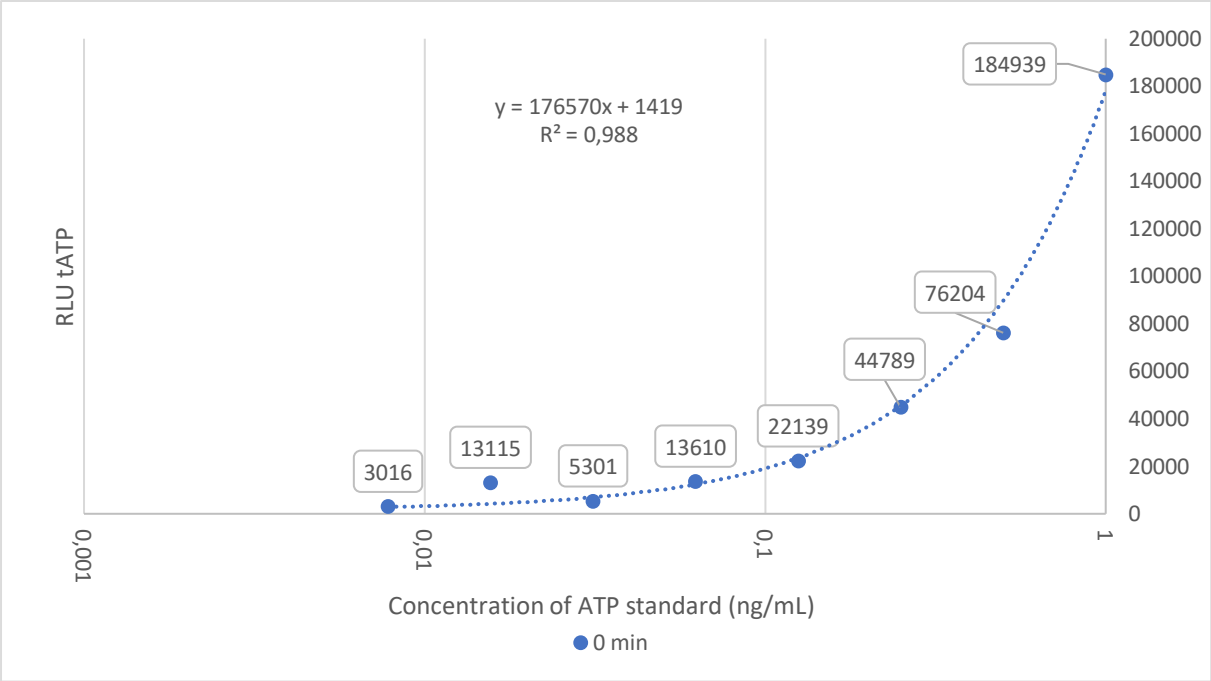

Figure 7: Standardization curve for the FLAA ATP standard

This plot visualises the good fit for standard that was from the DeltaTox kit, HY-LiTE ATP standard. The accuracy was high.

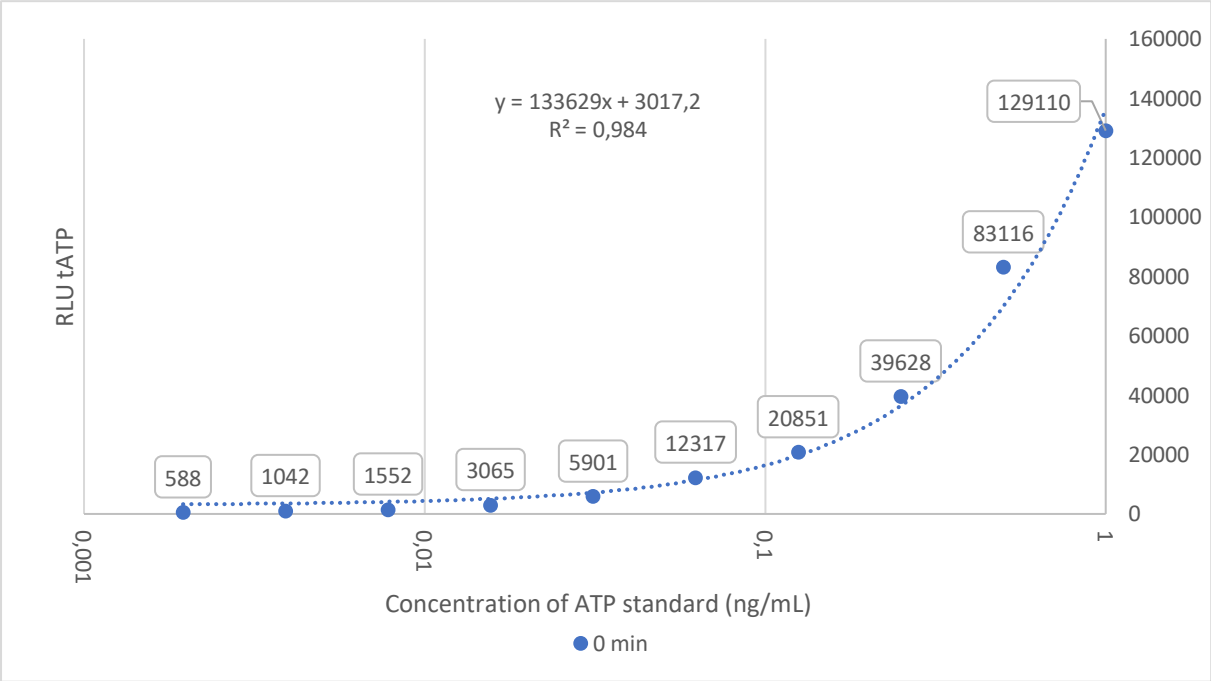

Figure 8: Standardization curve for the HY-LiTE ATP standard

5.2.2. Water samples

These results were obtained by following the steps in guide in the FLAA ATP Bioluminescent Assay kit.

Table 8: Obtained results for samples using the FLAA ATP Assay kit

Samples	Mean RLU	Standard deviation	ng/mL
Non-filtered biotope	228	108,.32	0,.00246
Strained biotope	141	47,.03	0,.00152
Filtered biotope	163	48,.50	0,.00176
Tap water home	533,.7	228,.91	0,.00571
Tap water school	528,.3	136,.55	0,.00577
Distilled water	175	103,.11	0,.00189

5.3.Results achieved by SystemSURE Plus kit

For obtaining these results, the SystemSURE Plus tATP kit and the related device were used.

5.3.1. Calibration

This plot visualises the good fit for the SystemSURE Plus kit. The equation showed, as in the previous case the high accuracy.

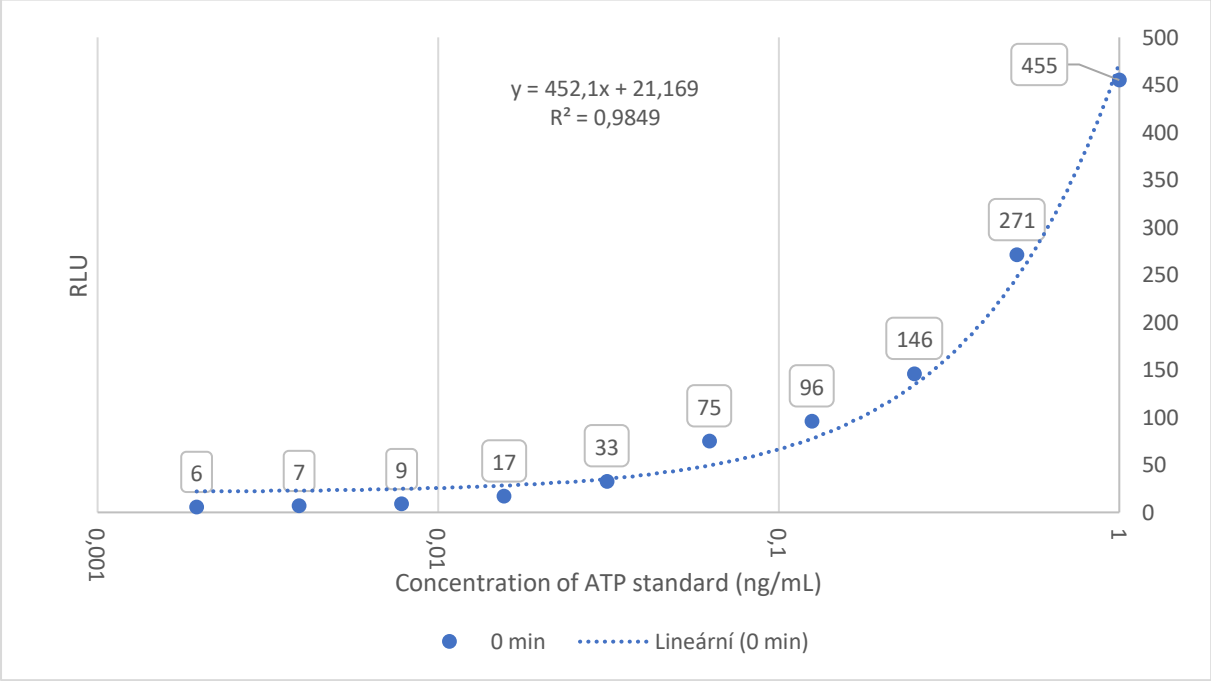

Figure 9: Standardization curve for the SystemSURE Plus kit

5.3.2. Water samples

The results for this water samples were measured by the SystemSURE Plus kit and device. These biotope samples were collected in front of the Faculty of Forestry and Wood Science, due to ice layer on the original biotope.

Table 9: Results of water samples using SystemSURE plus kit

Samples	Mean RLU	Standard deviation	ng/mL
Non-filtered biotope	128,.5	16,.26	0,.5648
Strained biotope	249,.5	33,.23	1,.0967
Filtered biotope	318	18,.38	1,.3978
Tap water home	34	19,.42	0,.1494
Tap water school	38,.3	2,.82	0,.1684
Distilled water	2,.6	0,.57	0,.0117

5.4. Comparison of the achieved results for water samples

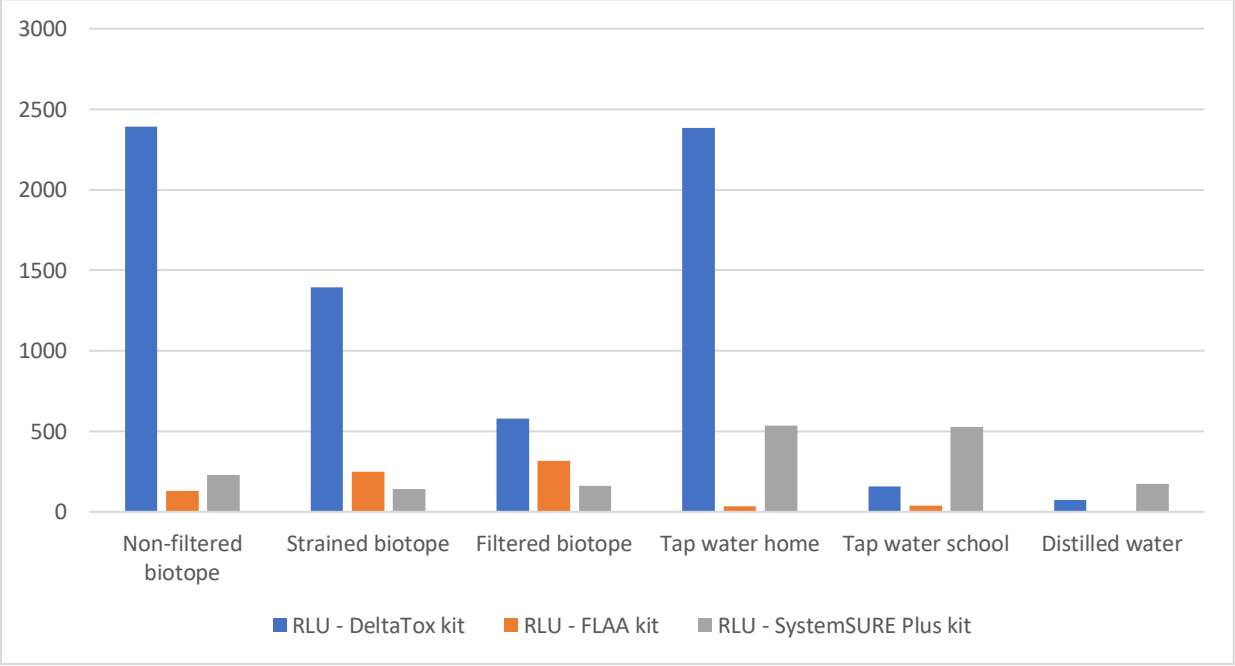

Figure 10: Graphical visualization of results for RLU

These results show that the FLAA ATP Bioluminescence Assay and the SystemSURE Plus kits measured close to each other. This could be due to the fact that their standards have to be freezed during delivery, while the DeltaTox kit standard and solutions is only cooled.

5.5. Results of *E. coli* cultivation and measurement

The results for RLU presented below were obtained by the SystemSURE Plus kit and device. From these measurements, ATP was calculated.

Table 10: Results for *E. coli*

Sample	Number of colonies /mL	CFU/mL	ATP concentration /mL	Number of cells /mL
Non-filtered biotope	375	3,.75x 10^6	5,.9x 10^4	5,.9x 10^7
Strained biotope	160	1,.6x 10^6	2,.5x 10^4	2,.5x 10^7
Filtered biotope	21	2,.1x 10^5	3,.3x 10^3	3,.3x 10^6
Tap water home	148	1,.48x 10^6	2,.3x 10^4	2,.3x 10^7
Tap water school	-*	-*	-*	-*
Distilled water	-*	-*	-*	-*

Note: *No results were obtained due to absence of visible *E. coli* growth on the agar.

The comparison of approximate number of *E. coli* cells calculated by plate count method to the ATP testing method came out to have strong correlation.

The correlation coefficient between CFU/mL and Number of cells/mL was 0.999, which indicates that the results are statistically significant.

5.6. Statistical results for water samples

In this study, the correlation between SystemSURE and FLAA water samples was examined, where correlation coefficient was -0.603. The analysis indicated that the relationship was not significant ($t = -1.512$, $df = 4$, $p = 0.205$). Thus, there was insufficient evidence to reject the null hypothesis that the true correlation is equal to 0. The 95% confidence interval for the correlation coefficient ranged from -0.950 to 0.408.

Similarly, the correlation between SystemSURE and DeltaTox water samples was explored, resulting in an estimated correlation coefficient of 0.043. Statistical analysis revealed a lack of significance ($t = 0.086$, $df = 4$, $p = 0.936$), suggesting that there is insufficient evidence to reject the null hypothesis of a true correlation of 0. The 95% confidence interval ranged from

-0.796 to 0.826 which leads to absence of a significant correlation between SystemSURE and DeltaTox water samples.

Lastly, the correlation between FLAA and DeltaTox water samples was investigated, with estimated correlation coefficient of 0.151. However, statistical analysis indicated a lack of significance ($t = 0.306$, $df = 4$, $p = 0.775$), suggesting insufficient evidence to reject the null hypothesis of a true correlation of 0. The 95% confidence interval ranged from -0.753 to 0.857. There is an absence of a significant correlation between FLAA and DeltaTox water samples.

6. Discussion

The bioluminescence testing with the use of ATP is very common in many fields, particularly in microorganism testing. Although, the plate-counting method remains due to accurate microbial identification, the ATP testing is primarily used in water management and food industry, where the cells can be measured quickly and efficiently.

In the healthcare environment it is crucial to effectively maintain infection control. Numerous studies have investigated the efficiency of the ATP measurement compared to traditional plate-counting methods in this sensitive environment. Presently, ATP is not a standardized method for assessing cleanliness in healthcare facilities. One primary challenge is due to high variance in benchmark values, which complicate the comparison of measurements with different measurement tools. Another limitation of this technique could be the residue of detergent, or disinfectants present on the surface (Nante, et al., 2017). Another study mentions that the main problem is due to lack of standardization at the international level (Amodio E. and Dino C., 2014). Despite these bottlenecks, the ATP bioluminescence has the biggest advantage in being able to show rapid results within seconds.

Several studies have investigated the efficiency of ATP bioluminescence testing in water sources. One study, that was done on water sources in hospitals, indicates that the ATP bioluminescence method is not recommended due to low correlations with microbiological cultures (Arroyo, et al., 2017). Alternatively, another study focusing on drinking water showed promising results. It was concluded that the ATP bioluminescence assay is relatively low-cost, fast and requires modest training in lab techniques. Moreover, it can be conventionally performed on site, and it is effective in evaluating the bacterial sloughing issue (Zhang, et al., 2019).

A study that was interested on monitoring wastewater revealed that the results revealed strong correlation between the culture-based methods and the ATP bioluminescence assay. This could indicate potential use of ATP bioluminescence assay as a process and effluent quality monitoring tool (Linklater and Örmeci, 2013). Although, another study, that was interested in water sludge, concluded that the water sludge blocked the light emitted during the testing,

which lead to incorrect results (Chu, et al., 2001). In this case, the different dilution ratios of water sludge have to be used, for obtaining correct results.

The studies have demonstrated, that for monitoring *E. coli*, the ATP bioluminescence assay have showed high efficiency. The only limitation for this method is its specificity, that can be used only on *E. coli* and cannot detect any other pathogens. This method of testing is typically done in food industry (Cao, et al., 2023, Sun, et al., 2022).

One study was concerned with the correlation between the ATP measurements and microbial contamination. There was a strong correlation between two ATP measurements on two different sites on the same fomite (0.800, $p < 0.001$) (van Arkel, et al., 2021).

In my opinion, the ATP bioluminescence method can be used most efficiently on testing of water quality. The results are obtained rapidly, which allows quick comparison with legislative standards. When the testing is completed on industrial device, it becomes highly efficient during field work to rapidly detect possible source of contamination. Therefore, it holds promise in the future research, particularly for comparing of results with a cultivation method. However, for surface testing it is not so efficient due to lack of standardized results, and inability to recognise different microbial species.

The results of RLU accurately reflected to concentration of the standards. This indicates that they are reliable for testing purposes. However, a significant limitation is the variability in RLU values measured by different kits. Therefore, calculations and standardization are crucial for ensuring consistent and comparable test results across various kits.

7. Conclusion and contribution of the thesis

The aim of this thesis was to assess the usability of ATP measurement from different kits.

The results obtained showed, that the standards used in kits can be reliable in testing of ATP. The preparation and test itself were easily done. One of the main factors during the testing, was to do it as quickly as possible for getting precise results. Time factor is crucial, due to the aging samples and thus, their ATP content.
The water samples results showed that the FLAA ATP Bioluminescence Assay and the SystemSURE Plus kits had similar results of RLU. The DeltaTox results had slightly higher numbers. This can be due to the delivery packaging, where the FLAA ATP Bioluminescence Assay and the SystemSURE Plus kits are frozen, while the DeltaTox kit is delivered at cooled temperatures only.

E. coli was cultivated and measured by the SystemSure Plus device. The obtained results showed that one CFU do not correspondent to one ME, which is the unit commonly used for the ATP results recalculation. Although, the correlation between CFU/mL and Number of cells/mL had showed to be high.

In conclusion, the main aim of this thesis was fulfilled, for where the standards from kits indicated to be reliable for testing of water quality.

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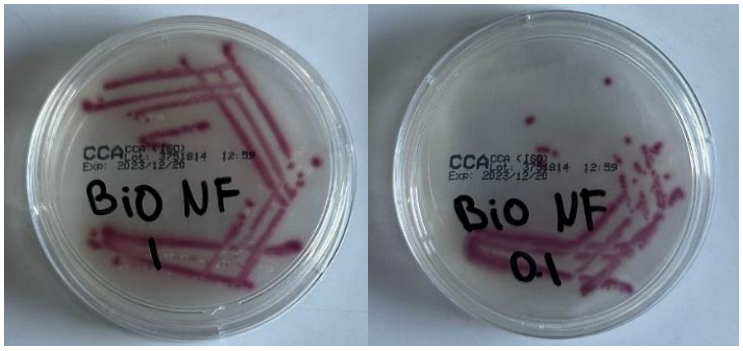

Figure 11: Non-filtered Biotope results for *E. coli* (photo: K.Khollová)

Figure 12: Distilled water results for *E. coli* (photo: K. Khollová)

Figure 13: Tap water results for *E. coli* (photo: K.Khollová)

Figure 14: Tap water home results for *E. coli* (photo: K.Khollová)

Figure 15: Strained Biotope results for *E. coli* (photo: K.Khollová)

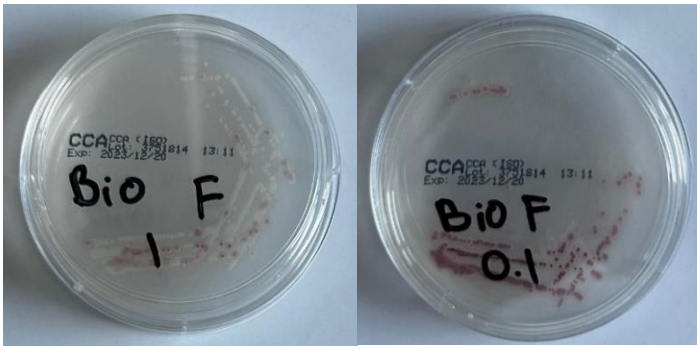

Figure 16: Filtered Biotope results for *E. coli* (photo: K.Khollová)