ATP measurements for monitoring microbial drinking water quality

Vang, Óluva Karin

Publication date:
2013

Document Version
Publisher's PDF, also known as Version of record

Technical University of Denmark

ATP measurements for monitoring microbial drinking water quality

Óluva K. Vang

DTU Environment
Department of Environmental Engineering

PhD Thesis
October 2013

ATP measurements for monitoring microbial drinking water quality

Óluva K. Vang

PhD Thesis
October 2013

DTU Environment
Department of Environmental Engineering
Technical University of Denmark

Óluva K. Vang

ATP measurements for monitoring microbial drinking water quality

PhD Thesis, October 2013

The synopsis part of this thesis is available as a pdf-file for download from the DTU research database ORBIT: http://www.orbit.dtu.dk

Address: DTU Environment
Department of Environmental Engineering
Technical University of Denmark
Miljoevej, building 113
2800 Kgs. Lyngby
Denmark

Phone reception: +45 4525 1600
Fax: +45 4593 2850

Homepage: http://www.env.dtu.dk
E-mail: reception@env.dtu.dk

Printed by: Vester Kopi
October 2013

Cover: Torben Dolin

Preface

This thesis presents the research of my PhD study carried out at the Department of Environmental Engineering at the Technical University of Denmark. Professor Hans-Jørgen Albrechtsen was the main supervisor; Development Engineer Anders Bentien at GRUNDFOS Holding A/S was co-supervisor at the onset of the PhD study, and Development Engineer Christian Smith was co-supervisor during the last part of the PhD study. The PhD project was funded by the Danish Council for Strategic Research through the project Sensors for Monitoring and Control of Water Quality (SENSOWAQ).

The thesis is based on three scientific papers:

I. Vang, Ó.K., Corfitzen, C.B., Smith, C., Albrechtsen H.-J. 2013. Evaluation of ATP measurements to detect microbial ingress in drinking water by waste water and surface water. Submitted.

II. Vang, Ó.K., Corfitzen, C.B., Spliid, H., Albrechtsen H.-J. 2013. Level of microbial ATP and free ATP in non-chlorinated drinking water - assessing microbial drinking water quality with ATP measurements. Submitted.

III. Vang, Ó.K., Corfitzen, C.B., Smith, C., Albrechtsen H.-J. 2013. An evaluation of reagents for the ATP assay for assessing microbial drinking water quality. Manuscript.

The papers will be referred to in the text by their roman numerals I, II and III (e.g. Vang *et al.*, I).

During my PhD I have presented research - comprising laboratory and field study experiments on ATP measurements in drinking water - at international conferences which has resulted in the following conference proceedings.

Vang, Ó.K., Corfitzen, C.B. and Albrechtsen H.-J. (2011) *Adenosine triphosphate measurements for real-time monitoring of microbial drinking water quality*. Faecal Indicators: problem or solution? 6-8 June 2011, Edinburgh, UK. Proceedings: P28 (poster presentation).

Vang, Ó.K., Corfitzen, C.B. and **Albrechtsen H.-J.** (2011) *Detection of microbial contaminations in drinking water using ATP measurements - evaluating potential for online monitoring*. Water Quality Technology Conference and Exposition 13-17 November 2011, Phoenix, Arizona. Proceedings: 1181-1186 (oral presentation).

Vang, Ó.K., Corfitzen, C.B.. and Albrechtsen H.-J. (2009) *Risk Assessment on Intrusion of Campylobacter jejuni and Indicator Organisms in Drinking Water Distribution Networks*. AWWA International Symposium on Waterborne Pathogens, 2-4 May 2010, Manhattan Beach, California. Proceedings: TUE3 - 31 (online - AWWA log-in required) (oral presentation).

Vang, Ó.K., Corfitzen, C.B. and Albrechtsen H.-J. (2009) *Survival of E.coli, coliform bacteria (K. pneumoniae) and the pathogen C. jejuni in drinking water in drinking water distribution networks*. 15th International Symposium on Health-Related Water Microbiology, 31 May- 6 June 2009, Naxos, Greece. Proceedings: 96-97 (oral presentation).

Mentioning of trade names and commercial products (reagents for the ATP assay and luminometers) in this PhD thesis does not constitute endorsement or recommendation for use.

September 2013

Óluva K. Vang

Acknowledgements

I would like to thank my supervisor Professor Hans-Jørgen Albrechtsen for his constructive comments, good ideas, continuous inspiration and interesting discussions throughout the project as well as pleasant company. Anders Bentien, who was my external co-supervisor at the onset of the PhD project, is greatly acknowledged for his participation and interest in the project. Christian Smith who took over as the external co-supervisor is greatly acknowledged for his enthusiasm and support during the last part of the PhD project.

Funding from The Danish council for Strategic Research and GRUNDFOS through the project SENSOWAQ (Sensors for Monitoring and Control of Water Quality) is greatly acknowledged. The Urban Water Technology Research School, in which I have been enrolled, is acknowledged.

A much appreciated thanks to Jos Rijkx (CEO Promicol, The Netherlands) and Promicol employees for an exciting collaboration on their ATP sensor prototype. The water companies, especially Lyngby Waterworks and Nordvand A/S, are acknowledged for providing access for drinking water sampling.

Thanks to Associate Professor Henrik Andersen, Professor Karsten Arnbjerg Nielsen (DTU Environment) and Professor Henrik Spliid (DTU Compute) on their advice and guidance on statistical matters. A much appreciated thanks to technicians Sabrina Nedell, Mona Refstrup and Lene Kirstjen Jensen for their assistance in the laboratory and with practicalities. Thanks to the administration at DTU Environment, especially Anne Harsting and Susanne Wichmann for always being helpful. Torben Dolin and Lisbet Brusendorff are acknowledged for their help and contribution to graphics for my thesis and articles.

Thanks to all my colleagues at the department for making daily life pleasant and easy going. To Charlotte my colleague, office mate and friend - thanks for your assistance with so many aspects throughout my PhD study, for being the positive person you are and for always being helpful. Thanks to Sarah for good times, interesting discussions and great support and encouragement.

Thanks to friends and family in Denmark and to my family in the Faroe Islands for their long distance support. Finally, the two most precious persons in my life – Eirik and my lovely daughter Fríða – to whom I am forever grateful for supporting me throughout the course of my PhD, accepting my long hours in the laboratory and dealing with my absence physically and mentally in the final phase of my PhD study – thank you!

Summary

Current standard methods for surveillance of microbial drinking water quality are culture based, which are laborious and time-consuming, where results not are available before one to three days after sampling. This means that the water may have been consumed before results on deteriorated water quality or potential contaminations are available. Moreover, the low frequency of grab sampling will most likely not even detect short-term contaminations.

Methodology and instrumentation for rapid detection and quantification of microorganisms have advanced significantly over the past decades. Such rapid methods are vital for an improved surveillance and distribution of clean and safe drinking water. One of these rapid methods is the ATP assay.

This thesis encompasses various methodological aspects of the ATP assay describing the principal and theory of the ATP assay measurement. ATP is the main energy carrying molecule in living cells, thus ATP can be used as a parameter for microbial activity. ATP is extracted from cells through cell lysis and subsequently assayed with the luciferase enzyme and its substrate luciferin, resulting in bioluminescence, i.e. light emission which can be quantified.

The overall aim of this PhD study was to investigate various methodological features of the ATP assay for a potential implementation on a sensor platform as a real-time parameter for continuous on-line monitoring of microbial drinking water quality.

Commercial reagents are commonly used to determine ATP in drinking water. For on-line continuous real-time monitoring it is essential to choose an adequate enzyme reagent in terms of limit of detection, stability in catalytic activity and an efficient extraction of microbial ATP from cells. Experiments with different types of commercial and R&D reagents for the ATP assay demonstrated differences in previously mentioned features, which all are required for a successful ATP measurement.

The ATP assay has been used to measure and quantify the active biomass in drinking water systems in numerous studies – as a parameter for treatment processes at waterworks, microbial quality in distributed water, detection of aftergrowth, biofilm formation etc.

This PhD project demonstrated that ATP levels are relatively low and fairly stable in drinking water without chlorine residual despite different sampling locations, different drinking water systems and time of year of sampling. Moreover, microbial ATP – opposed to total ATP – was also evaluated to be a

more accurate and dynamic parameter for monitoring microbial drinking water quality. The ATP assay also proved capable in detecting microbial ingress in drinking water by wastewater and surface water contaminants. These findings advocate the use of ATP as a real-time parameter for continuous on-line monitoring, where sudden and significant changes in microbial drinking water quality can be detected.

Initial experiments with an ATP sensor prototype for continuous real-time monitoring of drinking water definitely demonstrated a potential, with reproducibility in time-series on microbial activity in tap water monitored over the same time period on multiple days. Concerns related to the ATP sensor prototype were mainly mechanical instability and not the ATP assay itself.

The use of rapid methods, such as ATP, will most likely increase the extent and quality of monitoring microbial drinking water quality, where several of the existing methods have prospects to further automation and implementation on sensor platforms.

Danish summary

De nuværende standardmetoder til overvågning af mikrobiel drikkevandskvalitet er dyrkningsbaserede. De er arbejds- og tidskrævende, og resultater foreligger først en til tre dage efter prøvetagningen. På det tidspunkt kan drikkevand af forringet kvalitet eller endda forurenet drikkevand allerede været indtaget af forbrugerne. Ydermere er den lovpligtige prøvetagningshyppighed lav, og hvorved kortvarige forureninger sandsynligvis ikke detekteres.

Hurtigmetoder til at påvise og kvantificere mikroorganismer har gennemgået en betydelig udvikling de sidste årtier, både med hensyn til nye og forbedrede måleprincipper og udvikling af avanceret udstyr. Hurtigmetoder kan bidrage væsentlig til en forbedret overvågning og distribution af rent og sikkert drikkevand. En af disse hurtigmetoder er ATP-metoden.

Denne ph.d.-afhandling omfatter forskellige metodemæssige aspekter vedrørende ATP-metoden, herunder beskrivelse af metodeprincip og teori for selve ATP-målingen. ATP er det primære energibærende molekyle i levende celler, og kan således bruges som en parameter for mikrobiel aktivitet. ATP ekstraheres fra celler ved at destruere cellens membran (lysis), som dernæst analyseres ved brug af luciferase enzymet og det tilhørende substrat luciferin, hvilket resulterer i bioluminescence – dvs. lysudsendelse, der kan kvantificeres.

Det overordnede formål med dette ph.d.-studie var at undersøge ATP-metodens metodemæssige egenskaber med henblik på at implementere ATP-metoden på en sensor platform som en real-time parameter for kontinuert online monitering af mikrobiel drikkevandskvalitet.

Kommercielle reagenser bruges rutinemæssigt til at analysere for ATP i drikkevandsprøver. Med henblik på online kontinuert real-time måling er det essentielt at anvende den rigtige reagenstype med hensyn til detektionsgrænse, stabilitet af enzymets katalytiske aktivitet og effektiv ekstraktion af mikrobielt ATP. Undersøgelser med forskellige typer af kommercielle og R&D reagenser til ATP-målingen demonstrerede forskelle mellem reagenser med hensyn til de benævnte egenskaber, som alle er påkrævet for en succesfuld ATP-måling.

ATP-metoden har i adskillige studier været anvendt til kvantitative målinger af den aktive biomasse i forskellige drikkevandssystemer, for eksempel som parameter for behandlingsprocesser på vandværker, mikrobiel drikkevands-kvalitet i ledningsnettet, påvisning af eftervækst, biofilmdannelse osv.

Dette ph.d.-studie har vist, at ATP niveauet i drikkevand uden klortilsætning er forholdsvis lavt og stabilt ved forskellige prøvetagningslokaliteter,

drikkevandssystemer og over året. Derudover er mikrobielt ATP evalueret til at være en mere nøjagtig og dynamisk parameter i forhold til total ATP til at overvåge mikrobiel drikkevandskvalitet. Spildevands- og overfladevands-forureninger kunne påvises i drikkevand med ATP-metoden. Disse resultater understøtter anvendelsen af ATP som en real-time parameter til kontinuert online monitering til at påvise pludselige og væsentlige ændringer i mikrobiel drikkevanskvalitet.

Indledende forsøg med ATP implementeret i en sensor prototype til kontinuert real-time monitering af drikkevand viste potentiale med hensyn til reproducerbarhed af tidsserier for mikrobiel aktivitet i hanevand moniteret over samme periode over flere dage. Problematikker i forbindelse med ATP sensor prototypen var hovedsagligt forbundet med mekanisk ustabilitet og ikke selve ATP-metoden.

Anvendelsen af hurtigmetoder, som ATP-metoden, vil uden tvivl øge omfanet og kvaliteten af overvågningen af eksempelvis mikrobiel drikkevandskvalitet. Desuden har mange hurtigmetoder, herunder også ATP-metoden, gode perspektiver for yderligere automatisering og potentiel implementering på sensorplatforme.

Table of contents

Abbreviations

Abbreviation	Full name
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
AOC	Assimilable organic carbon
AODC	Acridine Oreange Direct Count
Ap_5A	Di(adenosine-5') pentaphosphate
ATP	Adenosine 5'-triphosphate
ATPγS	Adenosine 5′-O-(3-thio) triphosphate
CoA	Coenzyme A
CTP	Cytidine 5'-triphosphate
DAPI	4',6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
EC_A	Adenylate energy charge
GTP	Guanosine 5'-triphosphate
HPC	Heterotrophic plate counts
IMS	Immunomagnetic separation
ITP	Inosine 5'-triphosphate
L	Dehydroluciferin
L-AMP	Dehydroluciferyl-adenylate
Luc	Luciferase
LH_2	Luciferin
P_i	Inorganic phosphate
p_4A	adenosine 5'-tetraphosphate
PMT	Photomultiplier
O_2	Oxygen
$OxyLH_2$	Oxyluciferin
RNA	Ribonucleic acid
RLU	Relative light units
TDC	Total direct counts
UTP	Uridine 5'-triphosphate
VBNC	Viable but non-culturable

1. Introduction

Currently the most widely used indicator for assessing the microbiological quality of drinking water is heterotrophic plate counts (HPC) together with faecal indicators such as coliforms, *E. coli* and enterococci (Danish executive order on drinking water, 2011). These conventional culturable methods still are the golden standard for monitoring microbial drinking water quality (van der Kooij, 2003; Chowdhury, 2012; Hammes *et al.*, 2011). The advantage of these methods is their sensitivity, which especially applies for the faecal indicators. The major weakness of the culturable methods is the required incubation time, i.e. results are not available before one to three days after sampling.

Despite frequent monitoring of microbiological quality with the traditional culturable methods, the majority of the distributed drinking water is not monitored (WHO, 2013). Thus, grab sampling combined with the current standard culturable methods does not provide the complete picture of the development in terms of microbial quality – far from it. Consequently, it is not possible to ensure clean and safe drinking water at all times. This requires development of rapid methods for a continuous real-time monitoring of the microbial drinking water quality.

During the past decades microbiology has advanced significantly, which has resulted in an increase in methods and instrumentation for the detection, quantification and identification of microorganisms. This includes several rapid microbiological methods, e.g. polymerase chain reaction (PCR), flow cytometry (FCM) and biosensors to name a few (Clark *et al.* 2011; Hammes *et al.*, 2012; Kim *et al.*, 2013). Some of these rapid methods can provide quantitative results on microbial drinking water quality within minutes to hours as oppose to days. The application of many of these rapid methods is still confined to the laboratory. Sensors encompassing the combination of rapid methods, continuous on-line measurements and real-time alarm signalling would significantly contribute to the surveillance of microbial drinking water quality (Kazner *et al.*, 2009). Sensors located at e.g. waterworks, distribution network and sensitive consumers for monitoring microbial drinking water quality would allow water utility managers to be proactive rather than reactive, and for corrective actions to be made in a timely manner, if the drinking water quality is microbiologically compromised (Figure 1).

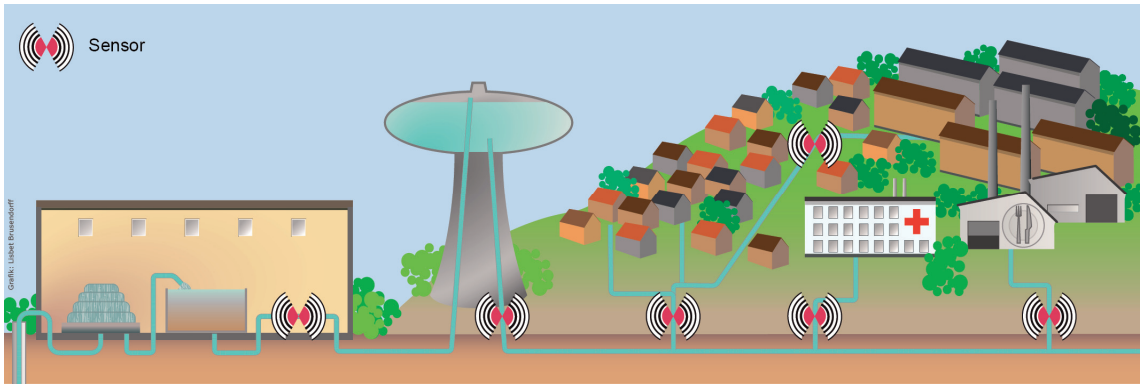

Figure 1: Overview of potential locations of an on-line sensor based on a real-time analysis method for continuous monitoring of microbial drinking water quality.

One 'new' emerging rapid method is the adenosine triphosphate (ATP) assay, which can be used as a parameter for microbial activity in drinking water. The assay has during the past decade gained renewed interest for assessing and characterising the microbiological state of drinking water – bulk phase, biofilms and as a parameter on efficiency of treatment processes at the waterworks (Delahaye *et al.*, 2003; van der Wielen and van der Kooij, 2010; Hammes *et al.*, 2010; Liu *et al.*, 2013). The absolute advantage of this assay is its rapid time for analysis and availability of results, which is in the order of minutes.

ATP is the main energy carrying molecule in all living cells. The ubiquitous presence of adenosine triphosphate (ATP) in all living cells, and the linear relationship between the amount ATP and the bioluminescent signal when assayed with the firefly enzyme luciferase (McElroy, 1947) is what makes ATP an useful parameter for assessing microbial activity in a sample. Principal in short, cellular ATP is extracted through cell lysis and subsequently assayed with luciferase and its substrate luciferin. The bioluminescence system of the luciferase assay, often also referred to as the Luc assay, has been extensively studied and has achieved a broad range of applications – industrial and within research. Holm-Hansen and Booth (1966) were the first to demonstrate the applicability of the ATP assay for microbial activity in aquatic samples.

There are several physical parameters on sensor platforms for real-time monitoring of drinking water, for example temperature, oxygen content, turbidity and pH (Ghazali *et al.*, 2010; Corfitzen and Albrechtsen, 2011). Though, rapid microbiological methods have as of yet not reached a stage, where they can be implemented and installed for on-line continuous real-time monitoring of microbial drinking water quality (Sheikh *et al.*, 2012).

1.1. Aims and approach

The overall objective of this PhD study was to investigate and enhance the understanding of the potential of ATP as a monitoring parameter for microbial drinking water quality. Also, to identify and provide information on essential criteria/requirements for the automation capability of the ATP assay in order for the assay to be implemented on a sensor platform for continuous real-time monitoring of microbial drinking water quality.

More specifically the aims were to:

1. outline criteria and evaluate requirements for the implementation of the ATP assay on a sensor platform for continuous sampling and analysis of drinking water samples, with regard to extraction efficiency, sensitivity and stability of catalytic activity of commercial reagents and stability of ATP standards.
2. evaluate reliability and accuracy of the methodology for quantifying microbial drinking water quality in terms of ATP concentration levels and potential variation in ATP between drinking water systems.
3. investigate the applicability of the ATP assay to detect potential contaminants in drinking water, interpretation of the response and sensitivity of ATP measurements, and differences in response between various types of contaminants.
4. evaluate the prospects for automation of ATP assay and a proof of concept based on initial trials with the ATP assay on a fully automated sensor platform.

These aims are achieved through laboratory scale investigations where the ATP assay is applied for determining the microbial quality in drinking water samples collected from waterworks, distribution networks and taps from various water supply systems in Denmark.

The thesis is organized in two parts – in the first part the results of this PhD study are set into context of the existing literature and knowledge on the application of the ATP assay for aquatic samples with special focus on microbial drinking water quality. The second part of the thesis comprises the papers listed in the preface.

2. Adenosine triphosphate

2.1. ATP and cellular nucleotides

Microorganisms require energy for growth and other metabolic processes within the cell. The most important energy-rich nucleotide in cells is the phosphate compound adenosine triphosphate (ATP), and it is the prime energy carrier in all living cells. Energy is conserved from the oxidation of (in)organic compounds (carbohydrates, proteins and lipids) and is stored in the cell mainly in the form of ATP (Brock and Madigan, 1991).

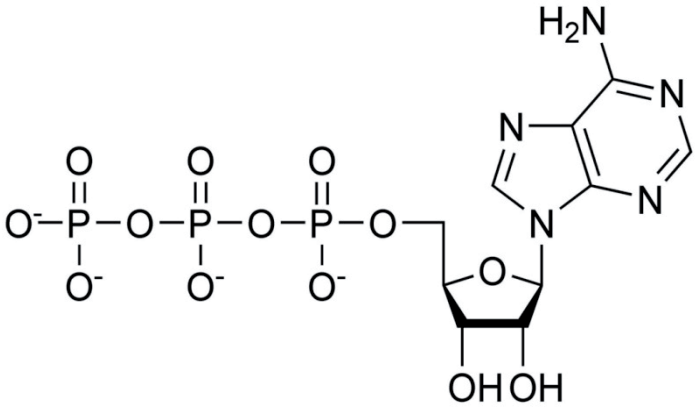

Figure 2: Chemical structure of adenosine triphosphate.

ATP consists of the ribonucleoside adenosine to which three phosphate molecules are bonded in series. Two of the phosphate bonds are phosphoanhydrides and have high free energies of hydrolysis greater than 30 kJ (Figure 2). ATP is formed from adenosine diphosphate (ADP) an inorganic phosphate (P_i). ATP is formed through catabolic reactions of cells (energy generation) and is used in anabolic reactions (energy consumption), i.e. when required the energy stored in ATP is used for cellular processes. ATP is then transformed back into ADP and P_i. ATP is generated during certain exergonic reactions and consumed in certain endergonic reactions (Brock and Madigan, 1991).

The nucleotides have a variety of functions in a living cell: 1) storage and transport of cellular metabolic energy, 2) synthesis of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), 3) activation and transfer of precursors for cellular biosynthesis (Karl, 1980).

2.2. Adenylate energy charge

Microbial ATP concentrations are closely related to biochemical activity and can change rapidly if environmental conditions are altered. The chemical energy in terms of intracellular ATP may rapidly change with turnover times of 0.1 to 1.0 sec for growing bacteria. Hence, the percentage of ATP in the overall energy flux can potentially be relatively small (Karl, 1980). The total adenylate energy charge (EC_A) reflects the energy status of a cell and is expressed by the following relationship between cellular concentrations of ATP, ADP and adenosine monophosphate (AMP) (Atkinson and Walton, 1967):

$$EC_A = \frac{[ATP] + 1/2\,[ADP]}{[ATP]+[ADP]+[AMP]}$$

EC_A is an expression for the amount of metabolic energy momentarily stored in the adenine nucleotide pool. Hence the theoretical range of EC_A ratios is from 0.0 (all AMP) to 1.0 (all ATP). Laboratory studies indicate that the EC_A ratio in growing cells is stabilized between 0.8 to 0.9 (Chapman *et al.*, 1971).

Changes in nucleotide concentrations may occur due to physiological stress and/or nutritional status of the environment (Karl and Holm-Hansen, 1978). A low EC_A quotient suggests that growth is no longer possible and that cells may be nutrient stressed (Jewson and Dokulil, 1982). A study on starving condition of *Pseudomonas sp*. demonstrated that initially there was a loss of ATP per cell during the first 8 days of starvation, followed by a gradual increase back to the original level of ATP per cell. Thus, indicating that cells were able to adapt and regenerate ATP to the initial level before starvation (Kurath and Morita, 1983). This is an advantageous attribute for cells in order to survive in the environments with changing nutrient availability.

There have been several studies on adenylate energy charge ratios in the aquatic environment - especially marine and fresh water environments (Karl, 1980). However, it has not been possible to find studies describing the EC_A ratio of drinking water bacteria. In a sample with a mixed bacterial population such as drinking water, it is likely that cells are on different growth stages. The combination of growing and dividing cells, where some have reached the stationary phase and others might be in a state of dormancy with very low activity, which means that some cells are contributing more than others to the ATP measurement. Hence, it is an average value of ATP which is assayed (Stanley, 1989a)

A decrease in EC_A ratio has been observed for samples which have been filtrated (0.81 to 0.68) or exposed to light/dark shocks (0.90 to 0.69) (Jewson and Dokulil,

1982). Especially the potential adverse effect of filtration on ATP and other nucleotides has been addressed in several studies. Centrifugation and filtration tended to decrease the ATP content (Jones and Simon, 1977). Vacuum filtration resulted in a decrease of total ATP, and the effect increased with increasing volume filtered (Karl *et al.*, 1981).Also, metabolic stress may be imposed by vacuum filtration resulting in a decrease in ATP concentrations, though the concentration of total adenine nucleotides was conservative, indicating a rearranging between concentrations of nucleotides rather than cell lysis (Karl and Holm-Hansen, 1978).

2.3. The ATP assay

2.3.1. Methods for determination of ATP and other nucleotides

ATP and other nucleotides can be measured with several methodologies which can be divided into following four groups of techniques (Khlyntseva *et al.*, 2009):

- Chromatographic methods
- Fluorescence methods
- Bioluminescence methods
- Sensors with immobilised luciferase

The chromatographic methods (ion exchange, reversed-phase or ion-pair reversed-phase high-performance liquid chromatography (HPLC) and thin-layer chromatography (for ATP)) can be used for simultaneous detection of nucleotides in a mixture (Khlyntseva *et al.*, 2009). Fluorescence can be used as a detection technique in the chromatographic determination of nucleotides, but also as an independent analytical technique. ATP determination with fluorescence is subdivided into following two groups - fluorescence enhancement and fluorescence quenching. Also, variety of sensors with immobilised luciferase have been developed which can detect ATP in the micro molar range (Khlyntseva *et al.*, 2009).

Bioluminescence is the emission of light in a biological system stimulated by an enzyme catalysed biochemical reaction (Leitão and da Silva, 2010).The most common methodology for detection of ATP is the bioluminescence method which uses the firefly luciferase enzyme (enzyme entry: EC 1.13.12.7), also referred to as the Luc assay. The Luc assay is advantageous in terms of high sensitivity (10^{-14} M ATP), selectivity and the relatively ease of use. Other adenine nucleotides, ADP and AMP, can be determined by converting them to ATP with adenylate kinase (ADK) and pyruvate kinase, and subsequently

determine with the standard reaction with luciferin/luciferase (Khlyntseva *et al.*, 2009). The principal of the Luc assay for determination of cellular ATP is as follows (Figure 3):

1. Extraction of ATP (cell lysis by e.g. reagent, heat treatment)
2. Reaction with luciferin/luciferase (bioluminescence)
3. Light emission measurement by a photomultiplier.

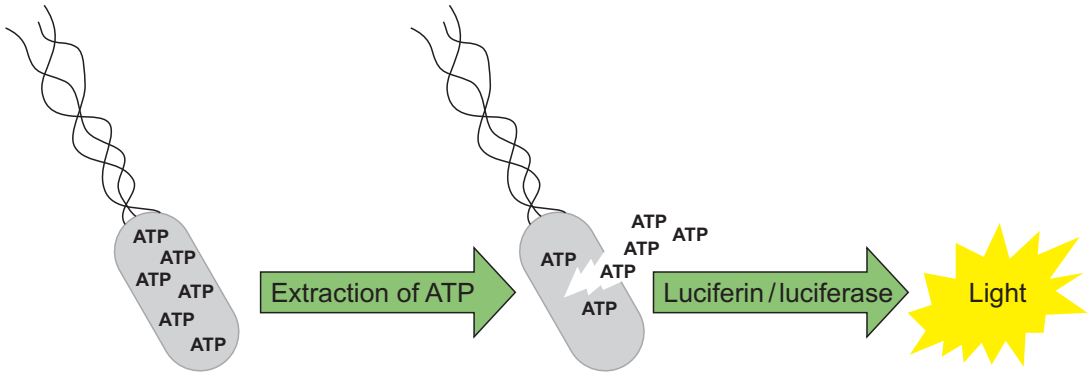

Figure 3: Principal of the Luc assay for determination of ATP, 1) extraction of ATP from cells, 2) reaction with luciferin/luciferase and 3) measurement of bioluminescence.

2.3.2. The Luc assay

Although there are many species of luminous beetles, the knowledge of the biochemistry of beetle bioluminescence has primarily been based on the firefly luciferase (Luc) from the *Photinus pyralis*, the common North American firefly, since it is the most efficient one (Fraga, 2008; Leitão and da Silva, 2010). The accepted name for the *P. pyralis* luciferase enzyme is Photinus-luciferin 4-monooxygenase (decarboxylating, ATP-hydrolysing) (Marques and da Silva, 2009) (Figure 4).

Figure 4: The firefly *Photinus pyralis* (Firefly, 2013).

The Luc bioluminescence system is a complex system and there has been extensive research on the mechanisms and reactions of the assay. The bioluminescence system is dependent on four components: oxygen (O_2), the Luc enzyme, the substrate luciferin (LH_2) and the complex ATP·Mg^{2+} (Lundin, 2000). The significant finding by McElroy (1947) demonstrated that the energy source for the bioluminescence system was the energy-rich molecule ATP, and that light emission was proportional to amount of ATP of a sample. Despite much progress during the past decades the knowledge about this system it is not yet fully understood (Fraga, 2008).

The Luc assay for determination of ATP is a two-step reaction. The first reaction includes the substrate luciferin (D-LH_2) and ATP-Mg^{2+}, where ATP is hydrolysed into AMP generating the enzyme-bound intermediate D-luciferyl-adenylate (D-LH_2-AMP) and inorganic pyrophosphate (PP_i, i.e. $P_2O_7^{4-}$) bound to Mg^{2+} (Fraga, 2008):

$$\text{Luc} + \text{D-LH}_2 + \text{ATP-Mg}^{2+} \leftrightarrow \text{Luc} \cdot \text{D-LH}_2\text{-AMP} + \text{PPi-Mg}^{2+}$$

The second reaction is the oxidation and decarboxylation of the formed intermediate D-LH_2-AMP resulting in AMP, CO_2 and oxyluciferin ($OxyLH_2$; 2-(6-hydroxybenzothiazolyl)-4-hydroxythiazole) which is the light emitter (*hv*) with photons of yellow-green light (550-570 nm) (da Silva and da Silva, 2011).

$$\text{Luc} \cdot \text{D-LH}_2\text{-AMP} + O_2 \rightarrow \text{Luc} + \text{AMP} + CO_2 + \text{OxyLH}_2 + hv$$

The above reaction happens in several steps.

Luciferin can be found in D or L optical isomers. Both isomers react with Luc and ATP·Mg^{2+}, but only D-LH_2 has O_2 consumption and the respective light emission.

The non-bioluminescent L-isomer behaves as an inhibitor of light emission (Fraga, 2008).

The activation reaction is very specific for ATP, not occurring with other nucleoside triphosphates as UTP (uridine 5'-triphosphate), CTP (cytidine 5'-triphosphate), GTP (guanosine 5'-triphosphate) and ITP (inosine 5'-triphosphate); only p_4A (adenosine 5'-tetraphosphate) is able to promote a weak bioluminescence. Besides p_4A, also free ATP, ATPγS (adenosine 5′-O-(3-thio) triphosphate) and Ap_5A (di(adenosine-5') pentaphosphate) can replace cellular ATP although with weaker efficiencies (Fraga, 2008).

The presence of the divalent cation Mg^{2+} is essential for the bioluminescence reaction, since the formation of ATP-Mg^{2+} complexes partially shields the negative charges and influences the conformation of the phosphate groups, and thus explains the requirement of this divalent cation in the reaction (Marques and da Silva, 2009).

2.3.3. Luc assay bioluminescence kinetics

In case of fixed concentrations of the two substrates luciferin and oxygen, the Luc assay can be treated as a simple one-substrate enzyme reaction and thus assumed to follow the Michaelis-Menten kinetics, where the reaction rate (v) or rate of ATP degradation can be expressed as:

$$\frac{v}{V_{max}} = \frac{S}{(S+K_m)}$$

v: reaction rate
S: substrate concentration (ATP)
V_{max}: maximum rate and maximum substrate concentration
K_m: Michaelis-Menten constant

Since the concentration of ATP usually is relatively low compared to the Michaelis-Menten constant, which is the substrate concentration at which the reaction rate is half of V_{max}, the rate of degradation can be simplified to:

$$v = k \cdot S$$

where k (k=V_{max}/K_m) is the first order rate constant for the degradation of ATP (Lundin, 2000).

The firefly luciferase reaction reaches maximum intensity within a second. The light emission will subsequently decay or remain relatively stable depending on the assay conditions (Guardigli *et al.*, 2011). The kinetics of the firefly luciferase reaction can be subdivided in two – those obtained with high and low luciferase concentrations. The kinetics obtained with high luciferase concentrations are

characterized by a rapid rise in light intensity to a maximum in the first few seconds, and a prompt decay to about 5-10% of the peak, followed by a slow decay that may last for hours or even days. Such flash reagents have a high sensitivity and are well suited for detection of low numbers of bacteria (1 amol ATP per cell).

A low concentration of luciferase gives a relatively stable light emission, where decay is proportional to the ATP concentration at ATP<1 μmol/L (ATP<<Km) (Guardigli *et al.*, 2011). Reagent types have been classified into three different groups based on their kinetic properties (Lundin, 2000):

- Stable-light emitting reagents with a decay rate of 0.5% min^{-1} and a detection limit of 1000 amol ATP.
- Slow decay reagents with decay rates of 10% min^{-1} and a detection limit of 10 amol ATP.
- Flash reagents with a decay rate of 235 % min^{-1} (90% of the light is emitted during the first minute) and a detection limit of 1amol ATP or less.

Stable-light and slow decay reagents can be added manually to the sample, while flash reagents require luminometers with automatic timed dispensing systems in order to measure the rapid light signal achieved with this reagent type.

2.3.4. Optimum assay conditions

With all the required precursors present for the bioluminescence reaction to occur, i.e. luciferase, luciferin, oxygen and ATP-Mg^{2+}, the parameters which influence the bioluminescence reaction the most are pH and temperature (Fraga, 2008).

Temperature influences reaction rates and consequently affects the rate of light emission. The optimum temperature for the reaction is 25°C for wild type luciferases (Ford and Leach, 1998), though room temperature in the range of 18-23°C is given for some commercial regents according to manufacturer protocols of reagents e.g. by Celsis and Promicol (Vang *et al.*, III).

The optimum pH for the reaction is pH 7.8, though for analytical purposes a range of pH 6 to 8 is given (Guardigli *et al.*, 2011). Tricine buffer, which has a pK_a of 8.15 offers the greatest buffering capacity of any common buffer and works well for firefly luciferase (Ford and Leach, 1998).

The typical emission spectrum for luciferase is in the yellow-green region (550–570 nm), with a peak at 562 nm at basic media (pH 7.5–7.8). Luciferase is a pH-

sensitive enzyme, and acid media (pH 5–6) can shift the emission to red (maximum at 620 nm). This can also be caused by higher temperatures, heavy metal cations and the substitution of LH_2, ATP or by replacing LH_2-AMP by several analogues. It is believed that conformational changes, which influence the active site microenvironment, are responsible for the different colour emission (Fraga, 2008; Marques and da Silva, 2009).

2.3.5. Luc assay inhibition

Besides the light-producing pathway luciferase catalysed bioluminescence also displays lateral reactions which may interfere with the activity of the Luc enzyme. These constitute substrate related compounds (luciferin analogues), intermediates and products of the Luc catalysed reactions (Leitão and da Silva, 2010).

The main Luc inhibitors are luciferin analogues including L-luciferin (L-LH_2), benzothiazole derivatives and nucleotides (Denburg *et al.*, 1969; Leitão and da Silva, 2010). A lateral reaction is where the complex luciferase D-LH_2-AMP also reacts with oxygen in a dark reaction leading to the oxidized product dehydroluciferyl-AMP (L-AMP). L-AMP is capable of reacting with PP_i-Mg^{2+}, which is released in the light producing pathway, forming dehydroluciferin and regenerating ATP. L-luciferin and dehydroluciferin are strongly inhibitory. Thus, it is of great importance to use very pure preparations of D-luciferin (Leitão and da Silva, 2010; Lundin, 2012). Also, the product oxyluciferin is a competitive inhibitor of luciferase (Leitão and da Silva, 2010).

Nucleotides on the other hand are usually relatively weak inhibitors. The most potent nucleotide inhibitors are uncomplexed AMP and ATP bound to the luciferase, and are competitive inhibitors with respect to ATP-Mg^{2+} (Lee *et al.*, 1970; Leitão and da Silva, 2010).

The product PP_i is also inhibitory of the Luc reaction. However, PP_i can simultaneously be a stabilizer or activator at low concentrations, as it removes the strong inhibitor L-AMP through its pyrophosphorolysis producing dehydroluciferin (abbreviated L) that is a less powerful inhibitor (Marques and da Silva, 2009) (Leitão and da Silva, 2010).

Many divalent metal ions can replace Mg^{2+} in the light reaction. Light production by various divalent metal ion-ATP complexes studied by Lee *et al.* (1970) showed that Mn^{2+} is as active as Mg^{2+}, and Co^{2+} slightly less active than Mg^{2+} in the luciferase-catalysed light production. Other divalent metal ions (Co, Zn, Fe, Cd, Ni, Ca, Sr) were also found to be capable of replacing Mg^{2+} to some extent in

the production of light (Lee *et al.*, 1970). Moreover, anions such as nitrate, thiocyanate, iodide and bromide are also inhibitors of the luciferase bioluminescent reaction (Lee *et al.*, 1970; Deluca *et al.*, 1979).

The assay design (with the standard addition) as well as type and formulation of commercial reagents are assumed to consider potential inhibition by adding additional stabilising components to the enzyme reagent mixture addition e.g. the chelating reagent ethylenediaminetetraacetic acid (EDTA) and coenzyme A (CoA) (Fraga *et al.*, 2005; Guardigli *et al.*, 2011), in order to reduce potential inhibition to a minimum.

2.3.6. Detection of light emission

Light emitted from the bioluminescent reaction consists of photons, and are registered with a luminometer (Figure 5). Light output is measured by integrating the area under the light emission curve for a set period of time (Jago *et al.*, 1989). A luminometer usually consists of a sample chamber for a cuvette, a detector (photomultiplier - PMT), signal processing and a signal output display.

Figure 5: Example of commercial available luminometer – Celsis Advance Coupe luminometer and Advance software (Celsis Advance.im version 3). Photo: Ó.K. Vang.

A PMT registers light photons either by counting individual photons or register the electrical current, when photons strike the PMT, where the output is photons per second or relative light units.

The PMT detects light emitted proportional to the concentration of the analyte e.g. ATP. There will also be a background detected which is caused by phosphorescence of plastics, impurities in reagents etc. The background level for

luminometers is usually lower than for other analytical techniques such as spectrophotometry and fluorometry (Jago *et al.*, 1989).

There are several factors which influence the sample light emission e.g. sample volume, reaction rates, concentration of reactants, reagent injection and mixing, cuvette material and size, and temperature (Jago *et al.*, 1989). The availability of a broad range of commercial reagents and photomultipliers generally make the ATP assay a fairly simple methodology, where all required components of the reaction are added in surplus to the sample, i.e. ATP is the limiting substrate and consequently the ATP concentration is proportional to the light emission.

2.4. Applications of the ATP assay

The Luc assay has a wide range of applications - biotechnology research, industrial applications and environmental monitoring. The two most widely used applications of the assay are the utilization of the luciferase gene as a reporter in molecular biology studies and bioimaging, the other is quantification of analytes connected to ATP or other participants of the light reaction (Marques and da Silva, 2009; Leitão and da Silva, 2010).

2.4.1. Gene expression and regulation

Molecular and cellular biologists use reporter genes to study gene expression and regulation. Reporter genes, in this case firefly luciferase, are introduced into cellular DNA and used to associate a specific gene function with luminescence. Bioluminescence is advantageous for gene expression studies because of its sensitivity, speed and easy methodology (Schmelcher and Loessner, 2008).

2.4.2. Hygiene monitoring

For many years the Luc assay has routinely been used for determination of ATP for microbiological control in a variety of consumer products, e.g. food, dairy, juice, brewery, pharmaceuticals and personal care products. The ATP assay is often used for sterility testing as absence/presence test for high throughput screening (HTS) products (Celsis, 2013; Promicol, 2013a).

2.4.3. Environmental samples

Holm-Hansen and Booth (1966) were the first to apply the assay for determination of ATP of microorganisms in aquatic samples, more specifically marine water. The assay has since then been used for determining biomass activity of microorganisms in marine and fresh water environments (Azam and

Hodson, 1977; Riemann *et al.*, 1979; Björkman and Karl, 2001, Fujii *et al.*, 2008).

The application of the ATP assay has also extended measuring active biomass in soil and sediments, sludge and wastewater and extended to other water types such as process water (cooling water) and drinking water (e.g. Cowan *et al.*, 2002; Jensen, 1989; Jørgensen *et al.*, 1992; Meesters *et al.*, 2003; van der Kooij, 1992).

2.4.4. Detection of specific microorganisms

Usually the ATP assay is applied as a measure microbial activity in general of a sample. However, the ATP assay can be used for specific detection of bacteria or yeasts by separating the species by filtration prior to the ATP measurement (Stanley, 1989b).

The ATP assay can also be combined with immunomagnetic separation (IMS) for detection of specific bacteria, e.g. *E. coli* or enterococci, where the specific bacteria are targeted with selective antibodies attached to magnetic beads (Lee and Deininger, 2004; Bushon *et al.*, 2009a; Bushon *et al.*, 2009b).

This combined methodology of ATP and IMS has been used for detection of *E. coli* in beach water samples (Bushon *et al.*, 2009a). The ATP/IMS assay correlated well with the conventional plate spreading, with a limit of detection of >10 CFU/100 mL. Quantitative results are available within a few hours; and if guideline values are exceeded, it is possible to alarm bathers significantly faster compared to with the traditional cultivation methods. There are several issues when considering the ATP assay in combination with IMS for detection of specific bacteria, i.e. specificity of antibodies, the magnetic capturing step and the washing steps influence the recovery rate. This method has not the required sensitivity for detection of *E. coli* in drinking water (<1/100 mL), and moreover, the protocol for this combined methodology is significantly more complicated than the ATP measurement for total microbial activity.

3. ATP assay for drinking water purposes

3.1. Microbial drinking water quality and public health

3.1.1. Microbial indicators and standard methods

Microbial water quality can vary rapidly, and short-term peaks in total bacterial numbers or pathogen concentration may be a risk to public health. Contaminated drinking water may have been consumed, since there is a delay of days of on the analysis time with the culture based methods, which are the current standard methods for surveillance of microbial drinking water quality (Figure 6). Also, contaminations of relatively short durations may not even be detected due to the low sampling frequency. Even with frequent grab sampling, the majority of the distributed drinking water is not monitored. Hence, confidence cannot be placed solely on end-product testing to determine the microbial safety of drinking water (WHO, 2013).

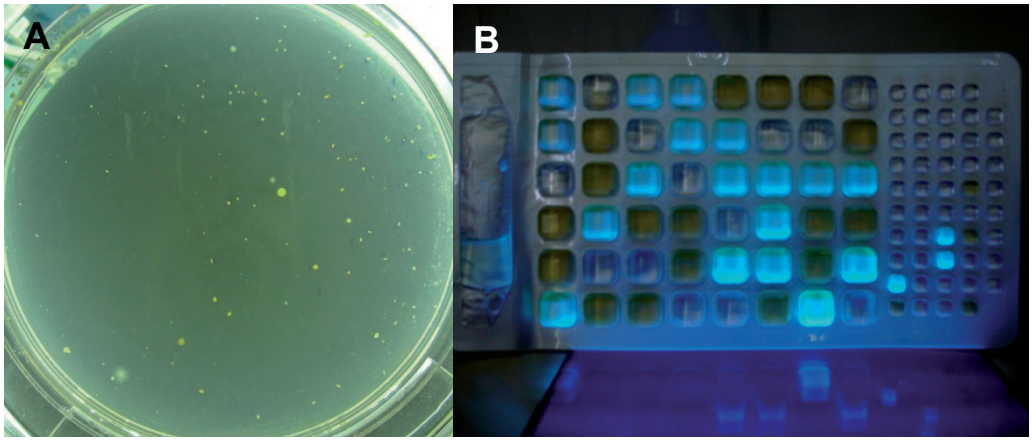

Figure 6: Examples of culture-based methods. A) HPC 22°C (yeast media, 68±4 h); B) Colilert-18 for detection of coliforms (yellow wells) and *E. coli* (fluorescent wells). Photos: Ó.K. Vang.

Continuous sampling combined with a real-time analysis such as the ATP assay would be a significant improvement with regard to surveillance of the microbial drinking water quality. This can potentially lead to the construction of an early warning system, where it will be possible to alarm the consumers in case of a microbial contamination of the water supply system.

This is especially relevant for water supplies which distribute drinking water without a disinfectant residual as e.g. in Denmark or the Netherlands (Smeets *et*

al., 2009). An on-line sensor for continuous monitoring combined with a real-time analysis would possible allow for detection of sudden changes in microbial quality, e.g. a contamination incident. Moreover, detection of pulse contaminations which might occur in between the regulatory controls could potentially be detected (Figure 7).

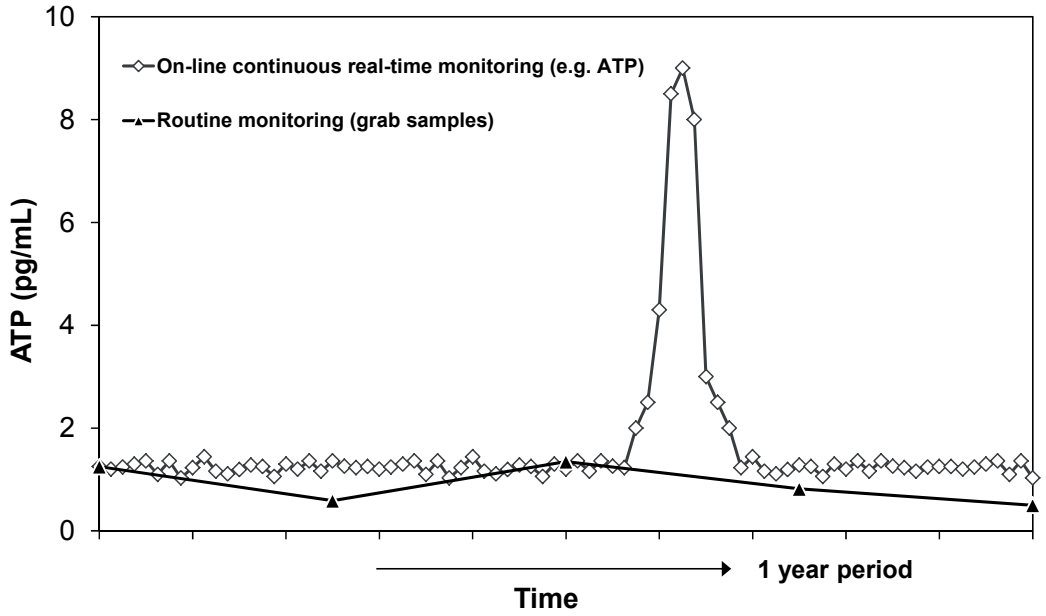

Figure 7: Conceptual drawing of a pulse contamination detected through on-line continuous monitoring with a real-time analysis (e.g. ATP) between regulatory controls (grab sampling).

3.1.2. ATP and microbial drinking water quality

The methodology of HPC measurements is influenced by cultivation media, incubation temperature and culturability of the indigenous bacteria in drinking water, where only a small fraction of total cells are quantified (often <1%) (Maki *et al.*, 1986; Siebel *et al.*, 2008). Moreover, culture based methods do not measure viable but non-culturable cells (VBNC). Measuring microbial ATP has been considered to be superior to the culture based methods due to the rapid availability of results and the fact that all microbes present in a sample both culturable and non-culturable cells are included in the measurement regardless of cellular health, growth requirement and nutrient availability (Venkateswaran *et al.*, 2003). Furthermore, in the case of clumped cells or particle associated cells, the ATP assay may prove to be a more accurate measure than plate counts (Venkateswaran *et al.*, 2003; Liu *et al.*, 2013b). An overview of the advantages and drawbacks of the ATP assay and the culture based methods are given in Table 1.

Table 1: A comparison of the ATP assay versus the traditional culture based methods - advantages and drawbacks.

	Advantages	Drawbacks
ATP assay	– measurement for all active cells (also VBNC-cells) – quantitative – results available within minutes (real-time analysis) – simple measurement procedure – small sample volumes – potential for sensor platform	– non-specific – not as sensitive as the culture based methods for specific indicators e.g. *E. coli*/coliforms – results cannot be converted to a cell count
Culture based methods	– living bacteria (cultivable) – quantitative – can detect indicator organisms (*E. coli*/coliforms) at low concentrations	– time/labour consuming – late results – VBNC-bacteria – heterotrophic plate counts (HPC): <1% detected – selective – low potential for sensor platform

The advantage of the culture based methods, especially faecal indicators such as coliforms and *E. coli*, is their sensitivity and specificity to detect e.g. one coliform/*E. coli* in 100 mL of drinking water with a background level of approximately 10^5 cells/mL (Vang *et al.*, II; Vital *et al.*, 2012).

There are several other rapid methods for assessing microbial quality in aquatic samples. An overview of technical specifications of ATP and other microbiological culture independent methods e.g. total direct counts (TDC), fluorescence in situ hybridisation (FISH), FCM and quantitative PCR (qPCR) is given in Table 2.

Table 2: Comparison of culture and culture independent methods for specific or non-specific detection of microorganisms (modified from Sheikh *et al.*, 2012).

	HPC	TDC (DAPI)	FISH	Flow cytometry	qPCR	ATP
Time	2-5 days	1 day	1-2 days	Minutes-hours	Hours	Minutes
Capital costs	Low capital costs	Large capital costs	Large capital costs	Large capital costs	Large capital costs	Large capital costs
Precision	Low	High (few cells/mL)	High (few cells/mL)	High	High	High
Specificity	Low	Low to medium	Medium to high	Medium to high	Medium to high	Medium to high
Level of expertise	Low	Medium	High	High	High	Medium
Type of information and sample	Grab samples – indicator organisms	Grab samples – all DNA	Grab samples – general or specific	Grab samples and potential for continuous measurements – general or specific	Grab samples – general or specific	Grab samples and potential for continuous measurements – general or specific

Common for many of culture independent methods is the fast availability of results, where several methods have potential for continuous monitoring. However, the cost per sample is higher compared to HPC and they require a higher level of technical know-how than the culture based methods.

3.2. Methodological characteristics/features of the ATP assay

3.2.1. Criteria and challenges

Criteria for an optimum assay measurement and quantification of ATP in drinking water samples are:

- Sensitive enough for quantifying low ATP concentrations (a limit of detection of at least 1 pg/mL)
- Stabile (repeatability) and reliable (accurate) measurement
- A ‘complete’ and fast extraction of cellular ATP
- Low interference of the ATP releasing agent
- Aseptic working conditions (Figure 8)
- Simple and easy measurement protocol.

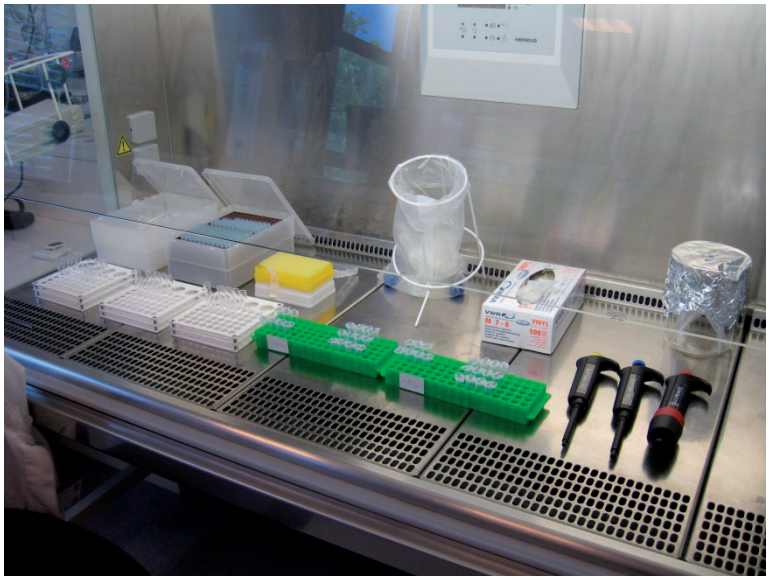

Figure 8: Sterile equipment and working conditions are required for ATP assay measurements. Photo: Ó.K. Vang.

Moreover, a variety of factors may affect the light emission and consequently influence the accuracy of the ATP assay:

- Potential inhibitors
- Temperature
- pH
- Stability in catalytic activity over time.

For example, light production of the ATP assay decreased with increasing salt concentrations in drinking water, with calcium as a main inhibiting compound (van der Kooij *et al.*, 2003). Such inhibition can be accounted for by using standard addition, which is explained further later.

Since ATP has a rapid turnover, it is important that conditions are maintained as constant as possible and kept equivalent to the natural environment of drinking water during sampling and analysis. Temperature and exposure to light might influence the ATP concentrations of the sample (Jewson and Dokulil, 1982; Berney *et al.*, 2006). The pH requirement for Danish drinking water is 7 to 8.5 (Danish executive order on drinking water, 2011), which is in the range of pH required for the ATP assay measurement.

The concept of developing an online sensor based on continuous ATP-measurements depends on understanding/knowing a number of methodological features e.g. reagent sensitivity, reagent stability, measurement stability.

If the ATP assay is to be transferred to a sensor platform for automatic sampling and ATP assay measurements there are some challenges in terms of stability of the catalytic activity of the luciferin/luciferase reagent.

3.2.2. ATP fractions and techniques

ATP occurs as both microbial ATP, which is related to active and viable cells, and a free (extracellular) ATP fraction, which is thought to be from cells in the die-of phase (Stanley, 1986). In, for example, marine and freshwater samples the concentration of free ATP is significant and often exceeds the microbial ATP concentration, hence in such samples it is important of relevance to quantify both microbial and free ATP (Azam and Hodson, 1977; Riemann *et al.*, 1979; Björkman and Karl, 2001). Also, drinking water may contain significant amounts of free ATP (Vang *et al.*, II; Hammes *et al.*, 2010; Vital *et al.*, 2012).

There are two approaches to measure ATP - to measure total ATP (microbial ATP+free ATP) or to determine the microbial ATP concentration. Often total ATP has been used as a parameter for microbial activity, i.e. free ATP is considered negligible (Magic-Knezev *et al.*, 2004; Eydal and Pedersen, 2007; van der Wielen and van der Kooij, 2010; Liu *et al.*, 2013). Other groups specifically quantify microbial ATP (Deininger and Lee, 2001; Delahaye *et al.*, 2003; Berney *et al.*, 2008; Velten *et al.*, 2007; Hammes *et al.*, 2010; Vital *et al.*, 2012). Only recently there has been an increasing focus on measuring free ATP in drinking water and its contribution to the total ATP pool (Vang *et al.*, II; Lautenschlager et al, 2010; Hammes *et al.*, 2010; Vital *et al.*, 2012).

The method for measuring total ATP is fairly straightforward, i.e. extraction and enzyme reagent are added to the sample without any pre-handling or pre-treatment of the sample. The concentration of microbial ATP can be determined indirectly as the difference between total ATP and free ATP (i.e. total ATP and free ATP are measured), where free ATP is measured by performing the ATP assay without addition of extraction reagent. Alternatively the microbial ATP can be measured directly, by adding a filtration step for isolation of the microbial cells, and extracting ATP from the cells on the filter (Delahaye *et al.*, 2003).

For drinking water specifically, the fraction of free ATP is in general low, however it varies between 7-100% (Vang *et al.*, II). Measurement campaigns on non-chlorinated drinking water (N=211) demonstrated that the concentration of free ATP exceeds the microbial ATP concentration in 35% of the samples. The

fraction of free ATP were especially significant in samples with very low (<1 pg/mL) microbial ATP concentrations (Vang *et al.*, II).

Investigations in this PhD study on microbial and free ATP concentrations in drinking water have further substantiated the importance of measuring microbial ATP rather than total ATP.

3.2.3. Extraction of ATP

A challenge in order to perform quantitative ATP analysis in drinking water samples, which contain relatively low quantities of ATP, is to have an as complete cell lysis as possible in order to get a high output and accurate measurement as possible.

A prerequisite for measuring ATP in environmental samples is the extraction of cellular ATP from microorganisms through cell lysis. The extraction step has a direct impact on the reliability and efficiency of the ATP assay. Some properties identified for the ideal extractant are (Stanley, 1986):

1) Instantaneous extraction of ATP
2) Extraction of all cellular ATP present in sample
3) Instantaneous and irreversible inactivation of all ATP hydrolysing enzymes in the sample
4) No inhibition/quenching effect on the enzyme reagent
5) No effect on the assay kinetics.

Extraction can be achieved in several ways (Stanley, 1986):

- Boiling buffer, e.g. Tris buffer with EDTA
- Various dilute acids, e.g. perchloric acid and trichloroacetic acid
- Organic compunds, e.g. dimethyl sulfoxide and butanol
- Surfactants such as Triton X-100 and benzalkonium chloride.

Current standard methods for the ATP assay are based on extraction of ATP in boiling Tris buffer (Eaton *et al.*, 2005; ASTM D4012-81, 2009). Also trichloroacetic acid (TCA) is often recommended as a reference method for other ATP releasing agents (Lundin, 2000). A convenient single-step boiling method with deionized water for extracting microbial ATP has been proposed as an alternative to e.g. perchloric acid and Tris-borate buffer. This boiling method did not show any interference with the bioluminescence and was effective in inhibiting ATPase (Yang *et al.*, 2002).

In most drinking water studies commercial ATP releasing agents, i.e. cold extractants, are used (Delahaye, 2003; Eydal and Pedersen, 2007; Hammes *et*

al., 2010; van der Wielen and van der Kooij, 2010, Vang *et al.*, III). The use of cationic reagents, which usually are quaternary ammonium salts, for the extraction of bacterial ATP has become a routine procedure already in the late 80'ties (Schram & Witzenburg, 1989), and are relatively easy to use compared, to the more complicated protocol of the boiling method.

ATP extraction efficiencies of six commercial and six R&D reagent kit were investigated by Vang *et al.* (III). Results showed significant differences in extraction of ATP (Figure 9). A more efficient and optimum extraction might be achieved by prolonging, for example, the time of extraction in order to get a higher yield of ATP (LeChevallier *et al.*, 2003; Hammes et. al, 2010).

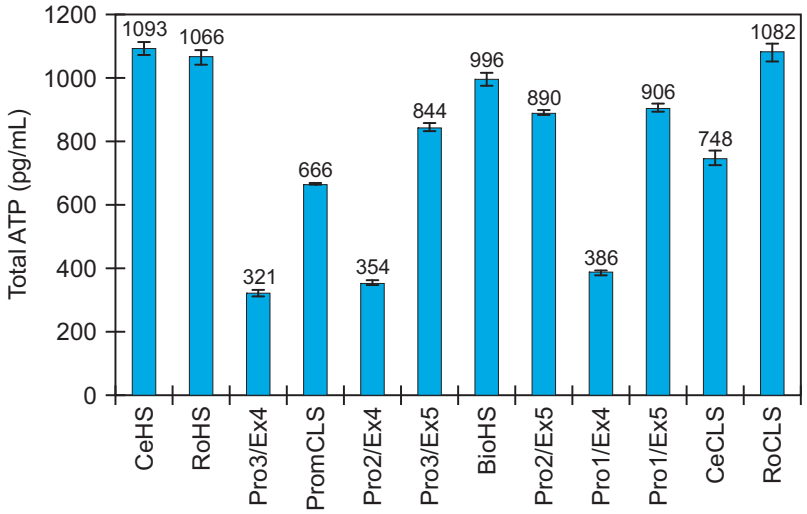

Figure 9: Extraction efficiency of six commercial reagents and six R&D reagents compared by measurement of total ATP concentrations in a drinking water sample spiked with yeast (Vang *et al.*, III).

3.2.4. Reagent sensitivity and stability

The luciferase enzyme has been cloned from several species, e.g. *Photinus pyralis*, *Luciola cruciata* and *Luciola lateralis*. The genes have been modified to increase thermostability, change emission spectrum, increase catalytic activity etc. (Lundin, 2000). For example, wild type luciferases are unstable above 25°C, and are completely inactivated within 5 min at 50°C (Guardigli *et al.*, 2011). The activity of a thermostable mutant luciferase was 75% of the initial activity at the same temperature.

Enzyme reagents exhibit different sensitivities depending on enzyme and substrate concentration, as mentioned previously. Flash reagents often have a higher sensitivity and correspondingly a lower limit of detection compared to stable light reagents (Vang *et al.*, III). However, the flash reagents are inherently

less stable in terms of catalytic activity of the reconstituted enzyme, which is the trade-off for a low limit of detection (Vang *et al.*, III).

It is essential that enzyme reagents to be used on a sensor platform remain relatively stable over time, since loss in catalytic activity of the enzyme will also lead to loss in sensitivity which has a direct impact on the limit of detection of the assay measurement. It is possible to calibrate with an internal ATP standard for the loss in in activity of the enzyme reagent as long as the limit of detection is maintained at a level that is satisfactory for the specific level of ATP in samples.

Limit of detection reported in literature on drinking water investigations are given in Table 3. It is evaluated that a limit of detection of 1 pg/mL or less is adequate for drinking water analysis.

Table 3: Limit of detection (LOD) of the ATP assay reported in drinking water studies. Molar concentrations are converted to grams by using the molar weight of ATP of 507.18 g mol^{-1}.

LOD		**Based on**	**Reference**
original value/unit	**(pg/mL)**		
- -	1 0.7	total ATP free ATP	Vang *et al.*, II
- -	0.2-8.9 0.3-1.4	total ATP free ATP	Vang *et al.*, III
1 ng/L	1	total ATP	Liu *et al.*, 2013a
0.0001 nM	0.05 pg/mL	total ATP	Hammes *et al.*, 2010
0.01 nM	5.1	total ATP	Hammes *et al.*, 2008
0.01 nM	5.1	total ATP	Velten *et al.*, 2007
-	1	total ATP	Ochromowicz and Hoekstra, 2005
0.2 pg	0.2	microbial ATP	Deininger and Lee, 2001
-	1	total	van der Kooij, 1992
0.14 pg/100 µL 0.24 pg/100 µL	1.4 pg/mL 2.4 pg/mL	total ATP total ATP	Marriott *et al.*, 1992

The limit of detection is lower for free ATP than for total ATP, since the signal to noise ratio is increased when measuring free ATP compared to total ATP, since only enzyme reagent is added in the assay measurement, i.e. there is no inhibition/quenching compared to addition of both ATP releasing agent and enzyme reagent, i.e. the total ATP measurement.

Experiments on stability of activity of reconstituted enzyme reagent stored at different conditions demonstrated that there was a denaturing of luciferin/luciferase reagent over time. After 19 days the activity was reduced to approximately 35-76%, depending on the storage (Table 4) (Vang *et al.*, not published). There was a higher loss in activity when stored a 1°C than at -20°C,

and moreover, the results demonstrated that activity of the reconstituted substrate/enzyme was best maintained when stored anaerobically at 1°C , i.e. an activity of approximately 75% after 19 days.

Table 4: Catalytic activity of a reconstituted luciferin/luciferase reagent (Roche) after 19 days stored at various conditions.

ATP standard (pg/mL)	Storage of enzyme reagent		
	Aerobic -20°C (%)	Aerobic +1°C (%)	Anaerobic +1°C (%)
10	66	61	75
500	53	35	76
1000	59	40	76

This means that type of enzyme reagent and storage conditions of the enzyme reagents are important to consider when implementing the ATP assay in a sensor for on-line continuous real-time monitoring.

3.2.5. ATP standards

When measuring ATP in e.g. environmental samples, the ATP concentrations should be given in moles or grams rather than relative light units, which are quite arbitrary, since these vary depending on reagents (sensitivity) and luminometers (Vang *et al.*, III; Jago *et al.*, 1989; Marriott *et al.*, 1992).

To convert the light signal (RLUs) into an ATP concentration, the sample has to be referred to an ATP standard, either with a standard curve or by standard addition. When using a calibration curve for converting RLU signals to an ATP concentration, it is likewise important to measure ATP standard controls throughout a day if e.g. the room temperature is not controlled or the activity of the substrate/enzyme is decreasing with time.

Manufacturers often recommend a commercial buffer for preparation of ATP standard dilutions. Though, it is important to be aware of that light emission varies for different matrices. As such, quantifying ATP in drinking water it is important to either use ATP standards prepared in the same matrix as drinking water or to use standard addition (van der Kooij *et al.*, 2003). The slope of the calibration curve differs when ATP standard dilutions are prepared e.g. in buffer, MilliQ-water or sterile filtrated autoclaved drinking water (Corfitzen, 2004).

ATP standards reconstituted in drinking water are stable for several years when stored at -80°C (Corfitzen, 2004). The reagent manufacturer BioThema has developed an ATP standard, which is stable at 4°C for 2 years (BioThema,

2013). In this PhD study, ATP standard salt of two manufacturers - Roche and Celsis - reconstituted in buffer, MilliQ-water or sterile filtrated autoclaved drinking water stored at 1°C were measured against standards stored at -80°C. Standards stored at 1°C were fairly stable over 28 days, where ATP standards prepared in sterile filtrated autoclaved water were more stable than standards in buffer (Vang *et al.*, not published).

In regard to a sensor based on the ATP assay, it is important that the liquid ATP standard reagent used as for standard addition in stable at relevant temperatures in order to get a reliable conversion of light emission to an ATP concentration.

For the quality control of concentrations of ATP standard dilutions, the light emission with ATP standard dilutions of newly prepared ATP standard salt are compared with that of an old ATP standard salt.

When analysing many samples, in e.g. the laboratory, there will be less work and less reagent use, if a standard curve is prepared and measured, rather than measure all samples with and without standard addition. Though calibration curves for both total ATP and free ATP have to be measured, since quenching by extractant and additional volume of reagents results in calibration curves with different slopes when determining total and free ATP concentrations.

The disadvantages of a calibration curve are:

- Preparing and validation of ATP standards is time consuming
- In case of few samples measurements it is also costly in additional reagent use for measuring ATP standards
- The calibration curve does not account for matrix effects or potential inhibition or quenching of the light signal from sample.

Standard addition is more appropriate when measuring a few samples. Also, for continuous monitoring of ATP concentrations it is appropriate to include a standard addition and thus accounting for diurnal temporal variations, and simultaneously taken into account any potential assay interferences (assay inhibition, turbidity and loss in enzyme activity over time.

If sample and media used for preparation of ATP standard have the same characteristics, i.e. if the sample is drinking water and the internal standard is reconstituted in drinking water (sterile filtrated and autoclaved), the ATP concentration is calculated by:

$$\mathrm{ATP_{sample}\ (pg\ ATP/mL)} = \frac{\mathrm{RLU_{sample}}}{\mathrm{RLU_{sample+IS}} - \mathrm{RLU_{sample}}} \cdot \mathrm{ATP_{IS}}$$

RLU_{sample}: the light emission from the sample
$RLU_{sample+IS}$: the light emission from the sample and the ATP standard
ATP_{IS}: concentration of the added ATP standard
ATP_{sample}: concentration of ATP in sample.

Considerations when using internal ATP standard are, for example, as a rule of thumb the IS should contain 10 times as much ATP as the sample, and it is important to avoid evaporation or contamination in order to maintain accuracy of the concentration of the IS (BioThema, 2013).

4. ATP levels in drinking water systems

Even though the ATP assay is not a novel assay, there is a novelty to its application for drinking water purposes. The assay has recently gained new interest for quantifying microbial activity in drinking water systems. This may be related to an increased awareness on the ATP assay applicability to measure and quantify microbial activity in drinking water. This may also be an effect of increased availability of many different commercial reagents for the ATP assay, where reagents are tailored for the specific application, i.e. distinguishing between clinical, food and water.

This chapter gives an overview of the various applications of the ATP assay in the drinking water system. The capability and dynamics of the ATP assay to quantify microbial activity in drinking water are described, also in relation to other microbiological methods.

4.1. Distributed drinking water

4.1.1. ATP levels in drinking water

ATP concentrations in drinking water are relatively low compared to other water types, e.g. marine, fresh, surface or waste water (Azam and Hodson, 1977; Riemann, 1979; Jørgensen *et al.*, 1992; Vang *et al.*, II). Most of the recent studies on ATP levels in drinking water have been carried out on Dutch or Swiss drinking water (Table 5).

Total and microbial ATP concentrations in drinking water (primarily without chorine residual) are remarkable similar across countries, water supply systems with different source waters and different treatment trains at waterworks. Though, for drinking water sampled from taps after overnight stagnation have demonstrated a significant increase in microbial ATP compared to water from the water mains, due to aftergrowth (Siebel *et al.*, 2008; Lautenschlager *et al.*, 2010, Vang *et al.*, II)

Seasonal variations in total ATP concentrations were observed by van der Wielen and van der Kooij (2010), while other investigations did not find any noticeable variations (Lautenschlager *et al.*, 2013; Liu *et al.*, 2013). Stable ATP concentrations throughout the distribution network, indicates a low and fairly stable baseline of ATP (van der Wielen and van der Kooij; Vang *et al.*, II; Vital et al, 2012; Lautenschlager *et al.*, 2013; Liu *et al.*, 2013a).

Table 5: ATP concentrations in drinking water. Values are stated as total ATP, concentrations in () represent microbial ATP.

Sample type	**Range (pg/mL)**	**Average (pg/mL)**	**Reference**
Waterworks, distribution network and tap water (stagnant)	<1-27 (<1.2- 27)	3.1 ±0.3 (1.7 ±0.2)	Vang *et al.*, II
Distributed water	-	(1.8 ±1.0)	Lautenschlager *et al.*, 2013
Distributed water	1-3	-	Liu *et al.*, 2013a
Waterworks	1-6	-	Liu *et al.*, 2013b
Distributed water	(approx. <5)	-	Vital *et al.*, 2012
Distributed water	0.8-12.1	-	van der Wielen and van der Kooij, 2010
Tap water (stagnant) Distributed water	- -	(6.32±4.92) (1.01±0.32)	Lautenschlager *et al.*, 2010
Distributed water	2.54-47.7	11.7±8.62	Siebel *et al.*, 2008
Distributed water, drinking water fountains and bottled water	0.016-0.055	-	Berney *et al.*, 2008
Treated water	<1-45	<5 in 75% of the samples	van der Kooij, 2003
Distributed water (with chorine residual)	(0.002-4.1) (0.002-0.2)	(0.3 ±0.7) (0.1 ±0.2)	Delahaye *et al.* , 2003
Distributed water	<1-23	-	van der Kooij, 1992

4.1.2. Correlation between ATP and other microbiological methods

ATP is often used as a microbiological parameter together with other microbiological methods to characterize microbial drinking water quality (Deininger and Lee, 2001; Siebel et al, 2008, Vital *et al.*, 2012) and to assess biological stability (Lautenschlager *et al.*, 2013; Liu *et al.*, 2013a).

There has been some focus on the correlation with HPC. Some studies have found a significant correlation between ATP and HPC in drinking water (Deininger and Lee, 2001; Delahaye *et al.*, 2003). However, more often there is

no correlation observed between ATP and HPC (Siebel *et al.*, 2008; Hammes *et al.*, 2010; Liu *et al.*, 2013a). It is essential to realise that these two microbiological parameters are very different; ATP is a total measurement, whereas HPC often only comprises <1% of the bacteria present in a sample (Maki *et al.*, 1986). Consequently, these two microbiological parameters should not necessarily correlate; in fact the opposite is more likely to be expected.

Other total counts methods such as microscopy and flow cytometry have often been found to have a significant correlation with ATP concentrations (e.g. Eydal and Pedersen, 2008; Siebel *et al.*, 2008; Hammes *et al.*, 2010). A correlation between total cell count methodologies might to some degree be expected, since these are measurements of total biomass. However, Liu *et al.* (2013) did not observe a correlation between flow cytometry and ATP. Flow cytometry has in a few recent studies been evaluated to be better at detecting small changes in biomass compared to ATP (Vital *et al.*, 2012; Liu *et al.*, 2013a).

Overall, it is important to recognize that the different methodologies provide different information on the microbiological state of drinking water. The different microbial parameters should be viewed as exactly that, different parameters, where it can be expected that they provide different but supporting information on e.g. microbial drinking water quality.

4.1.3. ATP per cell in drinking water

Total cell counts and ATP measurements have specifically been used to calculate the ATP per cell values. A value of 10^{-15} g ATP per bacterial cell has be given as an 'average' value (Stanley, 1989b). However, care should be taken of applying such a generic number, since ATP per cell is expected to vary depending on type of environment and nutrient availability. Several recent studies have demonstrated that the ATP content per cell for drinking water bacteria is one to two orders of magnitude lower (Table 6). This may be related to the fact that drinking water is an oligotrophic environment.

Table 6: ATP per cell reported in various drinking water studies. Values stated as total ATP/cell, concentrations in () represent microbial ATP/cell.

Country	Source water	Sampling location	TCC[a] method	ATP per cell ($x10^{-17}$ g/cell)	Reference
Bulk phase bacteria					
Denmark	Groundwater	Waterworks	Microscopy (DAPI)	(2.3-5.6)	Vang *et al.*, I
The Netherlands	River water (ARR[b]), groundwater	Waterworks	Flow cytometry	2.1-3.3	Liu *et al.*, 2013b
The Netherlands	River water Polder seepage water River water Polder seepage water	Waterworks Waterworks Distribution network Distribution network	Flow cytometry	(1.4-73) (1.9-17.4) (0.5-1.0) (1.2-2.7)	Vital *et al.*, 2012
Switzerland	-	Distribution network Waterworks	Flow cytometry	(5.42) (1.81)	Lautenschlager *et al.* 2010
Switzerland	Aquatic samples	Distribution network Alpine springs Fresh water Bottled water	Flow cytometry	(8.9)	Hammes *et al.*, 2010
The Netherlands	Groundwater Surface water	Waterworks Distribution network	Microscopy (AODC)	1.7-1.8	van der Wielen and van der Kooij, 2010
Switzerland	-	Distribution network	Flow cytometry	0.5-20.9	Siebel *et al.*, 2008
Switzerland	Mountain springs[c]	Distribution network	Flow cytometry	3.1-6.2	Berney *et al.*, 2008
Switzerland	Surface water	Treatment train at waterworks	Flow cytometry	(8-20)	Hammes *et al.*, 2008
Norway	Groundwater	Raw water	Microscopy (AODC)	22-52	Eydal and Pedersen, 2007
Surface associated bacteria					
The Netherlands	River water (ARR[b]), groundwater	Particle associated bacteria	Flow cytometry	3.8-5.4	Liu *et al.*, 2013b
Switzerland	-	Granular activated carbon filter	Flow cytometry	(6.7)	Velten *et al.*, 2007
The Netherlands	-	Rapid sand filters Granular activated carbon filters	Microscopy (AODC)	36 2.1	Magic-Knezev and van der Kooij, 2004
The Netherlands	River bank filtrate River water	Membranes (nanofiltration or reverse osmosis)	Microscopy (AODC)	2-70	Vrouwenvelder *et al.*, 1998

[a] total cell count

[b] artificial recharge and recovery

[c] the source water public drinking fountains was untreated mountain springs, while the source for the 'normal' tap water was not given in this study.

Average values of ATP per cell in drinking water vary mostly depending on the sampling location in the water supply system, and yet are very comparable. Two exceptions are noticeable, i.e. raw groundwater samples and samples after various treatment steps at the waterworks, where the ATP per cell was slightly higher than for the distributed water, indicating higher microbial activity.

ATP per cell in the bulk phase is generally less than bacteria associated with surfaces (e.g. filter material and biofilms) (Table 6), indicating planktonic cells to be less metabolically active than bacteria associated with surfaces, where the nutrient conditions are more favourable than in the bulk phase.

The amount of ATP per cell might also vary depending on the method used for total cell count, i.e. flow cytometry or microscopy, and additionally if there was live/dead differentiation of bacteria by using stains. Moreover, ATP per cell is in some studies are given as microbial ATP and in others as total ATP per cell. The most correct way to correlate these two parameters is applying microbial ATP, i.e. the active biomass in drinking water, and total cells counts with viability staining (membrane intact bacteria).

4.2. ATP as treatment process parameter

ATP has in a few studies been used to assess the efficacy of different treatment processes at waterworks as well as to characterise microbial behaviour in these systems in combination with other microbiological methods (e.g. FCM, HPC) (Hammes *et al.*, 2008; Vital *et al.*, 2012; Liu *et al.*, 2013a).

The importance of quantifying microbial ATP rather than total ATP has been emphasised, since free ATP may be present in significant amounts when drinking water treatment technologies such as ozonation or chlorine are used (van der – Kooij, 1992; Hammes *et al.*, 2008; Vital *et al.*, 2012).

ATP measurements have been applied to monitor the backwashing process of a rapid sand filter at waterworks, in order to assess the efficacy of the backwash, and to evaluate when the filter was clean from the backwashing treatment (Henriques and Schnipper, 2008). Additionally, ATP assay also proved applicable to assess when drinking after was had a normal microbial activity in terms of distribution after start-up of the backwashed filter.

4.3. Aftergrowth potential and biofilms

The ATP assay has demonstrated to be an applicable parameter to determine microbial growth potential (van der Kooij, 1995). The ATP assay is also one of the methods included in the preparation of a CEN standard to assess enhanced

microbial growth (biomass production potential - BPP) of materials in contact in contact with drinking water which is to be used in the European Acceptance Scheme (EAS) (van der Kooij *et al.*, 2006).

ATP measurements are used for monitoring in situ biofilms on pipe materials (Lethola *et al.*, 2004). The ATP assay has for example been used to measure microbial activity/density on PVC pipes in the distribution network (40 to 2000 pg total ATP/cm^2), and in biofilms on different types of materials and of various age (50 to 865 pg total ATP/cm^2; 6 months to 10 years) and biofilm formation rate in a model distribution network (van der Kooij, 1992; Hallam *et al.*, 2001; Boe-Hansen *et al.*, 2002).

Biomass on sections of plastic pipes of various age (1, 6 and 13 yr.) and a very old cast iron pipe (78 yr.) taken from a distribution network was quantified with ATP measurements (and HPC) (Figure 10) (Vang *et al.*, 2011). The ATP concentration was increasing with increasing age of the PE pipes, indicating higher cell number and/or higher metabolic activity with increasing age, while HPC concentrations were similar despite different ages. The biofilm of the old cast iron pipe had a significantly higher ATP concentration than the PE pipes, which can be explained by the relatively porous surface due to scaling etc., resulting in a larger surface area for attachment of bacteria.

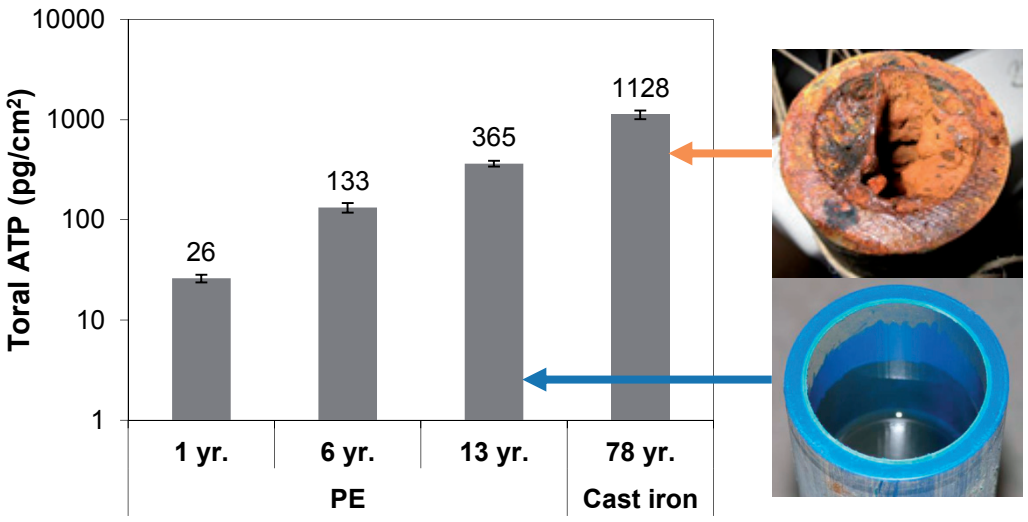

Figure 10: Biofilm densities on PE and cast iron pipes of different age from a distribution network supplying non-chlorinated drinking water (Vang *et al.*, 2011).

4.4. Detection of potential contaminations

4.4.1. Waste water and surface water contaminations

Using a rapid method such as ATP for real-time monitoring of microbial drinking water quality, it is relevant to know whether the method is capable of detecting potential drinking water contaminations and to which extent.

Very limited research has been on detection of potential contaminants in drinking water using the ATP assay. Only a few cases on drinking water spiked with *E. coli* have been investigated with the ATP assay; in one case an incubation step was included (Frundzyan and Ugarova, 2007; Ghazali *et al.*, 2010).

Experiments included in this PhD study encompassed two potential contamination situations – drinking water was contaminated with surface water from a pipe dig-out in relation to renovation the distribution network, and drinking water contaminated with wastewater because of pipe leakage/loss of pressure (Figure 11).

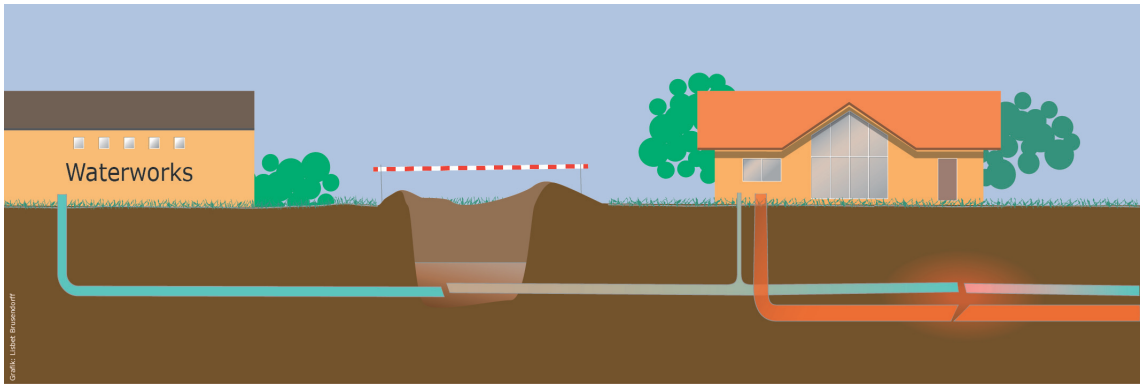

Figure 11: Potential drinking water contaminations with microbial ingress from surface water from a pipe dig-out and wastewater through leaking pipes.

Through several experiments, it was demonstrated that the ATP assay was able to detect wastewater diluted 10^3-10^4 times in drinking water, while surface water diluted 10^2-10^3 times in drinking water was detected (Figure 12).

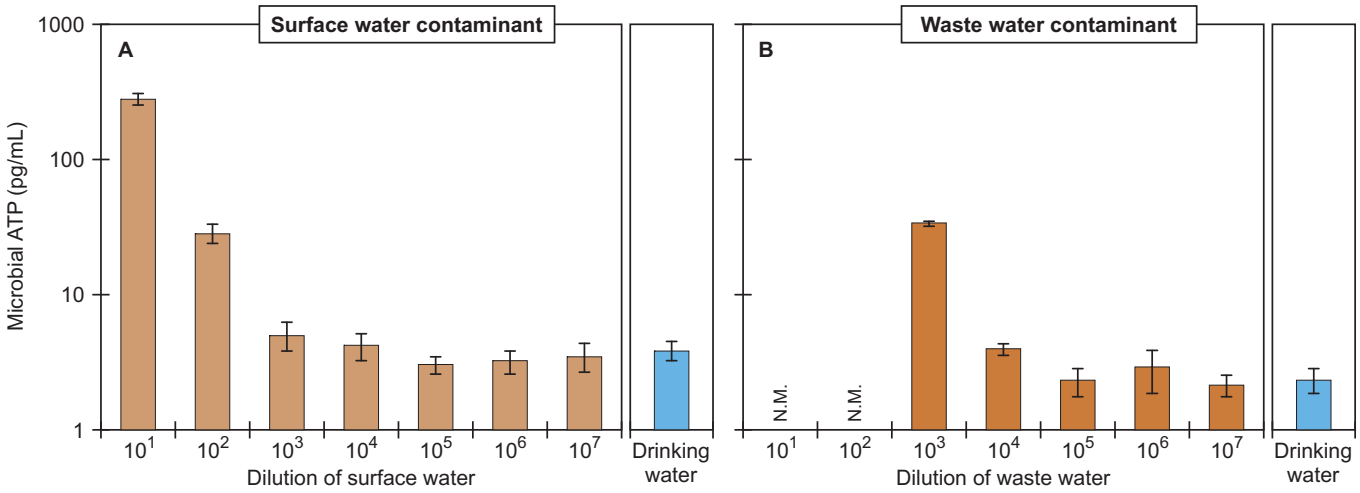

Figure 12: Response in microbial ATP in drinking water artificially contaminated with A) waste water and B) surface water.

Despite microbial ATP is considered a more accurate and dynamic parameter on microbial activity (Vang *et al.*, II), it did not improve the ATP assay's ability to detect the wastewater contaminant opposed to measuring total ATP in this specific experiment (Vang *et al.*, I). Applying more sensitive reagents for the ATP analysis did not reduce measurement variation for low concentrations of ATP, and consequently it did not contribute substantially to the ATP assay's ability to distinguish between contaminated and clean drinking water.

Compared to other microbiological methods the ATP assay was better at detected wastewater and surface water contaminations to a higher degree than TDC, though both HPCs and Colilert-18 exceeded the ATP assay in this respect (Vang *et al.*, I).

Several factors were identified as controlling for the ability of the ATP assay to detect contaminations, namely the microbial load of the specific contaminant, degree of dilution of the contaminant when assayed and the ATP level of the specific type of drinking water.

Monitoring microbial drinking water quality with ATP measurements displays a definite potential for real-time detection of microbial contaminants.

5. On-line sensors for monitoring microbial drinking water quality

The development of real-time methods and implementing these on sensor platforms for on-line continuous monitoring is the first step in the construction of an early warning system. A real-time monitoring and intelligent control of the water supply system would significantly improve the possibility to detect deteriorated microbial quality and microbial breakthroughs in distributed drinking water.

Online monitoring with real-time – or close to – microbial methods for detection of microorganisms in e.g. drinking water, is an area in a steady and exciting development. At-line systems, i.e. the system is located on-site, where the analysis also is performed systems, such as the fully automated Colifast system or the mbOnline COLIGUARD® which are used for fairly rapid detection (hours) of coliforms and *E. coli*, though the methodology principal of these systems are dependent on a certain time of incubation (Colifast, 2013; mbOnline, 2013). Furthermore, a first implementation of flow cytometry on an automated platform has recently been done, with the purpose of continuous monitoring of bacteria in drinking water (Hammes *et al.*, 2012).

5.1. Monitoring locations

The water supply system may comprise of a number of physical vulnerabilities, which might result in a deteriorated water quality or microbial ingress (Deininger *et al.*, 2011). A sensor based on a real-time analysis for continuous on-line monitoring of microbial drinking water quality has a definite potential for water supplies in delivering clean and safe drinking water. As previously presented in the introduction, a real-time on-line sensor for continuous monitoring of microbial drinking water quality combined with an early-warning system can be located in a number of locations – strategically selected – in the water supply system (Figure 13).

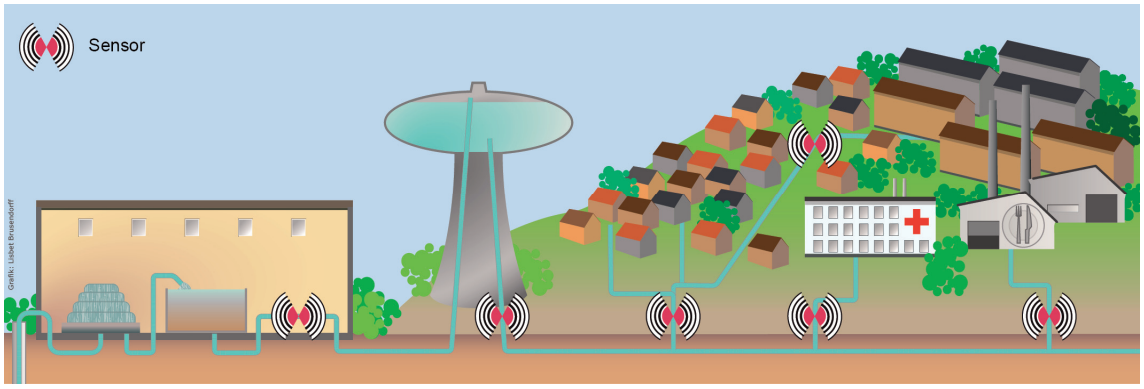

Figure 13: Overview of potential locations of an on-line sensor based on a real-time analysis method for continuous monitoring of microbial drinking water quality.

For each specific system it is necessary to identify optimum locations for a real-time microbiological sensor. A sensor for monitoring freshly produced drinking water leaving the water work, would detect a potential breach in hygienic barrier (treatment). Contaminants might enter the water supply system via leaks in a water tower/reservoir (e.g. bird faeces, surface water) or when renovating the distribution network (e.g. dirt, surface water). Hence, it would be apparent to locate a sensor at a water tower/reservoir outlet or on major pipe connections/branches. Moreover, such a sensor could also be located at the inlet to sensitive consumers such as hospitals, pharmaceutical and food industry (Figure 13).

Network modelling of the specific water supply system would be a tool for the identification of vulnerable locations and development of monitoring strategies with sensor technologies for the specific system.

5.2. ATP assay automation and sensor platforms

5.2.1. Design requirements and challenges

An automated sensor application would typically consist of following elements, a sampler, a sensor which is a detector of some sort, signal processing, and a display for results output and potentially configuration for remote signalling (Campbell, 2008). Some generic features of a sensor technology for monitoring microbial drinking water quality would be:

- Sensitive
- Robust and reliable
- Low level of maintenance
- As cheap as possible.

Despite the simplicity to perform the well-established methodology of the ATP assay either in the laboratory or on site with a hand-held luminometer, there are some difficulties to overcome when transferring the assay onto an automated sensor platform for continuous on-line monitoring. For example the substrate/enzyme reagent, reconstituted or not, has to have a catalytic activity throughout time which fulfils the required limit of detection. The challenge is that when reconstituted, the enzyme can be denatured quite fast (within a day), which can be calibrated for by using standard addition, again as long as sensitivity and the limit of detection required for drinking water is maintained, as previously. Other specific requirements for the automation capability of the ATP assay are:

- Accuracy and precision of sample and reagent dosing
- Cleaning of tubes/chambers to avoid carry over from previous sample
- Stability and repeatability of the photomultiplier)
- Mechanical stability and robustness
- Intelligent software program and on-line alarm system
- Low maintenance and inspection.

5.2.2. ATP sensor prototypes

The idea and concept for automation of the ATP assay for quantifying total active biomass in drinking water was partially investigated already in the early 1980'ties (Picciolo, 1981). The limit of detection for the assay used then was 10^5 cells/mL. Hence, at a concentration step was required, and much focus was put on the technique for concentrating ATP for this specific purpose. A proof of concept stage was not reached, since the project was terminated due to manpower constraints.

Collaboration between DTU Environment and the company CU Test resulted in a report for DANVA (The Danish Water and Wastewater Association) on a sensor prototype unit based on ATP measurements (Corfitzen *et al.*, 2007). The continuous time-series based on ATP measurements showed promising results for monitoring of microbial drinking water quality with ATP measurements. However, the design of this specific on-line ATP sensor was reconsidered and was still on a developing stage throughout the course of this PhD study.

Moreover, a fully automated *in situ* ATP analyser has been developed for measuring ATP in deep sea water (Aoki *et al.*, 2008). This microsystem is significantly different in size and design from the two previous systems

mentioned. The sensitivity of the system depends upon the design of the microfluidic device, and has a sensitivity of approximately 1 pg/mL.

5.2.3. Proof-of-concept with Promicol sensor prototype

A sensor prototype designed especially for the ATP assay (Figure 14) has been developed by Promicol (The Netherlands). The prototype is fully automated with regard to sampling, ATP assay measurements and cleaning of the system between sampling and ATP assay measurements.

Figure 14: Promicol ATP sensor prototype (Promicol, 2013b).

The sensor prototype also comprises an advanced software program designed for the ATP analysis in terms of data analysis. In a collaborative work with Promicol several features on its applicability to analyse ATP in drinking water were investigated. The research included measurements of ATP standard dilutions, repeatability, precision of the automatic dosing, potential carry-over between measurements, response to contaminated water and overall evaluation of robustness of the automated unit. Some of the results are presented here.

The Promicol ATP sensor prototype gave a linear response for ATP standard dilutions in the range of 0 to 1000 pg/mL, demonstrating its ability to measure and quantify ATP concentrations in the low and high range. Very similar to previous laboratory experiments on detection of waste water contamination in drinking water, the ATP sensor was also able detect a 10^3 dilution of wastewater in drinking water.

The precision of the automatic dosing system in the sensor needed improvement. A 1:1:1 volume relationship was programmed in the software for addition sample, enzyme reagent and extraction reagent, which was not achieved during use (Table 7). Also the he coefficient of variation (CV) was 15-22% for volume dosing, which needs improvement for more accurate ATP measurements and reproducible results.

Table 7: Variation and repeatability (CV) of dosing of sample, enzyme reagent and extraction reagent of ATP sensor prototype (N=10).

	Average (g)	**CV (%)**
Sample	0.082 ±0.012	15
Enzyme reagent	0.067 ±0.011	17
Extraction reagent	0.049 ±0.011	22

Continuous ATP measurements with the sensor prototype, programmed to sample and measure every 5 minutes with duration of 2.5 to 5 h, on microbial activity in tap water at the DTU Environment Department demonstrated that the ATP level increased over the time period 11 am to 12.30 pm (Figure 15). The same pattern was observed on three different days. On November 15th the RLU values very significantly higher compared to December 5th and 6th; reasons to this are unclear, but there may have been some instability to the instrument due to the recent relocation of the sensor. The peak in ATP concentrations around noon on all three days might indicate a re-suspension of loose-deposits or detachment of biofilm, due to the increased water demand over lunch time.

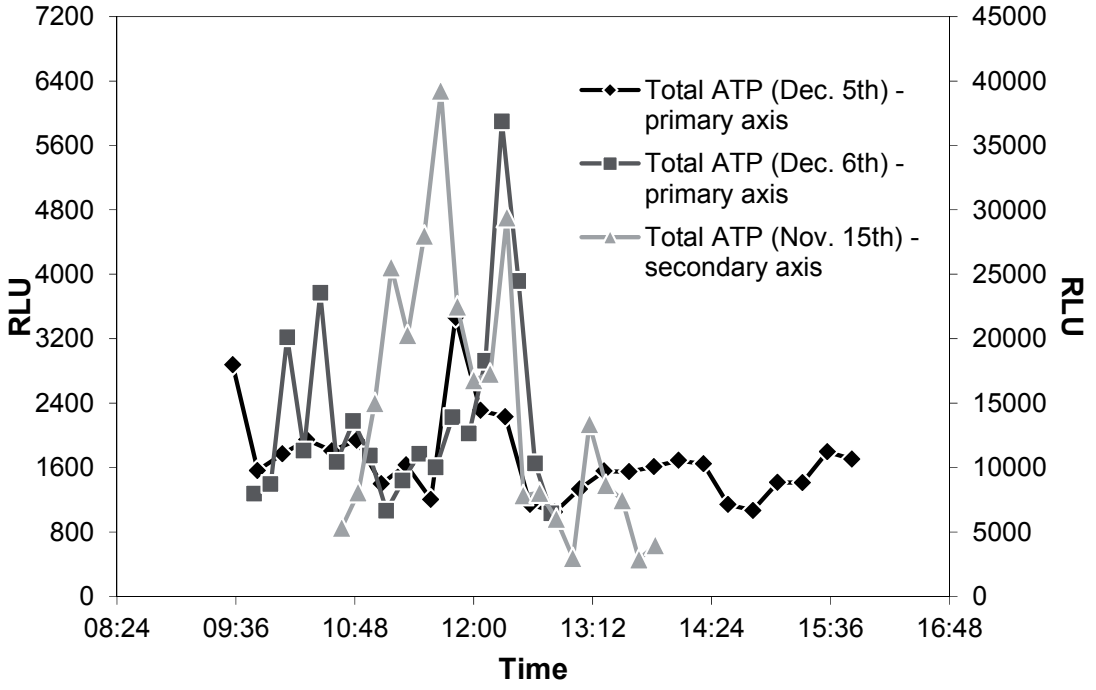

Figure 15: Time series of ATP measurements in tap water measured with a fully automated sensor prototype on three different days.

The continuous sensor prototype measurements were compared to simultaneous grab sampling and ATP measurements using another commercial ATP assay reagents and a commercial luminometer in the laboratory, which has been used for ATP measurements over several years and is quality controlled. The comparison verified that the observed increase in ATP was in fact a variation in microbiological activity, where the ATP concentration was in the range of 9.2 to 18 pg/mL from 11.47 pm to 12.37 pm (Figure 16).

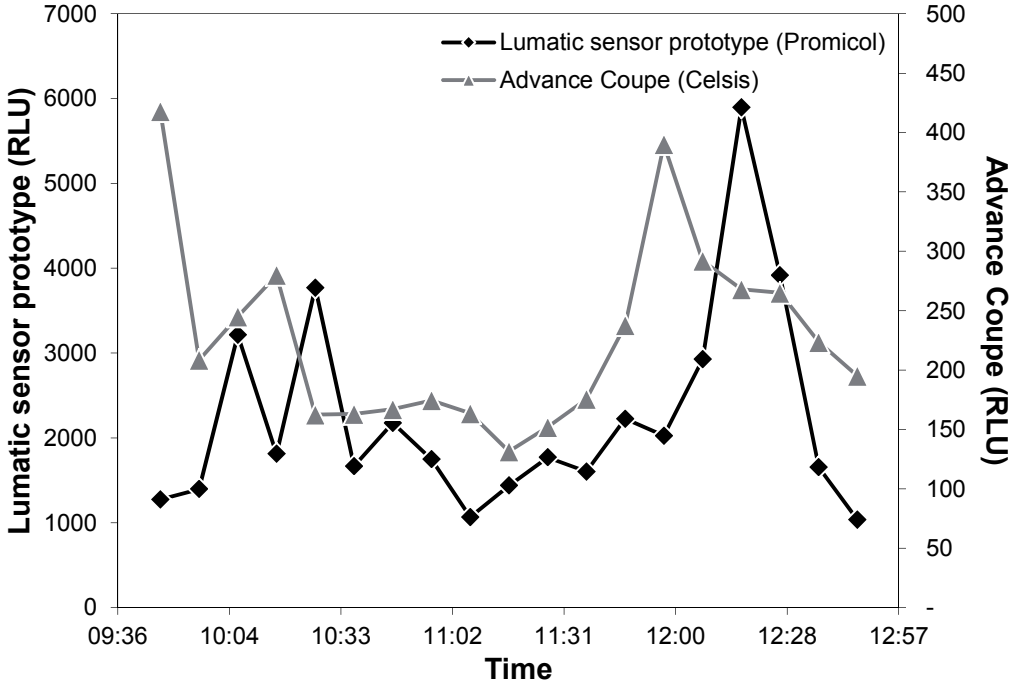

Figure 16: Time series of ATP measurements in tap water measured with a fully automated sensor prototype (Promicol and Promicol R&D reagents) and simultaneous grab sampling measured with a stand-alone luminometer (Celsis Advance Coupe and Celsis LuminEX/LuminATE).

Due to primarily mechanical instabilities the sensor prototype was evaluated not robust enough at the time being to be installed at a waterworks for continuous monitoring of ATP concentrations in drinking water. Hence, further experiments with the sensor prototype were ended. Amendments to the ATP sensor prototype have been initiated since the experiments included in this PhD study were performed.

The construction of the sensor can be divided into three, the physical/mechanical construction, the ATP assay design and the software program. The physical design of the sensor has many good features, e.g. the cleaning and flushing of the system between sampling and the ATP assay measurement, to avoid carry over from previous sample and cooling of reagents in order to prolong their catalytic activity. The design of the ATP assay included measurements of both total and free ATP; also it was possible to program standard addition for calibration of potential loss in catalytic activity of the reconstituted enzyme reagent. The software program developed was especially designed for its application, namely ATP measurements, with the option of programming alarm levels. Additionally, the sensor could be configured for remote on-line monitoring.

Overall, various aspects, required some adjustments/improvements in order to enhance measurement reliability as well as robustness of the sensor. Especially the mechanical feature required more stability. However, in terms of assay design and the initial results on continuous monitoring of tap water, the sensor prototype demonstrated promising aspects for the implementation of the ATP assay on a sensor platform for continuous real-time on-line monitoring of microbial drinking water quality.

6. Conclusions

This thesis describes the principal and theory of the ATP assay. Moreover it provides an overview of the assay's characteristic features, and how this assay has been used for the specific application of measuring and quantifying microbial activity in drinking water.

Despite the complexity to the ATP assay in reaction kinetics, potential inhibition, optimum assay conditions etc., there has been extensive research in order to understand these different aspects, making the ATP assay a fairly easy methodology with today's commercially available reagents and luminometers. This has contributed significantly to the usefulness of this parameter for measuring microbial activity in a broad range of applications, including measuring low levels of ATP concentrations - as in drinking water.

Most drinking water studies today apply different commercially available reagents for measurement of ATP. Work of this PhD study included investigation and evaluation of a variety of essential properties of the different types of commercial (and R&D) enzyme and extraction reagents used for quantifying ATP in drinking water. These exhibit different sensitivities, limit of detection, stability of catalytic activity and extraction efficiency. Careful considerations have to made, when choosing reagents and the design of assay, when applying the ATP assay either in the laboratory or in a potential sensor platform for real-time continuous monitoring of microbial drinking water quality.

Numerous drinking water campaigns revealed that the microbial ATP concentrations in non-chlorinated drinking water generally were low and relatively stable throughout distribution and over the year, which promotes microbial ATP as a parameter for rapid assessment of microbial drinking water quality. Also, the significance of quantifying microbial ATP rather than total ATP in drinking has been substantiated, where microbial ATP has been evaluated to be a more accurate and transparent parameter for microbial activity, potentially making it easier to detect small variation in microbial drinking water quality.

Applying the ATP assay for continuous monitoring of microbial drinking water quality in real-time was supported by its ability to detect potential contaminations of drinking water, where a significant increase in ATP concentrations was observed for drinking water contaminated with wastewater or surface water. Detection of contaminations in drinking water with ATP assay were controlled by the ATP load of the contaminant, the extent of dilution of the contaminant

when assayed and the background level of ATP in the specific drinking water at the given time.

A fully automated sensor prototype based on the ATP assay with continuous automatic sampling and ATP measurements of drinking water demonstrated reproducibility in time-series measurements of microbial activity in drinking water over the same time period on multiple days. This demonstrated a definite potential of the ATP assay to detect changes in microbial activity in drinking water in real-time with continuous measurements.

7. Significance of work and perspectives

Experiments on detection of potential real-life contaminants done in this PhD study are to our knowledge the first to demonstrate the response in ATP to microbial ingress from 'real' contaminants in drinking water matrices. This PhD project has also shown the diversity and applicability of commercial reagents for the ATP assay and which requirements these need to fulfil if used on a sensor platform for continuous monitoring of ATP concentrations in drinking water.

This study has further substantiated the interpretation of microbial ATP versus total ATP, where microbial ATP is evaluated to be a more accurate and dynamic measurement for monitoring of microbial drinking water quality. Additionally, initial trials with an ATP sensor prototype have demonstrated prospects for the ATP assay on a sensor platform for continuous real-time monitoring of microbial drinking water quality.

Future research should encompass further investigations of the automation perspectives for the ATP assay in field locations, e.g. at waterworks and on the distribution network. For further evaluation of the ATP assay as a parameter for monitoring of microbial drinking water quality it is imperative to achieve continuous time series of ATP levels in drinking water and thereof attaining insight into diurnal, weekly, yearly variations. This will support in identifying more precise baseline levels of ATP in drinking water, and subsequently also identifying alarm levels, i.e. when microbial drinking water quality is compromised.

Data on ATP levels achieved with a continuous sensor can be used for modelling of the water supply system for identification of optimum monitoring locations as well as to frame monitoring strategies with ATP in combination of other microbiological methods. Monitoring locations specifically identified for a real-time analysis, such as ATP, combined with early-warnings systems and intelligent control of the whole water supply system will markedly improve the water supply's reliability and security of distribution of clean and safe drinking water.

8. References

Aoki, Y., Fukuba, T., Yamamoto, T., Fujii, T. (2008) Development of 'IISA'-ATP system in deep sea environment. OCEANS 2008 - MTS/IEEE KOBE TECHNO-OCEAN, VOLS 1-3 Book Series: OCEANS-IEEE, 548-551.

ASTM D4012-81 (2009) Standard Test Method for Adenosine Triphosphate (ATP) Content of Microorganisms in Water, American Society for Testing and Materials (ASTM), standard designation: D4012 – 81 of 1997, reapproved in 2009.

Azam, F., Hodson, R.E. (1977) Dissolved ATP in the sea and its utilization by marine bacteria. Nature, 267, 696-697.

Atkinson D.E., Walton, G.M. (1967) Adenosine triphosphate conservation in metabolic regulation. Journal of Biological Chemistry, 242. 3239-3241.

Berney, M., Weilenmann, H.U., Egli, T. (2006) Flow-cytometric study of vital cellular functions in Escherichia coli during solar disinfection (SODIS). Micorbiology-SGM, 152, 1719-1729.

Berney, M., Vital, M., Hülshoff, I., Weilenmann, H.-U., Egli, T., Hammes, F. (2008) Rapid, cultivation-independent assessment of microbial viability in drinking water. Water Research 42, 4010-4018.

BioThema (2013) BioThema.com website, accessed 02.08.2013: http://biothema.se/atp-standards/

Boe-Hansen, R., Albrechtsen, H.-J., Arvin, E., Jørgensen, C., 2002. Dynamics of biofilm formation in a model drinking water distribution system. Journal of Water Supply: Research and Technology - AQUA, 51, 7, 399-406.

Björkman, K.M., Karl, D.M. (2001) A novel method for the measurement of dissolved adenosine and guanosine triphosphate in aquatic habitats: applications to marine microbial ecology. Journal of Microbiological Methods, 47, 2, 159-167.

Brock, T.D., Madigan, M.T. (1991) Biology of microorganisms. Prentice Hall international, Inc., Sixth Edition, United states of America.

Bushon, R.B., Likridopulos, C.A., Brady, A.M.G., (2009a) Comparison of immunomagentic separation/adenosine triphosphate rapid method to traditional culture-based method for *E. coli* and enterococci enumeration in waste water. Water Research 43, 4940-4946.

Bushon, R.B., Brady, A.M., Likridopulos, C.A., Cireddu, J.V. (2009b) Rapid detection of *Escherichia coli* and enterococci in recreational water using an immunomagnetic separation/adenosine triphosphate technique. Journal of Applied Microbiology, 106, 432-441.

Campbell, D.P. (2008) Interferometric biosensors. In: The significance of faecal indicators in water, Eds. D. Kay, C. Fricker, Royal Society of Chemistry, UK.

Celsis (2013) Celsis.com website, accessed 16.08.2013: http://www.celsis.com/

Chapman, A.G., Fall, L., Atkinson, D.E. (1971) Adenylate energy charge in *Escherichia coli* during growth and starvation. Journal of Bacteriology, 108, 3, 1072-1086.

Chowdhury, S. (2012) Heterotrophic bacteria in drinking water distribution system: a review. Environmental Monitoring Assessment, 184, 6087-6137.

Clark, S.T., Gilbride, K.A., Mehrvar, M., Laursen, A.E., Bostan, V., Pushchak, R., McCarthy, L.H. (2011) Evaluation of low-copy genetic targets for waterborne bacterial pathogen detection via qPCR. Water Research 45, 3378-3388.

Colifast (2013) Colifast.no website, accessed 15.09.2013:
http://www.colifast.no/technology.html

Corfitzen, C.B., Albrechtsen, H.-J. (2011) On-line kontinuert måling af drikkevandskvalitet. Naturstyrelsen, Miljømintsteriet (in Danish).

Corfitzen, C.B. (2004) Investigation of aftergrowth potential of polymers for use in drinking water distribution. PhD thesis, Environment & Resources, Technical University of Denmark.

Corfitzen, C.B., Albrechtsen, H.-J., Arvin, E., Ursin, C. (2007) Kontinuert, 'real-time' måling af ATP og fysiske parametre i drikkevand, Forsknings- og udredningsprojekt nr. 6, 2007, Dansk Vand- og Spildevandsforening, DANVA (in Danish).

Cowan, D.A.; Russel, N.J., Mamais, A.; Sheppard, D.M. (2002) Antarctic Dry Valley mineral soils contain unexpectedly high levels of microbial biomass. Extremophiles, 6, 5, 431-436.

Danish executive order on drinking water (2011) Bekendtgørelse om vandkvalitet og tilsyn med vandforsyningsanlæg, BEK nr. 1024 af 31/10/2011. Naturstyrelsen, Miljøministeriet, Denmark (in Danish).

Deininger, R.A., Lee, J. (2001) Rapid determination of bacteria in drinking water using an ATP assay. Field Analytical Chemistry and Technology, 5, 4, 185-189.

Delahaye, E., Welté, B., Levi, Y., Leblon, G., Montiel, A. (2003) An ATP-based method for monitoring the microbiological drinking water quality in a distribution network. Water Research 37, 3689-3696.

Deluca, M.; Wannlund, J.; McElroy, W.D. (1979) Factors affecting the kinetics of light emission from crude and purified firefly luciferase, Analytical Biochemistry, 95, 194-198.

Denburg, J.L.; Lee, R.T.; McElroy, W.D. (1969) Substrate-binding properties of firefly luciferase – I. Luciferin-binding site, Arch. Biochem. Biophys. 134 , 381–394.

Eaton, A.D., Clesceri, L.S., Rice, E.W., Greenber, A.E. (2005) Standard methods for the examination of water and waste water. American Health Association, 21st edition, USA.

Eydal, H.S., Pedersen, K. (2007) Use of an ATP assay to determine viable microbial biomass in Fennoscandian Shield groundwater from depths of 3-1000 m. Journal of Microbiological Methods 70, 363-373.

Firefly (2013) Firefly.org, website, accessed 05.09.20113:
http://www.firefly.org/firefly-pictures.html

Ford, S.R.; Leach, F.R. (1998) Improvements in the application of firefly luciferase assays, from Methods in molecular biology, Vol. 102, Bioluminescence methods and protocols, Ed. R.A. Larossa, Humana Press Inc, Totowa, NJ.

Fraga, H. (2008) Firefly luminescence: A historical perspective and recent developments, Photochem. Photobiol. Sci. 7, pp 146–158.

Fraga, S.R., Fernandes, D., Fontes, R., Esteves da Silva J.C. (2005) Coenzyme A affects firefly luciferase luminescence because it acts as a substrate and not as an allosteric effector. The FEBS Journal, 272, 20, 5206-5216.

Frundzhyan, V., Ugarova, N. (2007) Bioluminescent assay of total bacterial contamination of drinking water. Luminescence, 22, 241-244.

Ghazali, M., McBean, E., Whalen, P., Journal, K. (2010) Supporting a drinking water contaminant warning system using the adenosine triphosphate test. Canadian Journal of Civil Engineering, 37, 1423-1431.

Guardigli, M., Lundin, A. and Roda, A. (2011) ”Classical” applications of chemiluminescence and bioluminescence. In Chemiluminescence and Bioluminescence: Past, present and future. Ed. Roda, A.. pp. 162-190, Royal Society of Chemistry, Cambridge, UK.

Hallam, N.B., West, J.R., Forster, C.F., Simms, J. (2001) The potential for biofilm growth in water distribution systems. Water Research 35, 17, 4063-4071.

Hammes, F., Berney, M., Egli, T. (2011) Cultivation-independent assessment of bacterial viability. Advances in Biochemical Engineering/Biotechnology, 124, 123-150.

Hammes, F., Berney, M., Wang, Y., Vital, M., Köster, O., Egli, T. (2008) Hammes, F., Berney, M., Wang, Y., Vital, M., Köster, O., Egli, T., 2008. Flow-cytometric total bacterial cell counts as a descriptive microbiological parameter for drinking water treatment processes. Water Research 42, 269-277.

Hammes, F., Goldschmidt, F., Vital, M., Wang, Y., Egli, T. (2010) Measurement and interpretation of microbial adenosine tri-phosphate (ATP) in aquatic environments. Water Research 44 (13), 3915-3923.

Hammes, F., Broger, T., Weilenmann, H.-U., Vital, M., Helbing, J., Bosshart, U., Huber, P., Odermatt, R. P., Sonnleitner, B., (2012) Development and Laboratory-Scale Testing of a Fully Automated Online Flow Cytometer for Drinking Water Analysis. Cytometry Part A, 81A, 508-516.

Henriques, A.O., Snipper, G. (2008) ATP som måleparameter ved returskyl af filtre på vandværk. DTU Environment, Technical University of Denmark, M.Sc. thesis.

Holm-Hansen, O., Booth, C.R. (1966) Measurement of adenosine triphosphate in ocean and its ecological significance. Limnology and Oceanography, 11, 4, 510-519.

Jago, P.H., Simpson, W.J., Denyer, S.P., Evans, A.W., Griffiths, M.W., Hammond, J.R.M., Ingram, T.P., Lacey, R.F., Macey, N.W., McCarthy, B.J., Salusbury, T.T., Senior, P.S., Sidorowics, S., Smither, R., Stanfield, G., Stanley, P.E. (1989) An evaluation of the performance of ten commercial luminometers, Journal of Bioluminescence and Chemiluminescence, 3, 131-145.

Jensen, B. (1989) ATP-related specific heterotrophic activity in petroleum contaminated and uncontaminated groundwaters. Canadian journal of Microbiology, 35, 8, 814-818.

Jewson, D.H.; Dokulil, M. (1982) Adenylate energy charge measurements in freshwater microbial studies. Journal of Ecology, 70, 595-606.

Jones, J.G., Simon, B.M. (1977) Increased sensitivity in the measurement of ATP in freshwater samples with a comment on the adverse effect of membrane filtration. Freshwater Biology, 7, 253-260.

Jørgensen, P.E., Eriksen, T., Jensen, B.K. (1992) Estimation of viable biomass in wastewater and activated sludge by determination of ATP, oxygen utilization rate and FDA hydrolysis. Water Research, 26, 11, 1495-1501.

Karl, D.M. (1980) Cellular nucleotide measurements and applications in microbial ecology. Microbiological Reviews, 44, 4, 739-796.

Karl, D.M., Winn, C.D., Wong, D.C.L. (1981) RNA Synthesis as a measure of microbial growth in aquatic environments. I. Evaluation, verification and optimization of methods. Marine Biology 64, 1-12.

Karl, D.M., Holm-Hansen, O. (1978) Methodology and measurement of adenylate energy charge ratios in environmental samples. Marine biology, 48, 185-197.

Kazner, C., van den Hoven, T., Wielinga, R. (2009) Technau – a collective effort towards safe water supply. In Technau: Safe drinking water from source to tap, state of the art and perspectives. Eds. T. vand den Hoven & C. Kazner, IWA Publishing,, UK, 3-14.

Kim , W.-J., Kim, S., Kim, A.R., Yoo, D.J. (2013) Direct detection system for Escherichia coli using Au-Ag alloy microchips. Industrial and Engineering Chemistry Research, 52, 22, 7282-7288.

Khlyntseva, S.V., Bazel, Ya.R., Vishnikin, A.B., Andruch, V. (2009) Methods for the determination of adenosine triphosphate and other adenine nucleotides. Journal of Analytical Chemistry, 64 (7), 657-673.

Kurath, G., Morita, R.Y. (1983) Starvation-survival physiological studies of a marine Pseudomonas sp. Applied and environmental microbiology, 45(4), 1206-1211.

Lautenschlager, K., Boon, N., Wang, Y., Egli, T., Hammes, F. (2010) Overnight stagnation of drinking water in household taps induces microbial growth and changes in community, Water Research, 44, 4868-4877.

Lautenschlager, K., Hwang, C., Liu, W.-T., Boon, N., Köster, O., Vrouwenvelder, H., Egli, T., Hammes, F. (2013) A microbiology-based multi-parameter approach towards assessing biological stability in drinking water distribution networks. Water Research, 47, 3015-3025.

LeChevallier, M.W., Shaw, N.E., Kaplan, L.A., Bott, T.L. (1993) Development of a rapid assimilable organic carbon method for water. Applied and Environmental Microbiology, 59,5, 1526-1531.

Lee, J., Deininger, R.A. (2004) Detection of E, coli in beach water within 1 hour using immunomagnetic separation and ATP bioluminescence. Luminescence, 19, 31-36.

Lee, R.T.; Denburg, J.L.; McElroy, W.D. (1970) Substrate-binding properties of firefly luciferase: II. ATP-binding site. Archives of Biochemistry and Biophysics, Volume 141, Issue 1, 38–52.

Lehtola, M.J., Juhna, T., Miettinen, I.T., Vartiainen, T., Martikainen, P.J. (2004) Formation of biofilms in drinking water distribution networks, a case study in two cities in Finland and Latvia. Journal of Industrial Microbiology & Biotechnology, 31, 11, 389-494.

Leitão, J.M.M. and da Silva, J.C.G.E (2010) Firefly luciferase inhibition, Journal of Photochemistry and Photobiology B: Biology 101, pp 1-8.

Liu, G., Van der Mark, E.J., Verberk, J.Q.J.C., van Dijk, J.C. (2013a) Flow cytometry total cell counts: A field study assessing microbiological water quality and growth in unchlorinated drinking water distribution systems. BioMed Research International, Vol. 2013, Article ID 595872, 10 pages.

Liu, G., Ling, F.Q., Magic-Knezev, A., Liu, W.T., Verberk, J.Q.J.C., van Dijk, J.C. (2013b) quantification and identification of particle-associated bacteria in unchlorinated drinking water from three treatment plants by cultivation-independent methods. Water Research 47, 3523-3533.

Lundin, A. (2012) Comparison of purity and activity of D-luciferin from 8 manufacturers. Special Issue: Abstracts of the 17th International Symposium on Bioluminescence and Chemiluminescence, May 28th - June 2nd 2012, Guelph, Ontario, Canada - (ISBC 2012), Luminescence, 27, 2, 136-137.

Lundin, A. (2000) Use of firefly luciferase in ATP-related assays of biomass, enzymes and metabolites. Methods in Enzymology 305, 346-370.

Maesters, K.P.H.; van Groenstijn, J.W.; Gerritse, J. (2003) Biofouling reduction in recirculating cooling systems through biofiltration of process water. Water Research, 37, 525-532.

Marques, S.M. and da Silva, J.C.G.E. (2009) Firefly Bioluminescence: A Mechanistic Approach of Luciferase Catalyzed Reactions. IUBMB Life, 61(1): 6-17, January 2009.

mbOnline (2013) mbOnline website, accessed 15.09.2013: http://www.mbonline.at/en/products/online_measuring_equipment/mbonline_coliguard_analyzer

McElroy, W.D. (1947) The energy source for bioluminescence in an isolated system. Proceedings of the National Academy of Sciences of the United States of America, 33, 11, 342-345.

Magic-Knezev, A., van der Kooij, D. (2004) Optimisation and significance of ATP analysis for meassuring active biomass in granular activated carbon filters used in water treatment. Water Research 38, 3971-3979.

Maki, J.S., LaCroix, S.J., Hopkins, B.S., Stanley, J.T. (1986) Recovery and Diversity of Heterotrophic Bacteria from Chlorinated Drinking Waters. Applied and Environmental Microbiology 5 (5), 1047-1055.

Marriott, N.J., Barnes, S.P., Smith, R.N. (1992) A comparative evaluation of three commercially available luminometers and two ATP reagent kits. International Biodeterioration & Biodegradation, 30, 339-352.

Ochromowicz, K., Hoekstra, E.J. (2005) ATP as an indicator of microbiological activity in tap water. European Commission, DG Joint Research Centre, EUR 22157, Italy.

Picciolo, G.L., Chappell, E.W., Deming, J.W., Thomas, R.R., Nibley, D.A., Okrend, H. (1981) Firefly luciferase ATP assay development for monitoring bacterial concentrations in water supplies. United states Environmental Protection Agency, EPA-600/S2-81-014 (project summary).

Promicol (2013a) Promicol.nl website, accessed 16.08.2013: http://www.promicol.nl/applications

Promicol (2013b) Promicol.nl website, accessed 16.05.2013: http://www.promicol.nl/testkit?tkit=21

Riemann, B. (1979) The occurrence and ecological importance of dissolved ATP in fresh water, Freshwater Biology, 9, 481-490.

Schmelcher, M., Loessner, M.J. (2008) Bacteriophage: Powerful tools for detection of bacterial pathogens. In Principles of bacterial detection – biosensors, recognition receptors and microsystems. Eds.: M. Zourob, S. Elwary, S. Turner, Springer, UK.

Schram, E., Witzenburg, W.-V. (1989) Control of experimental factors involved in luminescent ATP assays. In: ATP Luminescence – rapid methods in microbiology, Eds. P.E. Stanley, B.J. McCarthty, R. Smither, Blacwell Scientific Publications, Oxford, 37-52.

Sheikh, Q.I.; Boxall, J.B.; Biggs, C.A. (2012) A review of potential culture independent biological detection methods for the water industry – challenges of moving beyond the research lab. In: The significance of faecal indicators in water, Eds. D. Kay, C. Fricker, Royal Society of Chemistry, UK.

Siebel, E., Wang, Y., Egli, T., Hammes, F. (2008) Correlations between total cell concentration, total adenosine tri-phosphate concentration and heterotrophic plate counts during microbial monitoring of drinking water. Drinking Water Engineering Science 1, 1-6.

Smeets, P.W.M.H., Medema, G.J., van Dijk, J.C. (2009) The Dutch secret: how to provide safe drinking water without chlorine in the Netherlands. Drinking Water Engineering Science 2, 1-14.

Stanley, P.E. (1986) Extraction of adenosine triphosphate from microbial and somatic cells. Methods in Enzymology, 133, 14-22.

Stanley, P.E. (1989a) A review of bioluminescent ATP techniques in rapid microbiology. Journal of Bioluminescence and Chemiluminescence, 4, 375-380.

Stanley, P.E., (1989b) A concise beginner's guide to rapid microbiology using Adenosine Triphosphat (ATP) and luminescence. In: ATP Luminescence – rapid methods in microbiology, Eds. P.E. Stanley, B.J. McCarthy, R. Smither, Blackwell Scientific Publications, Oxford, 1-10.

van der Kooij, D. (1992) Assimilable Organic Carbon as an indicator of Bacterial Regrowth. American Water Works Association (AWWA) Journal Research and Technology, 57-65.

van der Kooij, D. (1995) The AOC manual. Report KIWA, SWE 95.022.

van der Kooij, D. (2003) Managing regrowth in drinking-water distribution systems. In: Emerging Issues in Water and Infectious Diseases Series. Heterotrophic plate counts and drinking-water safety: The significance of HPC's for water quality and human health, 199-232.

van der Kooij, D., Baggelaar, P.,K., Veenendaal, H.R., Moulin, L., Corfitzen, C.B., Albrechtsen, H.-J., Holt, D., Hambsch, B. (2006) Standardising the biomass production potential method for determining the enhancement of microbial growth by construction products in contact with drinking water. Inter-laboratory testing.

van der Wielen, P.W.J.J., van der Kooij, D. (2010) Effect of water composition, distance and season on the adenosine triphosphate concentration in unchlorinated drinking water in the Netherlands. Water Research 44 (17), 4860-4867.

Vang, Ó.K., Corfitzen, C.B., Lindhardt, B., Albrechtsen, H.-J. (2011) Overlevelse af indikatororganismer i drikkevand. Naturstyrelsen (in Danish).

Vang, Ó.K., Corfitzen, C.B., Smith, C., Albrechtsen, H.-J. (I) Evaluation of ATP measurements to detect microbial ingress in drinking water by waste water and surface water. Submitted manuscript.

Vang, Ó.K., Corfitzen, C.B., Spliid, H., Albrechtsen, H.-J. (II) Levels of microbial ATP and free ATP in non-chlorinated drinking water - assessing microbial drinking water quality with ATP measurements. Submitted manuscript.

Vang, Ó.K., Corfitzen, C.B., Smith, C., Albrechtsen, H.-J. (III) Levels of microbial ATP and free ATP in non-chlorinated drinking water - assessing microbial drinking water quality with ATP measurements. Manuscript in preparation.

Velten, S., Hammes, F., Boller, M., Egli, T. (2007) Rapid and direct estimation of active biomass on granular activated carbon through adenosine tri-phosphate (ATP) determination. Water Research 41, 1973-1983.

Venkateswaran, K., Hattori, N., LA Duc, M.T., Kern, R. (2003) ATP as a biomarker of viable microrganisms in clean-room facilities. Journal of Microbiological Methods, 52, 367-377.

Vital, M., Hammes, F., Magic-Knezev, A., Ross, P., Rietveld, L., Hammes, F. (2012) Flow cytometry and adenosine tri-phosphate analysis: Alternative possibilities to evaluate major bacteriological changes in drinking water treatment and distribution systems. Water Research 46, 4665-4676.

Vrouwenvelder, H.S., van Paassen, J.A.M., Folmer, H.C., Hofman, J.A.M.H. (1998) Biofouling of membranes for drinking water production. Desalination, 118, 1-3, 157-166.

WHO (2013) Water safety plan manual (WSP manual): Step-by-step risk management for drinking-water suppliers, Water Safety Plans: Managing drinking-water quality for public health, accessed 21.08.2013:
http://www.who.int/water_sanitation_health/WHS_WWD2010_advocacy_2010_2_en.pdf

Yang, N.-C., Ho, W.-M., Chen, Y.-H., Hu, M.-L., (2002) A convenient one-step extraction of cellular ATP using boiling water for the luciferin-luciferase assay of ATP. Analytical Biochemistry, 306, 323-327.

9. Papers

I. Vang, Ó.K., Corfitzen, C.B., Smith, C., Albrechtsen H.-J. 2013. Evaluation of ATP measurements to detect microbial ingress in drinking water by waste water and surface water. *Submitted manuscript*.

II. Vang, Ó.K., Corfitzen, C.B., Spliid, H., Albrechtsen H.-J. 2013. Levels of microbial ATP and free ATP in non-chlorinated drinking water - assessing microbial drinking water quality with ATP measurements. *Submitted manuscript*.

III. Vang, Ó.K., Corfitzen, C.B., Smith, C., Albrechtsen H.-J. 2013. An evaluation of reagents for the ATP assay for assessing microbial drinking water quality. *Manuscript in preparation*.

I

Evaluation of ATP measurements to detect microbial ingress in drinking water by wastewater and surface water

Vang, Ó.K., Corfitzen, C.B., Smith, C., Albrechtsen H.-J.

Submitted manuscript

Evaluation of ATP measurements to detect microbial ingress in drinking water by wastewater and surface water

Óluva K. Vang*[1], Charlotte B. Corfitzen[1], Christian Smith[2] Hans-Jørgen Albrechtsen[1]

[1]Department of Environmental Engineering, Technical University of Denmark, Miljoevej, Building 113, DK-2800 Kgs. Lyngby.

[2]GRUNDFOS Holding A/S, Poul Due Jensens Vej 7, DK-8850 Bjerringbro

*Corresponding author: okva@env.dtu.dk

Abstract

Fast and reliable methods are required for monitoring of microbial drinking water quality in order to protect public health. Adenosine triphosphate (ATP) was investigated as a potential real-time parameter for detecting microbial ingress in drinking water which was artificially contaminated with wastewater or surface water. To investigate the ability of the ATP assay in detecting different contamination types, the contaminant was diluted with non-chlorinated drinking water. Wastewater, diluted at 10^4 in drinking water, was detected with the ATP assay, as well as 10^2 to 10^3 times diluted surface water. To improve the performance of the ATP assay in detecting microbial ingress in drinking water, different approaches were investigated, i.e. quantifying microbial ATP or applying reagents of different sensitivities to reduce measurement variations; however, none of these approaches contributed significantly in this respect. Compared to traditional microbiological methods, the ATP assay could detect wastewater and surface water in drinking water to a higher degree than total direct counts (TDCs), while both heterotrophic plate counts (HPC 22°C and HPC 37°C) and Colilert-18 (*E. coli* and coliforms) were more sensitive than the ATP measurements, though with much longer response times. Continuous sampling combined with ATP measurements displays definite monitoring potential for

microbial drinking water quality, since microbial ingress in drinking water can be detected in real-time with ATP measurements. The ability of the ATP assay in detecting microbial ingress is controlled by both the ATP load from the contaminant itself and the ATP concentration in the specific drinking water. Thus, a contamination is more easily distinguished with the ATP assay in line with a lower ATP concentration in the specific drinking water.

Key words: adenosine triphosphate, rapid method, bacteria, drinking water, contamination

1 Introduction

Nowadays microbial drinking water quality is most often monitored through grab sampling, and the current standard methods for regulatory control are usually culture-based. These traditional techniques are labour-intensive and time-consuming, and the results are usually first available 1 to 3 days after sampling. Since the residence time in distribution systems can often be relatively short, i.e. less than three days, and drinking water of poor microbial quality or even contaminated may have already been consumed when results are available. Furthermore, the low sampling frequency allows contaminations of short durations to pass undetected through the water supply system. Consequently, there is an acknowledged need for the further development of fast and reliable methods for measuring microbial drinking water quality in order to improve the protection of public health. Such rapid methods would be of value to water managers and utilities for better monitoring strategies and protection of drinking water from source to the consumer's tap.

Adenosine triphosphate (ATP) measurements provide results within minutes (Deininger and Lee, 2001; Delahaye et al., 2003; van der Wielen & van der Kooij, 2010) and is one of several approaches (Hammes et al., 2012; Stedmon et al., 2011) which are currently being investigated as a potential parameter for evaluating the microbial quality of drinking water. Continuous sampling combined with real-time analysis such as ATP would significantly improve surveillance of microbial drinking water quality and provide an early warning system, thus allowing health authorities and managers to react in a timely manner. This is especially important for water supplies which distribute drinking water without a disinfectant residual as in Denmark or the Netherlands, for example (Smeets et al., 2009).

ATP measurements include all active – and non-culturable cells – cells in a sample. Thus, they can provide a better estimate of the total active biomass in a drinking water sample than heterotrophic plate counts, where only a small fraction of total cells are quantified (often <1%) (Maki et al., 1986; Siebel et al., 2008). The ATP assay has proven to be applicable for measuring active biomass in various aquatic environments (Eydal and Pedersen, 2007; Aoki, 2008; Bushon et al., 2009), and ATP measurements have also been applied in determining microbial activity (bulk phase and biofilm) in drinking water systems in several investigations (Boe-Hansen et al., 2002; Delahaye et al. 2003; Lehtola et al., 2004; Hammes et al., 2010; van der Wielen and van der Kooij, 2010).

ATP measurements can be used as a parameter for assessing general microbial quality in distributed drinking water (van der Wielen and van der Kooij, 2010), although they are also applicable as a technical parameter of the efficiency of treatments processes at waterworks (Hammes et al., 2008; Vital et al., 2012; Liu et al., 2013a). Moreover, ATP measurements have also been used as a microbiological parameter for assessing biological stability and aftergrowth in drinking water (Siebel et al., 2008; Lautenschlager et al., 2010; Vital et al., 2012; Liu et al., 2013a).

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 contaminations caused by microbial ingress from wastewater or surface water, or how much of this water has to present before ATP concentrations are increased. This has been limited so far to investigations of drinking water spiked with *E. coli* combined with incubation of water samples (Fundzhyan and Ugarova, 2007; Ghazali et al., 2010). However, laboratory cultures vary significantly from ‘real-life’ contaminants in terms of bacterial and substrate composition, bacterial abundance and other substances such as particles and chemicals.

Thus, the overall aim of this study was to investigate and interpret the response in ATP when drinking water is subjected to microbial ingress, and more specifically to assess to what degree drinking water contaminated with wastewater or surface water can be detected with the ATP assay. Other aspects investigated were 1) how ATP fractions and reagent sensitivity influence the ATP assay’s ability to detect contaminations, 2) whether aftergrowth derived from a contamination can

be detected and 3) how the ATP assay performs compared with traditional methods in detecting microbial ingress in drinking water.

2 Materials and methods

2.1 Experimental conditions

To investigate the response in ATP to a contamination, drinking water was contaminated with untreated wastewater or surface water at tenfold dilutions of 10^1-10^7. Three separate experiments were performed with wastewater-contaminated drinking water in order to investigate various aspects of the contamination scenario (different ATP fractions, different ATP reagents, aftergrowth) as well as to evaluate the reproducibility of the ATP assay's performance. Groundwater-based drinking water (non-chlorinated) was collected from a local waterworks (Lyngby Vandværk), untreated wastewater from a wastewater treatment plant (Mølleåværket) and surface water from a pipe dig-out after a rainfall event – all of which were collected on the same day as the experiments were executed (Table 1).

Response in ATP from aftergrowth derived from a contamination incident was measured after 0, 6 and 26 hours and after 6 days' incubation at 10°C of drinking water contaminated with wastewater in 10^1-10^5 time dilutions.

Table 1: Characteristic parameters and parametric values for drinking water, surface water and wastewater used for experiments in this study.

		Drinking water[a)]	Surface water	Wastewater[b)]
pH		7.6	-	7.9
Oxygen	mg/L	8.7	-	-
NVOC[d)]	mg/L	1.9	-	-
Turbidity	NTU	0.2	-	-
BOD[e)]	mg/L	-	-	260
COD[f)]	mg/L	-	-	605
Total-N	mg/L	-	-	57
Total-P	mg/L	-	-	9.6
TSS[g)]	mg/L	-	-	384
TDC[h)]	cells/mL	$1.3\pm0.4 \times 10^5$	$1.6\pm0.4 \times 10^7$	$2.4\pm1.0 \times 10^8$
Microbial ATP	pg/mL	2.3-6.4	$2.8\pm0.3 \times 10^3$	$4.0\pm0.4 \times 10^4$
Coliforms	MPN/mL	<1	1.3×10^4	3.4×10^7
E. coli	MPN/mL	<1	30	2.0×10^7

[a,b)]Physical-chemical parametric values for drinking water and wastewater are obtained through check/audit monitoring by the official authorities; [c)]physical-chemical parametric values of wastewater correspond to 0-6.8 mm of precipitation; [d)]non-volatile organic carbon; [e)]biological oxygen demand; [f)]chemical oxygen demand; [g)]total suspended solids; [h)]total direct cell counts

2.2 ATP assay

Microbial ATP concentration was obtained through a two-step ATP measurement procedure. Total ATP was measured by adding an extraction reagent (cell lysis) and then a luciferin/luciferase reagent. Free ATP was measured without cell lysis by adding only the luciferin/luciferase reagent to the sample. Microbial ATP concentration was then calculated as the difference between total ATP concentration and free ATP concentration (microbial ATP = total ATP - free ATP). Samples were analysed using the LuminATE reagent kit (92687, Celsis), but in one of the experiments on drinking water contaminated with wastewater (see 3.3) the RapiScreen Health reagent kit (1230839, Celsis) was used. Both reagent kits contain an extraction reagent.

To analyse the presence of ATP, a water sample (0.1 mL) was added manually to a cuvette and the extraction reagent was automatically added via the dispensing system on the luminometer (excluded when measuring free ATP). After 10 seconds of extraction the luciferin/luciferase was also added automatically, and after a 2-second delay the light emission was measured and integrated over 10 seconds. Samples were measured in two or three replicates on a luminometer (Advance Coupe, Celsis, the Netherlands).

Sample and reagent volumes for both reagent kits followed the manufacturer's recommendations. With the LuminEX/LuminATE reagent kit this was a 100 µL sample, 100 µL of extraction reagent and 100 µL for the enzyme reagent, whereas for the RapiScreen Health reagent kit this was a 50 µL sample, 200 µL of extraction reagent and 100 µl for the enzyme reagent. The detection limits were 1 pg/mL for total ATP and 0.7 pg/mL for free ATP with the LuminATE reagent kit, and 0.3 and 0.5 pg/mL with the RapiScreen Health reagent kit.

Luminescence was measured in relative light units (RLUs), which were converted to ATP concentrations by a calibration curve of ATP standards measured on the same day as the experiment. Two different calibration curves were made – one for total ATP and one for free ATP. ATP standards were prepared with ATP-standard salt (92638, Celsis) reconstituted in a Lumin(PM) buffer (92678, Celsis) to a concentration of $1x10^6$ pg ATP/mL, while dilution series in the range 2.5-1,000 pg ATP/mL were made in autoclaved sterile filtrated tap water (i.e. ATP free water) as a base for an ATP-standard curve. For samples measured with standard addition, the internal ATP standard (25 µL; 10^4 pg/mL) was added after the extraction of microbial ATP from cells (van der Kooij et al.; 2006). After a delay of 7 seconds, the enzyme reagent was added.

All glassware used for collecting water and for the experiments was acid-washed and heated to 540°C (6 hours). ATP-free laboratory equipment and ATP-free pipette tips were applied in the experiments, and all samples were handled under aseptic conditions.

2.3 Microbial counts

Heterotrophic plate counts (HPC) were conducted by pour plating on yeast extract agar (01497, Fluka Analytical, Sigma-Aldrich). A 1 mL water sample was transferred to a sterile petri dish and mixed with liquid yeast extract agar at 45°C with three replicates for each dilution. The plates were incubated in the dark at 22°C (HPC 22°C) or at 37°C (HPC 37°C), and plate colonies were quantified after 68±4 and 44±4 hours, respectively (DS/EN ISO 6222:1999).

Escherichia coli (*E. coli*) and total coliforms were measured by IDEXX Colilert®-18 (ISO 9308-2:2012). The samples were incubated in trays (Quanti-Tray®/2000, IDEXX) for 20±2 hours at 37°C, after which yellow wells were counted and the most probable number (MPN) for total coliforms was

determined. Under UV light, fluorescing wells were counted and used to determine the MPN of *E. coli* in the samples.

2.4 Total cell count

The total number of microbial cells was counted after being stained with DAPI (4',6-diamidino-2-phenylindole) for five minutes in the dark. Samples (8-38 mL) were filtrated through a black 0.22 µm polycarbonate filter (GE Water & Process Technologies, cat. no. K02BP02500), which was wetted with a 2% solution of Tween-80 in order to reduce surface tension. Subsequent to the filtration of the sample, 0.5 mL of a DABCO solution (1,4-diazabicyclo-(2,2,2)-octan) was added to the filter in order to prolong the fluorescence of the stained cells, which were finally enumerated through the use of fluorescence microscopy (Olympus BH-2). A maximum of 200 bacteria were counted on 10 or 15 different grids on the filter. Samples were preserved with a buffered formaldehyde solution to a final concentration of 2% and stored at 4°C until analysed.

2.5 Statistics

Experimental data were analysed by a t-test with a significance level of 0.05 to calculate whether concentrations of bacteria measured by the different methods described above were significantly different in drinking water contaminated with wastewater or surface water as opposed to non-contaminated drinking water. All statistics were computed in Excel.

3 Results and discussions

3.1 Drinking water contaminated with wastewater

To investigate the ability of the ATP assay to detect contaminants in drinking water, total ATP was measured in drinking water contaminated with wastewater. Total ATP concentration in the non-contaminated drinking water (3.7±0.5 pg total/mL) was subtracted from total ATP measured in drinking water contaminated with wastewater subtracted the (Figure 1), which demonstrated a response for all dilutions (10^1 to 10^5) of wastewater in drinking water. Total ATP concentrations decreased tenfold for each tenfold dilution of wastewater for the three lowest dilutions (10^1, 10^2 and 10^3), therefore showing linearity for these dilutions. Total ATP concentration in the 10^3 dilution (19±1.5 pg total ATP/mL)

was significantly higher than in drinking water (3.7±0.5 pg total ATP/mL) ($p<0.05$). The signal for the two most diluted wastewater samples (10^4 and 10^5) was higher than in drinking water, although the difference was not statistically different ($p>0.05$). Hence, the ability of the ATP assay to detect wastewater contamination in this specific experimental set-up was between a 10^3 and a 10^4 dilution of wastewater – equivalent to 0.1-1 L in 1 m^3.

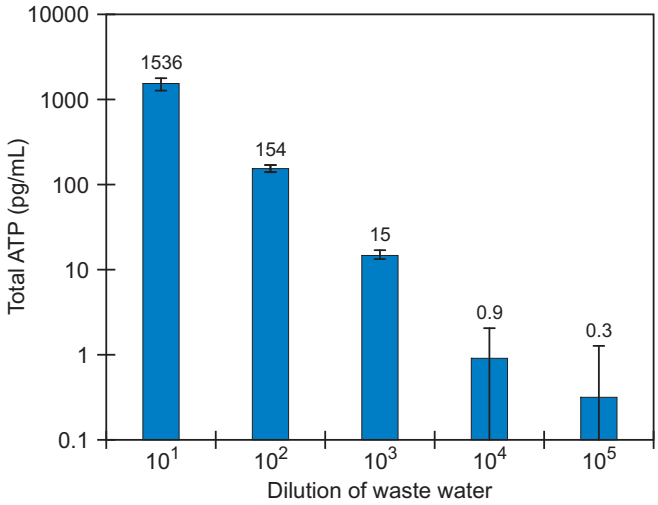

Figure 1: Total ATP concentrations in drinking water contaminated with wastewater. Background level of ATP (i.e. drinking water) is subtracted from samples contaminated with wastewater of various dilutions (LuminATE reagent kit).

The linear regression of total ATP concentrations in all five dilutions allowed for an estimation of the theoretical amount of wastewater detectable in drinking water at a $1.2x10^5$ dilution of wastewater ($x=b/a$; $a=15363±4.3$; $b=0.18±0.2$). ATP concentrations in the low dilutions were several orders of magnitude higher than in the high dilutions and as such had a relatively greater influence on the regression analysis, which in this specific experiment led to an overestimation of the ability of the ATP assay.

The bioluminescent reaction of ATP and luciferin/luciferase can be inhibited by, for example, luciferin analogues, nucleotides and a variety of anions and cations, some to a lesser extent than others (Denburg et al., 1969; Deluca et al., 1979; Karl, 1980). Samples were measured with standard addition to account for these potential matrix effects and potential inhibition by substances in the wastewater. This demonstrated a reduced light emission of 18% and 10% with high fractions of wastewater (10^1 and 10^2 dilutions) (Figure 1), but with such high concentrations of wastewater the response in ATP is expected to be substantially higher than for clean drinking water, leaving no doubt that water quality has been

compromised. Significant matrix effects or inhibition were not observed in either the clean or contaminated drinking water with wastewater diluted to 10^3 to 10^5 (2-8%). Reduced light emission due to matrix effects and/or inhibition is accounted for in ATP concentrations given in Figure 1.

To improve the efficiency of the ATP assay it seemed obvious to try to reduce measurement variations by either quantifying the microbial ATP fraction, and thus eliminate possible noise from free ATP, or by applying more sensitive reagents for the ATP analysis in order to better distinguish between ATP in drinking water and increased ATP levels caused by microbial ingress.

3.2 Significance of microbial ATP on method performance

To improve the sensitivity of the ATP assay, both total ATP and free ATP were measured to quantify the microbial ATP fraction (Figure 2-A), the concentration of which in the 10^3 dilution of wastewater (40±4.1 pg microbial ATP/mL) was significantly higher than in drinking water (6.4±0.9 pg microbial ATP/mL) ($p<0.05$), but not for further dilutions (10^4 to 10^7). As such, quantifying microbial ATP did not improve the ability of the ATP assay to detect wastewater compared to measuring total ATP, i.e. the ratio between the ATP concentrations in the 10^3 dilution of wastewater in drinking water and uncontaminated drinking water was not improved when quantifying microbial ATP compared to total ATP – a ratio of 6.3 in both cases. Free ATP concentration in the 10^3 wastewater dilution was also significantly different from the drinking water, demonstrating that the wastewater contamination contributed to increased levels of both microbial and free ATP.

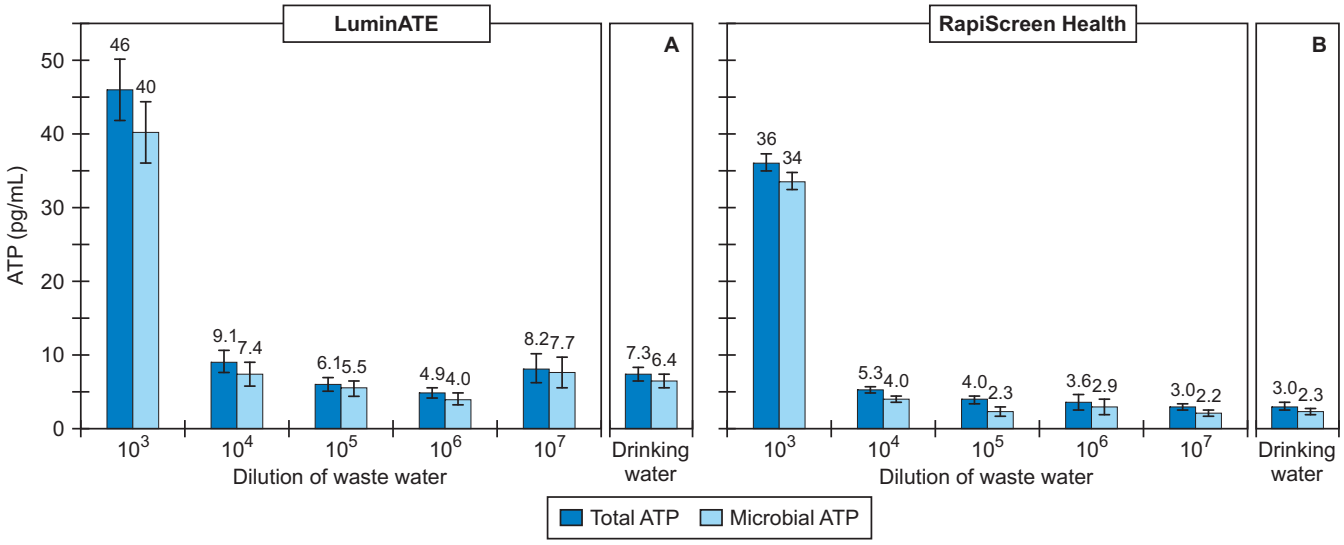

Figure 2: Total and microbial ATP concentrations in drinking water and drinking water contaminated with wastewater of various dilutions; A) LuminATE reagent kit was applied for ATP measurements and B) RapiScreen Health reagent kit was applied for ATP measurements.

ATP concentrations were different in drinking water samples collected on different days at 7.3 pg total ATP/mL (Figure 2-A) compared to 3.7 pg total ATP/mL in the previous experiment, i.e. approximately 50% higher. This is within expected natural variations in ATP concentrations in drinking water (Vang et al., II; van der Kooij, 1992; Berney et al. 2008; Hammes et al., 2010; Liu et al., 2013a). However, the level of ATP in drinking water, i.e. indigenous bacteria, influences the limit for detecting microbial ingress and as such the efficiency of the ATP assay.

When quantifying microbial ATP as the difference between total and free ATP, the standard deviation for microbial ATP included the standard deviation of both of these two measurements, so small fluctuations in microbial ATP may be concealed by measurement uncertainty.

3.3 Significance of reagent sensitivity on method performance

ATP measurements with good repeatability might improve the differentiation between contaminated water and drinking water, and thus contribute to the ATP assay's ability to detect microbial ingress. One approach to lowering measurement variation could be to apply more sensitive reagents for the ATP analysis, i.e. an increased signal with a better signal/noise ratio. Sensitivity varies significantly between various commercial luciferin/luciferase reagents, resulting

in large differences in RLU response; also some reagents are more suitable for measuring samples with low levels of ATP, as is the case for drinking water (Marriott et al., 1992; Vang et al., III). The RapiScreen Health reagent is more sensitive than the LuminATE reagent, i.e. more sensitive means more light is produced per ATP molecule present in the sample, and consequently a higher yield of relative light units (RLUs) is registered by the photomultiplier without acquiring a higher background signal. The coefficient of variation (CV) for both reagents was lowest in the less diluted wastewater (10^3) (Table 2), where ATP concentrations were relatively high and the CV was lower for the RapiScreen Health reagent (CV=3%) compared to LuminATE (CV=10%). The CV was also lower for the 10^4 dilution of wastewater when measured with RapiScreen Health than with LuminATE.

Table 2: Coefficient of variation (CV) of total and microbial ATP concentrations in drinking water and drinking water contaminated with wastewater of various dilutions measured with LuminATE or RapiScreen Health reagent kit.

	CV LuminATE		**CV RapiScreen Health**	
Dilution of wastewater	**Total ATP %**	**Microbial ATP %**	**Total ATP %**	**Microbial ATP %**
10^3	9	10	3	3
10^4	17	21	8	11
10^5	16	18	14	24
10^6	15	23	28	35
10^7	24	27	13	19
Drinking water	13	15	16	21

When ATP was measured with the RapiScreen Health reagent (Figure 2-B) the 10^3 dilution of wastewater in drinking water (34±1.2 pg microbial ATP/mL) was easily distinguished from drinking water, as in the two previous experiments. Moreover, microbial ATP concentration in the 10^4 dilution of wastewater (4.0±0.4 pg microbial ATP/mL) was significantly higher than in drinking water (2.3±0.5 pg microbial ATP/mL) ($p<0.05$). At higher dilutions (10^5, 10^6, 10^7) wastewater contamination was diluted to the ATP level exhibited by indigenous bacteria in the specific drinking water.

Thus, the ability of the ATP assay to detect wastewater in this experiment was improved to detect a 10^4 dilution of wastewater – equivalent to 100 mL in 1 m^3 of drinking water. However, although not statistically significant, ATP concentration in the 10^4 wastewater dilution was higher than in drinking water

for all three experiments where drinking water was contaminated with wastewater, indicating a tendency to detect up to a 10^4 dilution in this respect. Applying more sensitive reagents for the ATP analysis in this case did not substantially reduce the repeatability of replicates for samples with low ATP concentrations, i.e. in the drinking water range, and consequently did not improve the ability of the ATP assay to detect contaminants significantly.

Another aspect in evaluating the detection of contaminants with the ATP assay is wastewater concentration, which was lower (34 pg microbial ATP/mL) than in the previous experiment with LuminATE (40 pg microbial ATP/mL), so an increase in ATP load from wastewater contamination could not explain the ability to detect 10^4 diluted wastewater. On the other hand, microbial ATP concentration in drinking water was relatively low (2.3 microbial pg/mL) in this specific experiment compared to the previous experiment with the LuminATE reagent kit (6.4 pg microbial ATP/mL). The ratio of ATP between drinking water contaminated with wastewater and clean drinking water was highest with the RapiScreen Health reagent kit (14.4), thus demonstrating the importance of a low ATP concentration in drinking water. This contributes to the ability of the ATP assay with regard to uncovering the 10^4 dilution of in this specific experiment compared to previous experiments, and not the sensitivity of the reagent applied. As such, both the microbiological load of contaminant and the ATP concentration of indigenous bacteria in drinking water are two important controlling factors in the ATP assay's ability to detect potential contaminants in drinking water.

3.4 Detection of aftergrowth

The detection of aftergrowth was investigated by examining drinking water contaminated with wastewater incubated at a typical drinking water temperature (10°C). The contamination of a drinking water system will most likely add substrate to the system, besides bacteria, which can cause aftergrowth. The ATP levels in all dilutions (10^1 to 10^5) of wastewater remained unaltered after 6 and 26 hours. After 6 days, ATP concentrations had increased significantly ($p<0.05$) for all dilutions except in the 10^3 dilution (Figure 3).

The increase in ATP was highest for the lowest dilution (10^1) of wastewater (313 pg total ATP/mL) and decreased for each sample in this respect, i.e. 41 pg total ATP/mL for a 10^2 dilution, 3 pg total ATP/mL for a 10^4 dilution and 5 pg total

ATP/mL for a 10^5 dilution. Hence, it was possible to detect increased microbial activity presumably due to aftergrowth of bacteria emanating from contamination at a level as low as 10^5 dilution of wastewater. The larger increase in ATP concentrations in the less diluted samples indicated a higher aftergrowth of bacteria because of higher amounts of substrates in these samples due to a greater content of wastewater. Over the 6-day period ATP remained stable in drinking water (Figure 3).

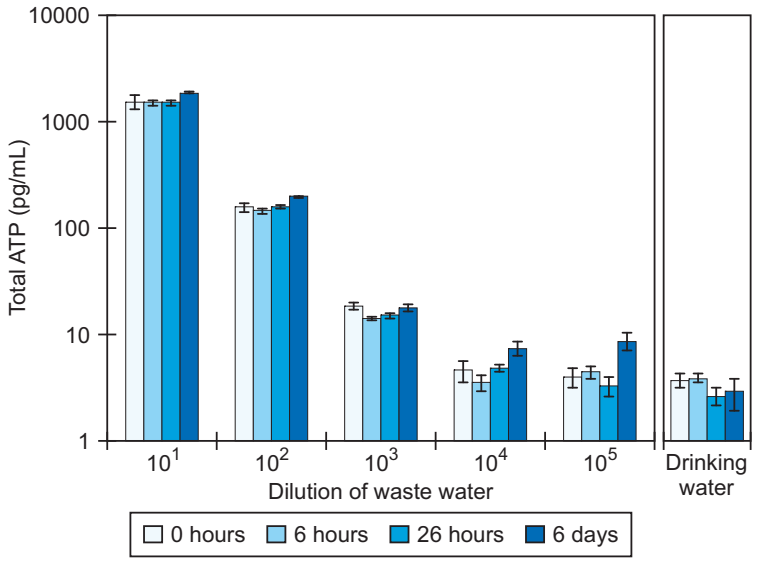

Figure 3: Total ATP concentrations in drinking water and drinking water contaminated with wastewater of various dilutions measured at 0, 6 and 26 hours and six days of incubation at 10°C.

Based on ATP per cell calculations ($2.7x10^{-17}$ g ATP/cell) an aftergrowth of 5 pg/mL corresponds to an increase of approximately $2x10^5$ cells, i.e. a huge increase in the number of cells results in a relatively small increase in ATP. This demonstrates that ATP assay may not be particularly sensitive to detect aftergrowth. Also a few studies comparing flow cytometry (FCM) and ATP measurements have reported stable ATP concentrations, despite increased cell numbers with FCM, indicating that ATP measurements alone might not be suited to evaluating biostability in drinking water (Vital et al., 2012; Liu et al., 2013a).

HPC 22°C measurements also demonstrated significant aftergrowth after just 6 hours incubation for the 10^3 wastewater dilution. After 26 hours of incubation all contaminant dilutions demonstrated significant aftergrowth in HPC 22°C (data not shown). In the case of real-life distribution network contamination with continuous flow and consumption, the signal from aftergrowth caused by this

event will most likely be diluted to the specific level of ATP in drinking water, since the increase in ATP was delayed for several days from the point and time of entry. On the other hand, in real life contaminated water might remain stagnant in pipes over several days, therefore allowing the aftergrowth of bacteria as a result of additional substrate from wastewater contamination, which in turn might cause recontamination of the network when hydraulic conditions allow.

3.5 Drinking water contaminated with surface water

In order to investigate other contaminant types, ATP was measured in drinking water contaminated with surface water (Figure 4). ATP concentrations decreased approximately tenfold for each tenfold dilution for the 10^1 to 10^2 surface water dilutions, but not at higher dilutions, since the microbial load of the surface water contaminant was diluted to the background level of ATP relevant to the specific drinking water. Microbial ATP concentrations were significantly higher in the 10^1 dilution (278 pg ATP/mL) and in the 10^2 dilution (24 pg ATP/mL) compared to clean drinking water (3.9 pg ATP/mL) ($p<0.05$). Microbial ATP concentration in the 10^3 and 10^4 dilutions were higher than in drinking water, though not significantly different. ATP concentrations in diluted surface water of 10^5, 10^6 and 10^7 were equivalent to ATP concentration in drinking water (Figure 4).

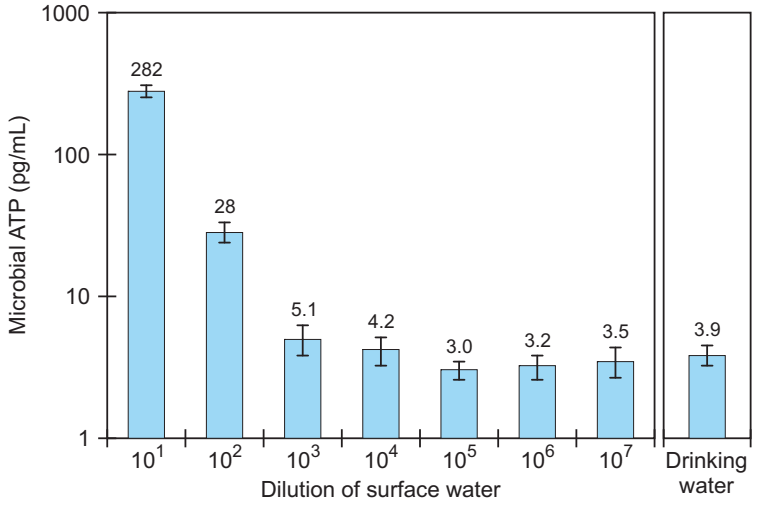

Figure 4: Microbial ATP concentrations in drinking water and drinking water contaminated with surface water of various dilutions.

Microbiological load will vary depending on the type of contaminant, and with a higher ATP concentration in wastewater than in surface water, wastewater could be detected at higher dilutions than surface water. High ATP loads from

contaminants and, for example, pulse contaminations will be detected, whereas continuous contamination with a low microbiological load will most likely not be detected with ATP measurements.

Another key parameter influencing the ability of the ATP assay to detect contaminations is the level of ATP in the specific drinking water, which may vary depending on type of water, treatment steps at the waterworks (Hammes et al., 2008) and time and potential spatial variations (van der Wielen & van der Kooij, 2010). Hence, concentrating a sample will increase the ATP concentration but will not necessarily improve method sensitivity, since the ratio of ATP in the contaminant and in the background will remain constant.

The ability in detecting contaminations with ATP measurements is somewhat lower than the results of current laboratory investigations, where ATP concentration in non-contaminated drinking water is known. Due to the natural variations of ATP in drinking water (van der Kooij, 1992; van der Kooij, 2003; van der Wielen and van der Kooij, 2010; Hammes et al., 2010; Liu et al., 2013a) it is important to identify a baseline in this respect for the specific water supply system. When monitoring microbial drinking water quality through grab sampling, ATP concentration in non-contaminated drinking water is not known, so in order to identify a given ATP concentration as a contamination it needs to be significantly higher than the expected natural variation of ATP in drinking water (Vang et al., II). Conversely, continuous online monitoring with ATP measurements will establish a baseline and provide information on potential changes in ATP throughout a day, for example, and as such it will be more efficient at identifying sudden increases in ATP triggered by possible contaminants. Overall, it is essential to establish baseline levels of ATP for the specific system, in order to most efficiently distinguish a contaminant in drinking water.

3.6 ATP versus traditional microbiological methods

Total direct cell counts

Total direct cell counts (TDC) detected a 10^3 dilution of wastewater ($2.40x10^5$ cells/mL) and up to a 10^2 dilution of surface water ($1.62x10^5$ cells/mL), i.e. concentrations were significantly higher than in drinking water ($1.17x10^5$ - $1.23x10^5$ cells/mL) ($p<0.05$) (Figure 5).

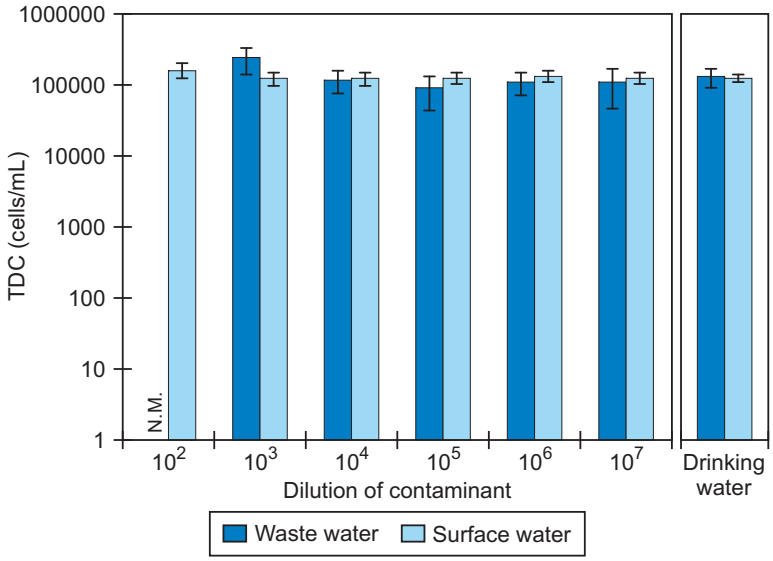

Figure 5: Total direct counts in drinking water and drinking water contaminated with wastewater or surface water of various dilutions (N.M.: not measured).

TDC and ATP concentrations, to a wide extent, should correlate, since both are a measure for total biomass. However, the ratio between concentrations in the 10^2 dilution of surface water and drinking water was 1.3 for TDC and 7.3 for microbial ATP, while the ratio for the 10^3 dilution of wastewater and drinking water was 1.8 for TDC and 4.9, 6.3 and 14.4 for microbial ATP (based on the three different experiments). Thus, the ATP assay was better than TDC in differentiating between clean drinking water and the two types of contaminants in drinking water, since the ratio between concentrations in contaminated drinking water and clean drinking water was considerably greater for ATP than for TDC. The sensitivity of TDC might be improved by increasing the precision of the method by increasing the number of grids being counted on the filter and the concentration of cells on each grid, since the CV of TDC was 36-55% for drinking water samples contaminated with wastewater and 17-22% for surface water contamination. Bacterial numbers may have been underestimated with TDC, due to multiple cells per particle (Camper et al., 1986; Liu et al., 2013b). This also demonstrates the advantage of the ATP assay compared to TDC, since ATP from bacteria in the bulk phase and particle-associated bacteria is expected to be extracted and measured.

Based on the measured data, the ATP content per cell was calculated at 3.5±0.6 and 5.6±1.8 $x10^{-17}$ g ATP/cell. This is in the same order of magnitude as reported by others for drinking water bacteria (Magic-Knezev and van der Kooij, 2004;

Velten et al., 2007; Berney et al., 2008; Hammes et al., 2008; Siebel et al., 2008; van der Wielen & van der Kooij, 2010; Vital et al., 2012).

Heterotrophic plate counts

For wastewater contamination, HPC 22°C was significantly higher than for drinking water at the 10^7 dilution of wastewater and HPC 37°C at the 10^6 dilution ($p<0.05$) (Figure 6-B), i.e. the HPC method was able to detect a 100 to 1000 times higher dilution of wastewater than the ATP assay. HPC 22°C decreased exponentially from the 10^2 to the 10^5 dilutions of surface water in drinking water(Figure 6-A), and the 10^4 dilution (156 CFU/mL) was significantly higher than the HPC 22°C in drinking water (16 CFU/mL) ($p<0.05$).

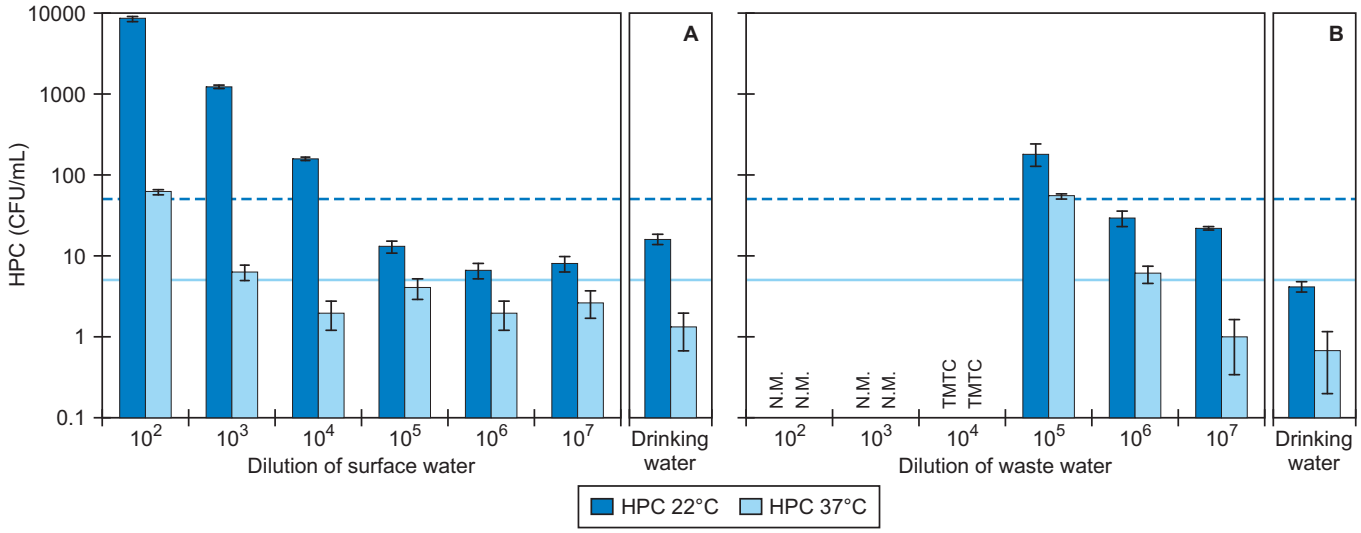

Figure 6: Concentration of heterotrophic plate counts (HPC 22°C and HPC 37°C - DS/EN ISO 6222:1999) in drinking water and drinking water contaminated with A) surface water of various dilutions or B) wastewater of various dilutions. Dotted line and full line indicate threshold values for HPC 22°C (50 CFU/mL) and HPC 37°C (5 CFU/mL) in finished drinking water, respectively (Danish executive order on drinking water, 2011). N.M.: not measured; TMTC: too many to count.

For HPC 37°C the 10^3 dilution of wastewater (6 CFU/mL) was significantly higher than in drinking water (1 CFU/mL) ($p<0.05$). Thus, surface water contamination was detected up to a 10^4 dilution for HPC 22°C and up to a 10^3 dilution for HPC 37°C. Consequently, the HPC method in this case was more efficient than the ATP assay in identifying both wastewater and surface water contamination in drinking water.

E. coli and coliforms

Coliforms (7 MPN/100 mL) and *E. coli* (2 MPN/100 mL) were detected in the 10^7 dilution of wastewater in drinking water (Figure 7-B). Thus, measuring *E. coli* and coliforms surpassed the ATP assay and other traditional microbiological methods applied in this study. It was possible to detect *E. coli* (30 MPN/100 mL) in the undiluted surface water, while it was <1 MPN/100 mL in all the dilutions (10^1 - 10^7) in drinking water (Figure 7-A). Conversely, coliforms were detected in the 10^4 dilution (2.0 MPN/100 mL). Hence, pathogenic bacteria may be present in drinking water without observing increased ATP concentrations.

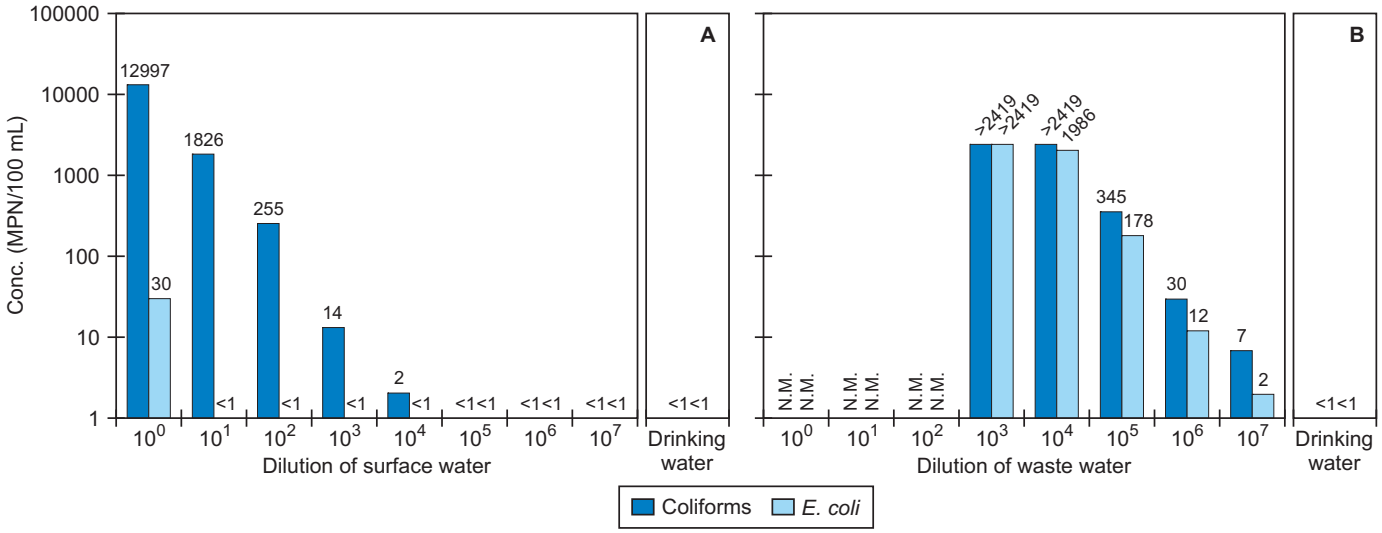

Figure 7: Concentrations of coliforms and *E. coli* (Coliert-18) in drinking water and drinking water contaminated with A) surface water of various dilutions or B) with wastewater of various dilutions.

The different methods provide information on various microbiological characteristics and give supporting information on the microbiological state of drinking water. The methods have different pros and cons regarding response time, indicators vs. total bacteria, total counts vs. metabolically active cells vs. viable but non-culturable cells as well as different workloads depending on the method employed.

The reproducibility of results through repeated experiments with wastewater and by also comparing different contaminants proves that ATP can be an efficient and robust parameter for assessing microbial drinking water quality. Nevertheless, despite the ability of the ATP assay to detect various types of microbial ingress in drinking water, the methodology also has its limitations. For instance, ATP assay is a non-specific method and represents the activity of all

bacteria – indigenous bacteria in drinking water and potential bacterial ingress – i.e. pathogenic bacteria can be present without eliciting a response in ATP. Furthermore, an increase in ATP might not always be caused by contamination. The resuspension of sediments in water pipes or the detachment of biofilm might also lead to increased ATP concentrations. Despite ATP being considered a robust monitoring parameter for microbial drinking water quality, a significant increase in ATP should be accompanied with methods for detection of specific bacteria (e.g. the indicators coliform, *E. coli*) in order to validate whether or not contamination has occurred. Hence, the best approach for monitoring microbial drinking water quality, in order to enhance water security and safety, is by combining rapid methods with methods targeted for specific bacterial detection. The advantage of ATP measurements compared to other methods used in this study is response time, whereby the ATP assay provides results within minutes as opposed to days, which is the case for Colilert-18, HPC 22°C and HPC 37°C. Even though both Colilert-18 and the HPCs were better at detecting microbial ingress than the ATP assay, ATP definitely has potential as an early warning parameter because of its fast response time (real-time), especially when the contaminant concentration is high, and it may also be applicable to detect pulse contaminations, i.e. a contamination of short duration with high ATP concentrations. The ATP assay is able to deliver fast, reliable and quantitative results on the microbiological state of drinking water in a contamination incident, and as such it has clear potential for the continuous real-time monitoring of microbial drinking water quality.

4 Conclusions

This study demonstrated that the ATP assay can provide fast and reliable results for detecting microbial ingress in drinking water, and thus it has potential as a parameter for the continuous real-time monitoring of microbial drinking water quality. More specifically, the conclusions of this experimental study were:

- The ATP assay was able to detect up to a 10^4 dilution of wastewater in drinking water.
- Quantifying microbial ATP did not significantly improve the ability of the ATP assay in identifying the wastewater contamination compared to measuring total ATP.
- Applying more sensitive reagents for the ATP analysis did not reduce the standard deviation of ATP measurements significantly for low concentrations of ATP, and consequently it did not contribute substantially to the ATP assay's ability to detect contaminations in drinking water.
- The ATP assay was able to detect a surface water dilution of 10^2 to 10^3 in drinking water.
- Increased ATP concentrations were observed after 6 days incubation for the 10^5 dilution of wastewater in drinking water because of additional substrate from the wastewater which led to bacterial aftergrowth.
- The ATP assay was better at differentiating between the wastewater and surface water contaminations and clean drinking water compared to TDC. Although the HPCs and Colilert-18 exceeded the ATP assay with regard to detection of waste water and surface water in drinking water, ATP measurements are nevertheless advantageous with regard to response time.
- The ability of the ATP assay in detecting a contaminant is controlled by both the microbial load of the specific contaminant as well as the ATP level of the specific type of drinking water.

Acknowledgements

This work was supported by the Danish Council for Strategic Research and the SENSOWAQ (Sensors for Monitoring and Control of Water Quality) project. Thanks go to the Urban Water Technology Research School. Thanks and to laboratory technicians Mona Refstrup and Sabrina Nedell for their valuable assistance in the laboratory. Torben Dolin and Lisbet Brusendorff are acknowledged for their assistance on graphics. Finally, thanks are extended to Lyngby-Taarbæk Forsyning A/S and Nordvand A/S for supplying us with drinking water, wastewater and surface water.

References

Aoki, Y., Fukuba, T., Yamamoto, T., Fujii, T., 2008. Development of 'IISSA'-ATP system in deep sea environment. OCEANS 2008 - MTS/IEEE KOBE TECHNO-OCEAN, VOLS 1-3 Book Series: OCEANS-IEEE, 548-551.

Berney, M., Vital, M., Hülshoff, I., Weilenmann, H.-U., Egli, T., Hammes, F., 2008. Rapid, cultivation-independent assessment of microbial viability in drinking water. Water Research 42, 4010-4018.

Boe-Hansen, R., Albrechtsen, H.-J., Arvin, E., Jørgensen, C., 2002. Bulk water phase and biofilm growth in drinking water at low nutrient conditions. Water Research 36, 4477-4486.

Bushon, R.B., Likridopulos, C.A., Brady, A.M.G., 2009. Comparison of immunomagnetic separation/adenosine triphosphate rapid method to traditional culture-based method for E. coli and enterococci enumeration in waste water. Water Research 43, 4940-4946.

Camper, A.K., LeChevallier, M.W., Broadaway, S.C., McFeters, G.A. (1986) Bacteria associated with granular activated carbon particles in drinking water. Applied and Environmental Microbiology, 52, 3, 434-338.

Deininger, R.A., Lee, J. (2001) Rapid determination of bacteria in drinking water using an ATP assay. Field Analytical Chemistry and Technology, 5, 4, 185-189.

Delahaye, E., Welté, B., Levi, Y., Leblon, G., Montiel, A., 2003. An ATP-based method for monitoring the microbiological drinking water quality in a distribution network. Water Research 37, 3689-3696.

Denburg, J.L.; Lee, R.T.; McElroy, W.D. (1969) Substrate-binding properties of firefly luciferase – I. Luciferin-binding site, Arch. Biochem. Biophys. 134 , pp 381–394.

Deluca, M.; Wannlund, J.; McElroy, W.D. (1979) Factors affecting the kinetics of light emission from crude and purified firefly luciferase, Analytical Biochemistry, 95, pp. 194-198.

Eydal, H.S., Pedersen, K., 2007. Use of an ATP assay to determine viable microbial biomass in Fennoscandian Shield groundwater from depths of 3-1000 m. Journal of Microbiological Methods 70, 363-373.

Ghazali, M., McBean, E., Whalen, P., Journal, K., 2010. Supporting a drinking water contaminant warning system using the adenosine triphosphate test. Canadian Journal of Civil Engineering 37, 1423-1431.

Hammes, F., Berney, M., Wang, Y., Vital, M., Köster, O., Egli, T., 2008. Flow-cytometric total bacterial cell counts as a descriptive microbiological parameter for drinking water treatment processes. Water Research 42, 269-277.

Hammes, F., Goldschmidt, F., Vital, M., Wang, Y., Egli, T., 2010. Measurement and interpretation of microbial adenosine tri-phosphate (ATP) in aquatic environments. Water Research 44 (13), 3915-3923.

Hammes, F., Broger, T., Weilenmann, H.-U., Vital, M., Helbing, J., Bosshart, U., Huber, P., Odermatt, R. P., Sonnleitner, B., 2012. Development and Laboratory-Scale Testing of a Fully Automated Online Flow Cytometer for Drinking Water Analysis. Cytometry Part A, 81A, 508-516.

Karl, D.M. (1980) Cellular nucleotide measurements and applications in microbial ecology. Microbiological Reviews, 44, 4, 739-796.

Lehtola, M.J., Juhna, T., Miettinen, I.T., Vartiainen, T., Martikainen, P.J., 2004. Formation of biofilms in drinking water distribution networks, a case study in two cities in Finland and Latvia. Journal of Industrial Microbiology & Biotechnology 31 (11), 389-494.

Liu, G., van der Mark, E.J., Verberk, J.Q.J.C., van Dijk, J.C. (2013a) Flow cytometry total cell counts: A field study assessing microbiological water quality and growth in unchlorinated drinking water distribution systems. BioMed Research International, Vol. 2013, Article ID 595872, p 1-10.

Liu, G., Ling, G.Q., Magic-Knezev, A., Liu, W.T., Verberk, J.Q.J.C., van Dijk, J.C. (2013b) Quantification and identification of particle-associated bacteria in unchlorinated drinking water from three treatments plants by cultivation-independent methods. Water Research, 47, 3523-3533.

Magic-Knezev, A., van der Kooij, D., 2004. Optimisation and significance of ATP analysis for measuring active biomass in granular activated carbon filters used in water treatment. Water Research 38, 3971-3979.

Maki, J.S., LaCroix, S.J., Hopkins, B.S., Stanley, J.T., 1986. Recovery and Diversity of Heterotrophic Bacteria from Chlorinated Drinking Waters. Applied and Environmental Microbiology 5 (5), 1047-1055.

Marriott, N.J., Barnes, S.P., Smith, R.N. (1992) A comparative evaluation of three commercially available luminometers and two ATP reagent kits. International Biodeterioration & Biodegradation, 30, 339-352.

Siebel, E., Wang, Y., Egli, T., Hammes, F., 2008. Correlations between total cell concentration, total adenosine tri-phosphate concentration and heterotrophic plate counts during microbial monitoring of drinking water. Drinking Water Engineering Science 1, 1-6.

Smeets, P.W.M.H., Medema, G.J., van Dijk, J.C, 2009. The Dutch secret: how to provide safe drinking water without chlorine in the Netherlands. Drinking Water Engineering Science 2, 1-14.

van der Kooij, D. (1992) Assimilable organic carbon as an indicator of bacterial regrowth. American Water Works Association (AWWA), Vol. 84, No. 2, 57-65.

van der Kooij (2003) Managing regrowth in drinking-water distribution systems. In: Emerging Issues in Water and Infectious Diseases Series. Heterotrophic plate counts and drinking-water safety: The significance of HPC's for water quality and human health, 199-232.

van der Kooij, D., Baggelaar, P.K., Veenendaal, H.R., Moulin, L., Corfitzen, C.B., Albrechtsen, H.-J., Holt, D.; Hambsch, B. (2006) Standardising the biomass production potential method for determining the enhancement of microbial growth by construction products in contact with drinking water - inter-laboratory testing; EC Grant Agreement nbr. S12, 403889.

van der Wielen, P.W.J.J., van der Kooij, D. (2010) Effect of water composition, distance and season on the adenosine triphosphate concentration in unchlorinated drinking water in the Netherlands. Water Research 44 (17), 4860-4867.

Vang, Ó.K., Corfitzen, C.B., Spliid, H., Albrechtsen, H.-J. (II) Levels of microbial ATP and free ATP in non-chlorinated drinking water - assessing microbial drinking water quality with ATP measurements. Submitted.

Vang, Ó.K., Corfitzen, C.B., Smith, C., Albrechtsen, H.-J. (III) Levels of microbial ATP and free ATP in non-chlorinated drinking water - assessing microbial drinking water quality with ATP measurements. Manuscript in preparation.

Velten, S., Hammes, F., Boller, M., Egli, T. (2007) Rapid and direct estimation of active biomass on granular activated carbon through adenosine tri-phosphate (ATP) determination. Water Research 41, 1973-1983.

Vital, M., Hammes, F., Magic-Knezev, A., Ross, P., Rietveld, L., Hammes, F. (2012) Flow cytometry and adenosine tri-phosphate analysis: Alternative possibilities to evaluate major bacteriological changes in drinking water treatment and distribution systems. Water Research 46, 4665-4676.

II

Levels of microbial ATP and free ATP in non-chlorinated drinking water – assessing microbial drinking water quality with ATP measurements

Vang, Ó.K., Corfitzen, C.B., Spliid, H., Albrechtsen H.-J.

Submitted manuscript

Levels of microbial ATP and free ATP in non-chlorinated drinking water - assessing microbial drinking water quality with ATP measurements

Óluva K. Vang[1,*], Charlotte B. Corfitzen[1], Henrik Spliid[2], Hans-Jørgen Albrechtsen[1]

[1]Department of Environmental Engineering, Technical University of Denmark, Miljoevej, Building 113, 2800 Kgs. Lyngby, Denmark.

[2]Department of Applied Mathematics and Computer Science, Technical University of Denmark, Matematiktorvet, Building 324, 2800 Kgs. Lyngby, Denmark.

*Corresponding author: okva@env.dtu.dk

Abstract

ATP has been used in several investigations to assess microbial drinking water quality. Microbial ATP can be determined by different approaches. When extracted on a filter after sample filtration (0.45 µm), microbial ATP concentrations were found to be 30-40% lower than when determined as the difference between the measured total and free ATP concentrations. Filtration in terms of different filter pore sizes and different filtration fluxes did not significantly affect the free ATP concentration compared to free ATP in non-filtrated samples, though repeatability was generally improved for the non-filtrated samples. Levels and variations in microbial ATP concentrations in non-chlorinated drinking water were analysed in 211 ATP assay measurements over a four-year period, with sampling from two waterworks, three distribution systems and tap water sampled following overnight stagnation. Microbial ATP concentrations were in the range of <1.2 to 22 pg/mL and were generally low for waterworks and distribution networks, namely <5 pg/mL in 75% of the samples at one waterworks and all three distribution networks. On average the microbial

ATP fraction contributed by 53% to the total ATP pool, but with significant variations. The microbial ATP concentration was highest in tap water following overnight stagnation, while average microbial ATP concentrations were higher at the waterworks than for distributed drinking water. The percentage of free ATP was significant (>50% in 35% of the samples) in non-chlorinated drinking water, with an inconsistent ratio to microbial ATP. The fraction of free ATP contributed significantly more in drinking water (47%) compared to other environmental samples such as surface water (19%) and wastewater (10%). Consequently, it is important to consider the contribution of free ATP to an accurate interpretation of ATP as a monitoring parameter of microbial drinking water quality.

Key words: Microbial ATP, free ATP, drinking water, bacteria, filtration

1 Introduction

Adenosine triphosphate (ATP) has proven to be an efficient indicator of changes in microbial drinking water quality in terms of active, living cells at waterworks and in distribution systems (van der Kooij, 1992, van der Kooij, 2003, Hammes et al., 2008; van der Wielen and van der Kooij, 2010; Vital et al., 2012). The ATP analysis is fairly simple and is advantageous in providing fast and reliable quantitative results.

ATP is the prime energy carrying molecule in all living cells, and the ATP content of depends on the size and physiological state of the cell (Stanley, 1989). Consequently, the ATP content of a water sample depends not only on microbial density but also on the microbial community and nutrient availability. As such, ATP concentrations cannot be converted directly to a cell number. Nevertheless, ATP can be interpreted as a measure for cell activity as well as for the abundance of bacteria (Berney et al., 2008).

The ATP assay has been used mainly in research to characterise microbial drinking water quality (Lee and Deininger, 1999; Delahaye et al., 2003; Eydal and Pedersen, 2007; Berney et al., 2008; Hammes et al. 2010), but is also used as a monitoring parameter of microbial drinking water quality, for example in the Netherlands (van der Kooij, 2003; van der Wielen & van der Kooij, 2010).

The amount of free ATP (total ATP = microbial ATP + free ATP) in samples has been of particular concern regarding the ATP assay. Free ATP can be released from cells in the die-off phase (Stanley, 1989), but in many cases it is considered negligible due to the general assumption that ATP is rapidly degraded by transphosphorylases and ATPases after cell death (Azam & Hodson, 1977). The abundance and stability of free ATP in environmental samples have been addressed in several studies, although with ambiguous results. For example, free ATP concentrations were considered to be low and negligible in endolithic communities in the Antarctica (Tuovila and LaRock, 1987) and in cold soils (Roser et al., 1993; Cowan et al., 2002; Cowan and Casanueva, 2007), while free ATP in seawater was found in significant concentrations of 0.1-0.6 μg/L, i.e. up to more than 90% (Azam & Hodson, 1977; Hodson et al., 1981) and varied in fresh water lakes between 0.1 and 3.8 μg/L, i.e. free ATP constituted 14-76% of the total ATP (Riemann, 1979).

Often, total ATP is measured as the parameter for microbial drinking water quality (Eydal and Pedersen, 2007; van der Wielen and van der Kooij, 2010; Liu et al., 2013). However, a few studies over the past few years have considered the contribution of the free ATP fraction in drinking water, and have specifically quantified microbial ATP (Berney et al., 2008; Hammes, 2010; Vital, 2012; Lautenschlager et al., 2013). In non-chlorinated distributed drinking water in Switzerland, the percentage of free ATP was relatively high (0-97%) (Hammes et al., 2010).

The high proportion of free ATP may be related to the method used in this study, since filtration and other sample pre-treatments before ATP analysis may change ATP concentrations due to cell rupture and the release of ATP (van der Wielen and van der Kooij, 2010). The presence of significant and varying amounts of free ATP questions the suitability of using total ATP as a monitoring parameter for microbial drinking water quality. Obviously, understanding the abundance of free ATP in drinking water is essential for the interpretation of results as well as for assay design.

In general, two approaches are used to measure ATP, namely measuring total ATP or quantifying microbial ATP. Measuring total ATP is fairly straightforward, with no pre-handling or pre-treatment of the sample required (van der Wielen and van der Kooij, 2010; Liu et al., 2013). Microbial ATP can be determined in two ways: either as the difference between total ATP and free

ATP, i.e. total ATP and free ATP are measured individually, or by direct measurement of microbial ATP, which requires a filtration step (e.g. Delahaye, 2003; Hammes, 2010). Discrepancies between the approaches in various studies and research groups means that there is no consensus on how or which fraction of ATP to measure, which calls for further investigation and clarification on the size of the microbial ATP and free ATP amounts relative to total ATP in drinking water.

Thus, the main objectives of this study are to investigate the size distribution of microbial and free ATP in drinking water and to determine their influence on the interpretation of ATP as a parameter for microbial drinking water quality. The study is based on ATP measurements of drinking water samples – groundwater-based and non-chlorinated drinking water. In addition, the methodology for determining free ATP and microbial ATP is addressed, specifically in relation to the effect of filtration on free and microbial ATP concentrations with regard to accuracy and repeatability.

2 Materials and methods

2.1 ATP assay for drinking water samples

Total ATP in drinking water samples was assayed by adding an ATP releasing agent (cell lysis) and the subsequent addition of the enzyme luciferase and its substrate luciferin, resulting in light emission. Free ATP was measured without cell lysis by adding only enzyme reagent to the sample. Microbial ATP concentration was determined as the difference between total ATP and free ATP concentration (microbial ATP = total ATP - free ATP).

All samples were measured with the LuminEX/LuminATE reagent kit (92687, Celsis). Sample volumes, used with the LuminEX/LuminATE reagent kit, were a 100 µL sample, a 100 µL extraction reagent and a 100 µL enzyme reagent (according to the manufacturer's recommendations). Drinking water samples were added manually to a cuvette, while extraction and enzyme reagents were added automatically with the dispensing system on the luminometer. The extraction reagent was not added when measuring free ATP. After 10 seconds of extraction the enzyme reagent was added, and after 2 seconds the light emission was measured and integrated over a further 10 seconds.

Luminescence was quantified as relative light units (rlu), which were converted to ATP concentrations through an ATP standards calibration curve measured on the same day as the samples. Two calibration curves were prepared – one for total ATP and one for free ATP – due to quenching/inhibition caused by the extraction reagent and dilution effects caused by the additional volume of extraction reagent when measuring total ATP. ATP standards were prepared with ATP standard salt (92638, Celsis) reconstituted in Lumin(PM) buffer (92678, Celsis) ($1x10^6$ pg ATP/mL). The stock solution was diluted further in autoclaved sterile filtrated (0.20 µm) tap water in line with a series of ATP standards in the range of 2.5-1,000 pg ATP/mL.

The limit of detection (LOD) of total ATP and free ATP was determined by linear regression of several calibration curves in the range of 0-10 pg/mL (Vang et al., III). The average LOD was 1 pg/mL for total ATP (N=20 calibration curves) and 0.7 pg/mL for free ATP (N=15 calibration curves). The limit of detection of microbial ATP was calculated to be 1.2 pg/mL (Vang et al., III). Samples were measured in duplicate or triplicate with an Advance Coupe luminometer (Celsis, The Netherlands). All glassware for collecting water was acid-washed and heated to 540°C (for 6 h), ATP-free laboratory equipment was used and ATP-free tips were used for pipetting. All samples and sample processing were handled using sterile techniques.

2.2 Techniques to determine free and microbial ATP

2.2.1 Determination of free ATP

To investigate whether filtration affected the repeatability and accuracy of free ATP concentration, drinking water samples were filtrated with filter pore sizes of 0.20 µm (26 mm cellulose acetate membrane syringe filter, Sartorius Minisart, 16534-K) and 0.45 µm (25 mm cellulose acetate membrane, Advantec MFS Inc., 25CS045AS) at different filtration pressures (flux of 226 versus 1358 L/(m^2·h)). The filtrate was measured by adding both extraction and enzyme reagent, and the results were compared to measuring free ATP with the addition of only the enzyme reagent to a non-filtrated sample, as described in section 2.1. The effect of filter pore size on free ATP concentration was investigated by filtrating a 1 mL water sample for approximately 5 seconds (equivalent to a flux of 1358 L/(m^2·h)) through a 0.20 or 0.45 µm pore size filter, where the concentration of free ATP was measured in 100 µL of the filtrate. Filtration pressure was investigated by filtrating a 1 mL sample through a 0.20 µm pore size filter for approximately 30

seconds (equivalent to a flux of 226 L/(m^2·h)) compared to a filtration of 5 seconds (equivalent to a flux of 1358 L/(m^2·h)). To investigate whether free ATP was retained by the filter, ATP standards of 10 and 100 pg ATP/mL were filtrated (0.20 µm), measured and compared to measurements of non-filtrated ATP standards. Microbial ATP concentrations were determined as the difference between free ATP – established using the different techniques described above – and total ATP following the protocol described in section 2.1. Samples in this specific investigation included groundwater (Sample 1), water sampled after pre-filter (Sample 2) and water sampled after UV disinfection (Sample 3) at a waterworks (Sjælsø Waterworks, Nordvand A/S).

2.2.2 Determination of microbial ATP

Microbial ATP concentrations in drinking water samples were determined directly after manual filtration through a 0.45 µm syringe filter (25 mm cellulose acetate membrane, Advantec MFS Inc., 25CS045AS) with subsequent extraction of microbial ATP from the filter: 1) 10 mL of sample was filtrated, 2) the filter was dried by flushing with air three times through the filter with a syringe, 3) a 1 mL sterile syringe with 1 mL extraction reagent was attached to the syringe filter, 4) the syringe piston was then pulled back to suck up air above the filter, 5) filter was then soaked with 0.5 mL extraction reagent, which was slowly pushed through the filter for 1 minute to extract microbial ATP, 6) the remaining extraction reagent of 0.5 mL was slowly pushed through the filter to remove all extracted microbial ATP from the filter, 7) the filter was dried by flushing air through the filter three times with a syringe, to remove all of the sample, 8) then steps 3 to 8 were repeated, and finally 9) the 2x1 mL extraction reagent with extracted microbial ATP was pooled for ATP analysis, where a subsample was analysed for microbial ATP following the total ATP protocol (see section 2.1). The direct determination of microbial ATP extracted on a filter was compared to microbial ATP determined as the differences between total and free ATP, where free ATP was measured in the filtrate after filtering the sample. For this specific investigation, drinking water and drinking water spiked with wastewater in tenfold dilutions (10^0-10^5) were measured with the two different protocols for determining microbial ATP. N=17, with duplicate sample measurements.

2.3 Dataset of ATP measurements in Danish drinking water

From 2008 to 2012 a total of 222 drinking water samples were analysed for ATP concentrations on the day of sampling. Samples of drinking water based on groundwater and without disinfectant residual were analysed for total and free ATP (see section 2.1). The total ATP concentration was below the detection limit of 1 pg/mL in 11 samples. The total ATP concentrations and the corresponding free ATP concentrations in these samples were not included in the data treatment, i.e. N=211.

The drinking water was sampled at two waterworks: WW 1 (Lyngby Waterworks; N=5) and WW 2 (Sjælsø Waterworks; N=35), three distribution networks: Net 1 (Copenhagen; N=64), Net 2 (Odense 2010, N=34) and Net 3 (Odense 2009-2010, N=61), and Tap 1 (Lyngby, N=12) representing water sampled on two different days from six different taps as a first flush after overnight stagnation. Samples included in Net 1 were collected on four different days (August and December) from a specific region of the distribution network in Copenhagen. Samples included in Net 2 and Net 3 were all taken from the distribution network of Odense, where samples for the measurement campaign of Net 2 were all collected within one day and represent a specific pipe section of approximately 3 km of the Odense distribution system. Samples included in Net 3 covered a larger area of the distribution network, and sampling was carried out on seven different days over a two-year period.

2.3.1 Estimating free ATP below LOD

In 63 drinking water samples (N=211), free ATP concentrations were below the limit of detection (LOD: 0.7 pg ATP/mL). In order to assess the microbial ATP in these samples, the free ATP concentrations below LOD were estimated through the correlation between free ATP and total ATP concentrations above LOD (N=153). The correlation was significant ($P<0.0001$, $\alpha=0.05$) with a Pearson's r of 0.68, and the slope of the linear regression analysis was ≈0.30 ±0.02 (Figure 1). Thus, free ATP concentrations below the LOD were estimated at 30% of the sample's total ATP concentration.

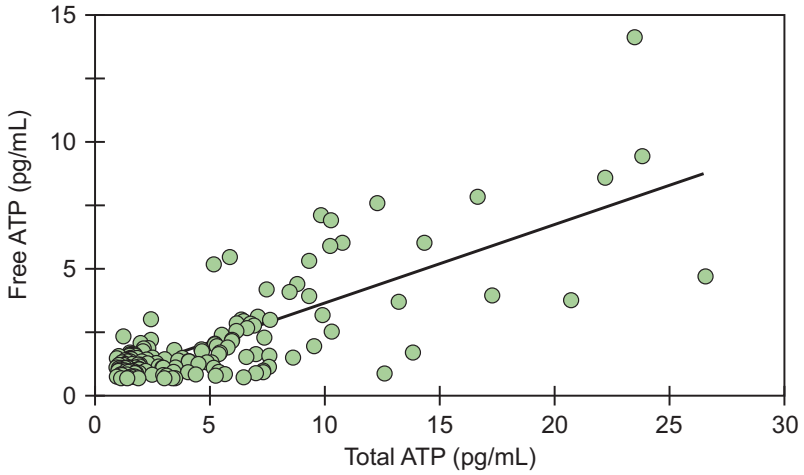

Figure 1: Linear model for the correlation between free ATP and total ATP, to determine the percentage of free ATP in samples which have free ATP below the LOD (0.7 pg ATP/mL). Slope of linear regression analysis designates that free ATP should constitute 30% of total ATP concentration in samples where free ATP is <LOD, though a maximum of 0.7 pg/mL (i.e. equal to LOD) is assigned to samples with a free ATP concentration below LOD.

In cases where 30% of total ATP was above the LOD, free ATP was set equal to 0.7 pg/mL, i.e. the limit of detection of free ATP. Microbial ATP was then determined by subtracting the estimated free ATP from total ATP. In 14 samples measured free ATP concentrations were higher than total ATP, and since the microbial ATP concentration was very small, it was consequently set equal to zero. ATP concentrations followed a log-normal-like distribution; therefore, a geometric mean with 95% confidence interval was calculated for total, free and microbial ATP. Statistical calculations were made using GraphPad Prism (version 5.00, 2007).

3 Results and discussion

3.1 Techniques for determining ATP in drinking water

3.1.1 Effect of filtration on microbial ATP

Microbial ATP concentrations, based on extraction of ATP in the filter after sample filtration, were in average 35% lower (N=13, 4 outlier values were excluded) than when determined as the difference between the total ATP and free ATP measurements (Figure 2). Hence, direct extraction on a filter resulted in a loss of microbial ATP.

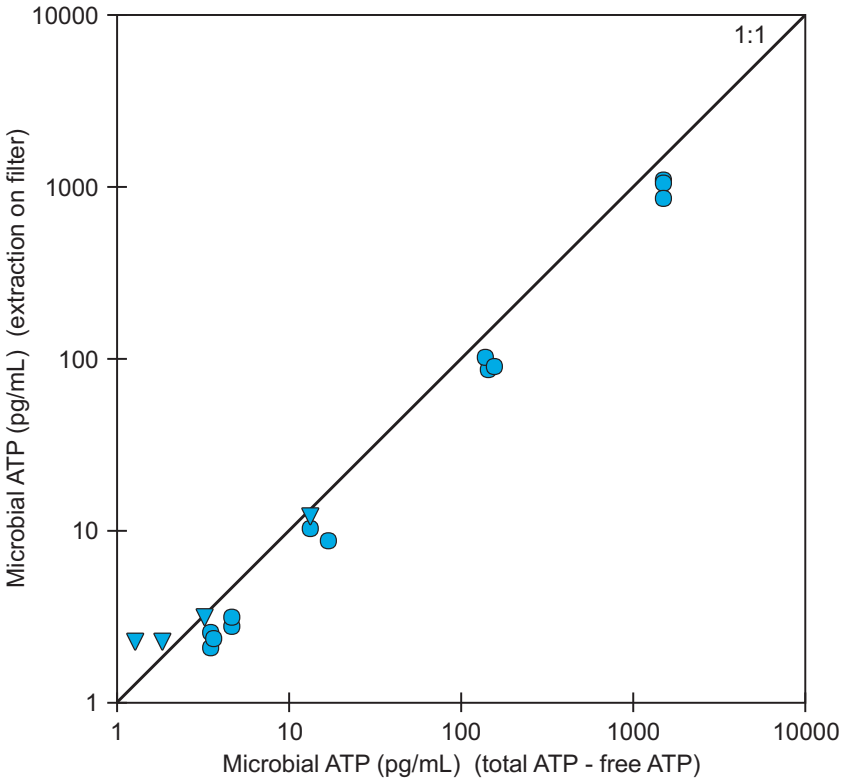

Figure 2: Comparison between the two ways employed to measure microbial ATP concentrations, either extracted on a filter or microbial ATP determined as the difference between total ATP and free ATP. The samples included drinking water spiked with wastewater. N=17, two replicate measurements. Triangle: outlier-value (N=4).

The smaller amount of microbial ATP observed with this filtration procedure could be due to incomplete extraction of ATP from cells in the filter or insufficient release of the extracted microbial ATP from the filter. Additionally, bacteria may pass through the filter, where a fraction of 46% of tap water bacteria has been reported as being able to pass through a 0.45 µm pore size filter (Wang et al., 2007), which was the filter pore size used in this investigation. However, free ATP concentrations in the filtrate of a 0.45 µm filter pore size measured by adding both extraction reagent and enzyme reagent were not significantly higher compared to the free ATP concentration of a non-filtrated sample (Figure 3).

The filtration step may also induce metabolic stress, thus resulting in reduced ATP concentrations (Jewson and Dokulil, 1982). The potential adverse effect of filtration on ATP and other nucleotides has been addressed, whereby both centrifugation and filtration tend to decrease the ATP content (Jones & Simon, 1977) because of oxygen and nutrient depletion (Stanley, 1986). Conversely, metabolic stress may be imposed by vacuum filtration, resulting in a decrease in

ATP concentrations, though the concentration of total adenine nucleotides has been noted as conservative (Karl & Holm-Hansen, 1978), therefore indicating a rearranging between concentrations of nucleotides rather than cell lysis. As such, the loss of ATP in this specific filtration procedure could have been caused by any of the abovementioned issues, or even in combination with one another. Especially, the combination of stress induced cells and filterable cells in a self-perpetuating process would result in less microbial ATP.

An alternative approach is both to extract microbial ATP from microorganisms on the filter and to measure bioluminescence on the filter, which is the principal function of the Filtravette by New Horizons Diagnostics (Delahaye et al., 2003). If this approach is used, it is important to use a filter with an adequate filter pore size, i.e. 0.2 µm or less (Wang et al., 2007). Furthermore, the choice of an appropriate filter material has to be considered, since bacteria can be trapped inside cellulose filters as opposed to polycarbonate filters (Hobbie et al., 1977), which obviously is problematic, since luminescence is measured above the filter. Microbial ATP determined as the difference between total and free ATP requires somewhat more reagent, since both free ATP and total ATP often are measured in a minimum of two replicates. On the other hand, the filtration approach increases measurement costs in terms of the membrane filter, and it is significantly less laborious and time-consuming to determine microbial ATP based on measuring total and free ATP rather than the filtration protocol described above. Consequently, further determinations of microbial ATP concentrations in drinking water samples in our study were determined as the difference between total ATP and free ATP concentrations.

3.1.2 Effect of filtration on free ATP

The accuracy of the free ATP measurement is important when microbial ATP concentrations are determined as the difference between total and free ATP concentrations. To investigate whether filtration (filter pore size and filtration pressure) influenced the repeatability and accuracy of the free ATP concentration, the free ATP concentration of filtrated samples were compared to free ATP measured by only adding the enzyme reagent to a non-filtrated sample (Figure 3).

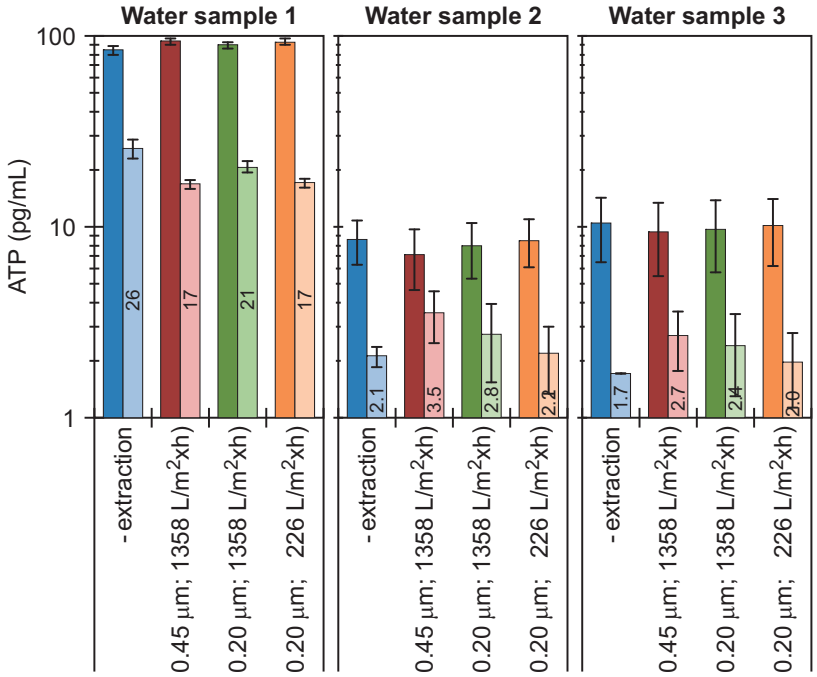

Figure 3: Effect of filter pore size and filtration pressure (flux) on the concentration of free ATP (filtrate) compared to free ATP measured with addition of enzyme reagent only (light coloured bars) in three drinking water samples. Microbial ATP (dark-coloured bars) was determined as the difference between total ATP and free ATP. N=1 with two replicate measurements for the blue and orange columns; N=3 with two replicate measurements for the red and green columns. The concentration of free ATP is given on each light-coloured bar.

Drinking water samples with both relatively high (Sample 1) and low (Sample 2 and 3) ATP concentrations were analysed. The microbial ATP concentration in sample 1 was 84-93 pg ATP/mL, depending on the protocol for free ATP, 7.2-8.6 pg /mL for sample 2 and 9.4-10.4 pg/mL for sample 3. Free ATP concentrations in the filtrate of the three filtration protocols were slightly higher than free ATP measured in the non-filtrated sample for water samples 2 and 3 at relatively low ATP concentrations, indicating that the filtration may have converted microbial ATP into free ATP due to cell lysis, or alternatively that bacteria were passing through the filter (Björkman and Karl, 2001; Wang et al., 2007). However, the concentrations of free ATP were not significantly different ($p<0.05$) between filter pore size (0.45 µm or 0.2 µm) or filtration flux (1358 or 226 L/(m^2·h)) compared to free ATP in the non-filtrated sample. Also, there was a poorer repeatability of samples 2 and 3 for the filtration protocols compared to the non-filtrated sample (Figure 3), which could indicate interference by the filtration step. As opposed to samples 2 and 3, the highest concentration of free ATP for sample 1 was measured in the non-filtrated sample, and it was significantly higher ($p>0.05$) than two of the filtration protocols – 0.45 µm pore size filter

(1358 L/(m^2·h)) and 0.20 µm pore size filter (226 L(m^2·h)) This might indicate a small accumulation of free ATP in the filter or perhaps attachment to particles/cells in the sample, and thus not filtrated. A significant loss of free ATP due to filtration was observed by Eydal and Pedersen (2007).

The filtration of ATP standards of 10 and 100 pg ATP/mL compared to non-filtrated ATP standards was not statistically different ($p>0.05$). The recovery (%) of filtrated ATP standard dilutions of 10 and 100 pg ATP/mL was 91% and 94%, respectively, which may indicate a small retention or sorption of free ATP to the filter material. Free ATP molecules are strongly negatively charged and may be attached to positively charged particles or dissolved compounds (Riemann, 1979). Environmental samples, such as drinking water, might behave differently when filtrated compared to the filtration of ATP standards, without intact cells or particles to the same extent as drinking water. Consequently, filtering a sample might slightly underestimate the concentration of free ATP when measuring free ATP in the filtrate. The consequence is less accurate microbial ATP concentration, since it will be slightly higher when determined as the difference between total ATP and free ATP.

Overall, concentrations between the filtrated and non-filtrated samples were not significantly different in two out the three samples; nevertheless, there was a tendency toward improved repeatability for non-filtrated samples. Hence, it is evaluated that the sample is less prone to any potential interference (e.g. cell lysis, sorption) if it is not filtrated, and accuracy and repeatability of free ATP is best preserved if assayed by only adding enzyme reagent to the sample.

3.2 ATP levels in drinking water

3.2.1 Total and microbial ATP in non-chlorinated drinking water

The geometric mean of total ATP concentration in the non-chlorinated drinking water was 3.1 ±0.3 pg total ATP/mL (95% confidence interval) (Table 1), with a range of <1 to 27 pg/mL and with only 11 samples (4%) below the detection limit of 1 pg/mL.

Table 1: Geometric mean of total, free and microbial ATP concentrations with 95% confidence intervals, minimum and maximum concentrations and average fraction size as a percentage of all samples (N=211) and for microbial ATP concentrations ≥1.2 pg/mL (LOD) (N=122).

	Geo.mean (pg ATP/ml)	95% conf.interval	Min[a)] (pg ATP/ml)	Max (pg ATP/ml)	Percentage %	Min %	Max %
All samples (N=211)							
Total ATP	3.1	±0.3	<1.0	27	100	-	-
Free ATP	1.2	±0.1	<0.7	14	47	7	100
Microbial ATP	1.7	±0.2	<1.2	22	53	0	93
Microbial ATP ≥1.2 pg/mL (LOD) (N=122)							
Total ATP	5.1	±0.6	1.7	27	100	-	-
Free ATP	1.4	±0.2	<0.7 [a)]	14	30	7	72
Microbial ATP	3.5	±0.4	1.2	22	70	28	93

[a)]Below LOD for the specific fraction of ATP.

The concentration range is similar to total ATP concentrations in Dutch drinking water, with 0.8 to 12.1 pg total ATP/mL in distributed water, originating from groundwater or surface water and distributed without chlorine residual (van der Wielen and van der Kooij, 2010). Moreover, total ATP concentrations in distributed drinking water of 20 water supplies in the Netherlands were in the range of <1 to 23 pg/mL (van der Kooij, 1992), and total ATP concentrations in drinking water leaving 243 treatment facilities (the Netherlands) had a concentration below 5 pg/mL in approximately 75% of samples, of which 15% were below 1 pg/mL (van der Kooij, 2003). On the other hand, somewhat lower total ATP concentrations were measured in distributed drinking water in the Netherlands – in most cases the concentration was less than 3 pg/mL (N=260) (Liu et al., 2013). Also, Berney et al. (2008) measured considerably low total ATP concentrations of 0.016-0.055 pg/mL in bottled water, drinking water fountains and tap water (Zürich, Switzerland).

The geometric mean for microbial ATP concentrations was 1.7 ±0.2 pg/mL (95% confidence interval) (Table 1) in the investigated drinking water (N=211), hence similar to distributed drinking water (1.8 ±1.0 pg/mL) in Zürich (Lautenschlager et al., 2013). The lowest concentration of microbial ATP was below the LOD of 1.2 pg/mL and the highest 27 pg microbial ATP/mL. Average microbial ATP concentration for five locations (WWs 1, 2 and Nets 1, 2 and 3) was ≤5 pg/mL

(Figure 4-A), and 75% of microbial ATP concentrations were <5 pg/ml for WW 1 and Nets 1, 2 and 3. This is similar to values reported by other studies; for example, non-chlorinated drinking water leaving two waterworks in Amsterdam, where the source water was river water and polder seepage water, had microbial ATP concentrations of less than 5 pg microbial ATP/mL (Vital et al., 2012). Furthermore, microbial ATP concentrations in distributed water in Paris (with chorine residual) were in the range of 0.002-4.1 pg/mL, where most of the samples had a concentration less than 1 pg/mL (Delahaye et al., 2003).

Interestingly, total and microbial ATP concentrations in drinking water primarily without chorine residual are very similar across countries and water supply systems with different water sources and different treatment trains. This may be related to the fact that average concentrations of cells in non-chlorinated drinking water also appear to be similar for Danish (1.2-1.3 $x10^5$ cells/mL), Dutch (0.94-1.06$x10^5$ cells/mL) and Swiss (0.5-2$x10^5$ cells/mL) drinking water (Vang et al., I; Vital et al., 2012).

3.2.2 Variations in microbial ATP – waterworks, distribution network and tap water

Average microbial ATP concentrations at the two waterworks were higher than in the three different distribution networks (Figure 4-A). Microbial ATP constituted on average 53% of total ATP (Table 1), ranging from 0 to 93% (supplementary information, Figure S1-A). The microbial ATP concentration was <1.2 pg/mL (LOD) in a significant number of samples (N=89) (grey-shaded area in Figure 5). Excluding concentrations of <1.2 pg/mL from the dataset provides a significant change in the geometric mean (3.5 pg/mL) as well as in the average fraction of microbial ATP (70%) (Table 1), indicating that for microbial concentrations above LOD, the microbial fraction is also more significant. The fraction of microbial ATP varied depending on location (Figure 4-B), with a broader range in the distributed drinking water (6-70% for the 25-75%-quartile) than in water from the two waterworks (56-88% for the 25-75%-quartile). A higher microbial ATP concentration and a higher fraction of microbial ATP at the waterworks could be explained by a recent backwash of the filter, cleaning of the water tank or other physical interruptions /processes which can lead to increased bacterial numbers and higher metabolic activity due to more favourable nutrient conditions (H. Henriques and G. Schnipper, unpublished; S.C.B. Christensen, unpublished).

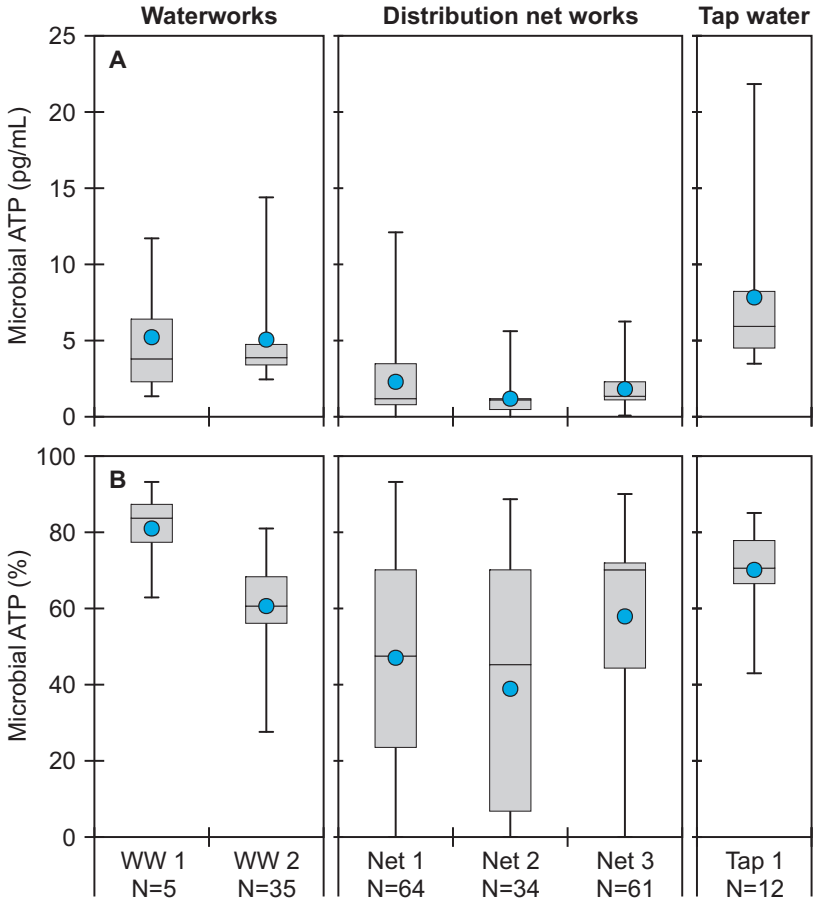

Figure 4: A) Microbial ATP concentrations and B) Percentage of microbial ATP in non-chlorinated drinking water samples (total N=211) from the various locations showing the 25%-quartile, median and 75%-quartile in a Box-Whisker plot. Whiskers denote minimum and maximum concentrations/percentages. Blue circles represent arithmetic mean concentrations/percentages of microbial ATP. Number of samples at each location is specified at the bottom of the diagram.

Drinking water samples collected as a first flush early in the morning (Tap 1) had the highest arithmetic mean of microbial ATP of7.9 ±5.8 pg/mL, ranging from 2.2 to 21.9 pg/mL (Figure 4-A). These relatively high microbial ATP concentrations, compared to waterworks and distributed water, can be explained by overnight aftergrowth in stagnant water, which may reflect both increased cell numbers and a higher metabolic activity (Karl, 1980). These results corroborate the findings of Siebel et al. (2008), observing 2.5-47.7 pg total ATP/mL in drinking water without prior flushing, and by Lautenschlager et al. (2010), who found higher microbial ATP concentrations in tap water after overnight stagnation (6.32 ±4.92 pg/mL) than after 5 minutes of flushing (1.01 ±0.32

pg/mL), where increased cell numbers and increased ATP per cell content in stagnated tap water demonstrated aftergrowth due to overnight stagnation (Lautenschlager et al., 2010).

Microbial ATP concentration in this study in general did not vary greatly despite large differences regarding geography, sample type, i.e. waterworks versus distribution network, or time of year during the sampling period of approximately four years. Seasonal variations in total ATP concentrations were observed in a study on Dutch non-chlorinated drinking water, where concentrations in distributed drinking water were significantly higher in the summer/autumn than in winter (van der Wielen and van der Kooij, 2010). However, total/microbial ATP concentrations were stable throughout the distribution network (van der Wielen and van der Kooij, 2010; Lautenschlager et al, 2013), which is also in agreement with the findings of the present study. A stable and relatively low microbial ATP concentration baseline may indicate biologically stable drinking water (Vital et al., 2012), but more importantly this also promotes microbial ATP as a suitable parameter for monitoring microbial drinking water quality, where significant changes in the quality as well as potential contaminations of drinking water can be detected easily by ATP measurements.

3.2.2 Significance of free ATP in drinking water relative to microbial ATP

Free ATP in drinking water samples from various sites in Denmark – waterworks, distribution networks and in-house installations – had a geometric mean of 1.3 ± 0.1 pg/mL (95%-confidence interval) (Table 1) in the period 2008-2012 (N=211). The concentration of free ATP was below the detection limit of 0.7 pg/mL in 39 samples, i.e. the concentration of microbial ATP was more or less equivalent to the total ATP concentration in 18% of the drinking water samples measured.

In order to improve the LOD for the determination of free ATP in drinking water (as well as total ATP), it is possible to optimise the ATP assay protocol with regard to temperature, sample volume and extraction time, for example, to increase the luminescence signal (LeChevallier et al., 2003; Hammes et. al, 2010). Another option is to use a reagent of higher sensitivity, thus resulting in a lower LOD, since the signal-to-noise ratio is increased (Vang et al., III; Marriott et al., 1992).

The fraction of free ATP in non-chlorinated drinking water ranged from 7 to 100% (supplementary information, Figure S1-B). Despite relatively low concentrations of free ATP in many drinking water samples, the fraction of free ATP was >50% in 74 drinking water samples (35%). In 63 of these samples the microbial ATP concentration was <1 pg/mL. Hence, the fraction of free ATP is significant primarily when the microbial ATP concentration is relatively low, which was especially the case for distributed water in this study (Net 1, Net 2 and Net 3) (supplementary information, Figure S1-B). Of course, these values are expected to be subject to some degree of uncertainty, since they are close to or below the limit of detection.

The fraction of free ATP was higher than expected, and somewhat higher than found by Hammes et al. (2010), where only 16% of the samples had more than 50% free ATP. However, water types other than only drinking water were included in that specific study. Lautenschlager et al. (2013) reported a large fraction of free ATP (42% in average) for relatively low microbial ATP concentrations (1.8±1.0 pg/mL) in distributed water, which are comparable to the findings in our study.

A comparison of ranked microbial ATP concentration with corresponding free ATP concentration (Figure 5) demonstrated no consistent ratio between the two fractions in our drinking water samples, a finding also observed by Hammes et al. (2010). However, concentrations of free ATP were highest in samples which also had a relatively high concentration of microbial ATP. The concentration of free ATP was >5 pg/mL in 13 samples, nine of which were sampled at the WW 2 (Figure 5). This indicates that the treatment of drinking water at the waterworks results to some extent in elevated concentrations of free ATP.

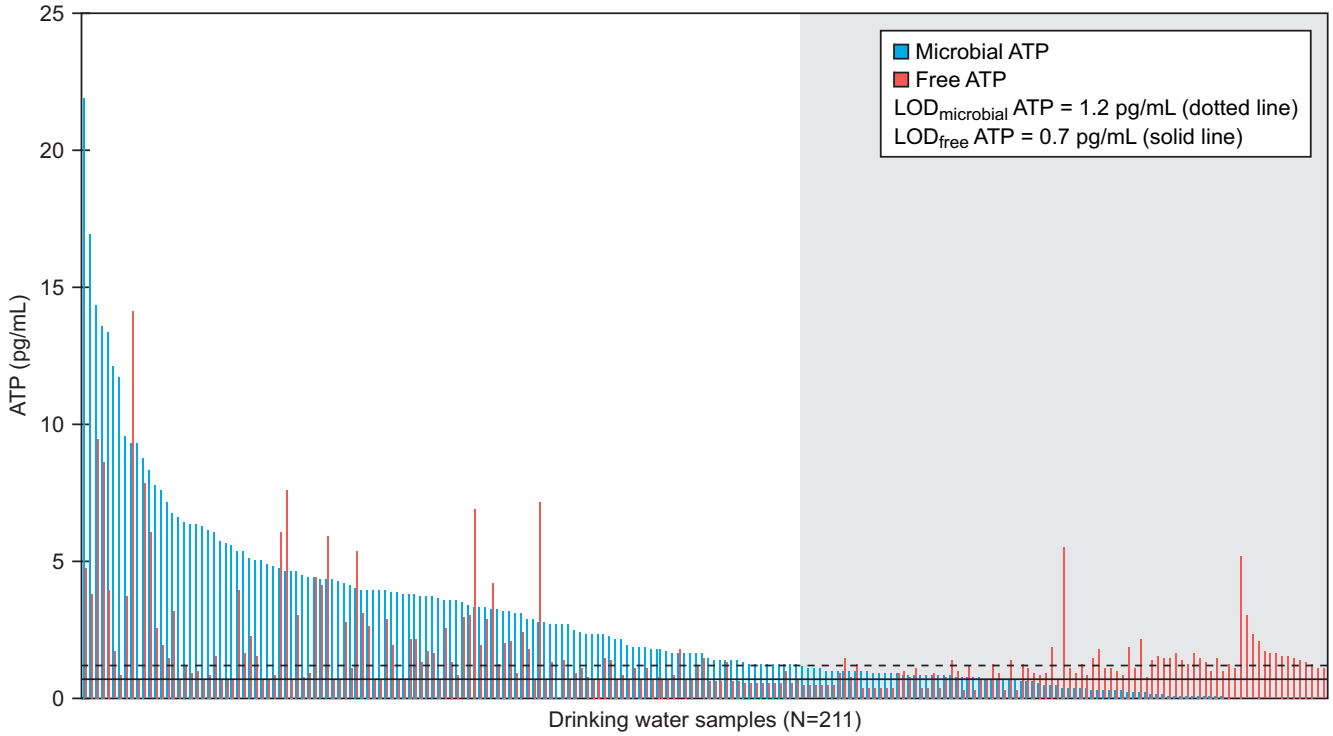

Figure 5: Ranked microbial ATP concentrations (blue bars) and the corresponding free ATP concentrations (red bars) in drinking water samples (N=211). Solid line (horizontal): limit of detection (LOD) for free ATP. Dotted line: theoretical LOD for microbial ATP. Grey-shaded area designates microbial ATP concentrations below the LOD of 1.2 pg/mL (N=89).

The fraction of free ATP in stagnated tap water (Tap 1) was 30% but 51% in distributed water, which again demonstrates that free ATP is less substantial in samples with relatively higher microbial ATP activity. This is in accordance with previous findings, namely a fraction of free ATP in stagnated water of 26% and 51% in distributed water (Lautenschlager et al., 2010). However, another study showed a significantly lower fraction of free ATP, which was always below 5% in tap water sampled as a first flush (Siebel et al., 2008).

Disinfection of drinking water by e.g. chlorination or UV-treatment, or the method applied for determining ATP, e.g. filtration and heat treatment, can increase the fraction of free ATP (van der Kooij, 1992, Hammes et al., 2008; van der Wielen and van der Kooij, 2010; Vital et al., 2013; Liu et al., 2013). However, none of these aspects can explain the significant contribution of free ATP in our study, since Danish drinking water is not chlorinated and the methodology for determining total and free ATP did not include any pre-treatment prior to the ATP measurement. It is highly relevant to distinguish between microbial and total ATP in water samples from treatment plants employing disinfection processes such as ozonation and chlorination (Hammes et

al., 2008; Vital et al., 2012), where free ATP obviously will be present in significant amounts. However, our study demonstrated that free ATP can also be present in significant amounts in drinking water which has not been disinfected at the waterworks or which does not have a chlorine residual in the distributed water.

Free ATP appears to be relatively stable in an oligotrophic environment such as that found in drinking water, and it is not rapidly utilised by exoenzymes or the bacteria present (Azam and Hodson, 1977), which might be because these bacteria have a low activity or might even be in a state of dormancy. Thus, free ATP may persist for a longer time in drinking water than observed for marine environments (Azam and Hodson, 1977). A continuous die-off of cells releasing ATP into the external environment might cause a background level of free ATP in drinking water, which is not constant to the amount of microbial ATP. Microbial ATP and total ATP correlated significantly (R^2=0.87), though free ATP was >1pg/mL in 116 samples (55%), and using total ATP as a parameter for monitoring the relatively low concentration of microbial activity in drinking water can in many cases overestimate the microbial activity of a sample. Furthermore, since the ratio between microbial ATP and free ATP varies, small – but significant – changes in microbial activity may not be detected because of a potential large amount of free ATP, which may conceal changes in microbial ATP when only measuring total ATP. Overall, total ATP might conceal fluctuations in drinking water quality, whereas microbial ATP might be a more dynamic parameter for monitoring the relatively low ATP concentrations found in drinking water.

3.3 ATP fractions in various types of water

Drinking water contains relatively low concentrations of ATP compared to other water types (Riemann et al., 1979; Jørgensen et al., 1992). Total ATP concentrations in drinking water were 1,000 times lower than in surface water and 10,000 times lower than in wastewater (Table 2). Furthermore, the concentration of free ATP was higher in surface water and wastewater than in drinking water. The fraction of free ATP in drinking water constituted on average 47% of total ATP, while the free ATP fraction was less significant in surface water (19%) and wastewater (10%) (Table 2).

Table 2: Concentrations of total and free ATP and the percentage of free ATP in various water types – drinking water, surface water and wastewater.

	Total ATP (pg ATP/ml)	Free ATP (pg ATP/ml)	Percentage of free ATP (%)	N
Drinking water[a)]	3.1 ± 0.3	1.2 ± 0.1	47	211
Surface water	3.5 ± 0.3 x10^3	0.7 ± 0.3 x10^3	19	1
Wastewater	33.6 ± 4.5 x10^3	3.4 ± 0.7 x10^3	10	3

[a)]Geometric mean with 95%-confidence interval

Also, other environmental samples exhibit free ATP in significant amounts, even exceeding microbial ATP (Azam and Hodson, 1977; Björkman and Karl, 2001), while it is considered negligible in other environments (Cowan and Casanueva, 2007). As such, free ATP has a larger impact on total ATP in drinking water than in other environmental samples such as surface water and wastewater. Hence, measuring microbial ATP will firstly provide a more accurate measure on microbial activity in drinking water samples and secondly make it easier to observe small variations in microbial activity, thereby also making it easier to distinguish potential contaminations.

4 Conclusions

Concerning the methodology for quantifying microbial ATP in drinking water, filtration and extraction on the filter resulted in 30-40% lower microbial ATP concentrations compared to microbial ATP determined as the difference between measured total and free ATP concentrations. Filtration with different filter pore sizes and filtration fluxes did not significantly affect the free ATP concentration compared to free ATP in non-filtrated samples at low microbial ATP concentrations (approximately 10 pg/mL), though repeatability was actually worse. Hence, drinking water samples were not filtrated but were measured directly without the addition of an extraction reagent when determining concentrations of free ATP.

From investigations levels and variations in microbial ATP concentrations and the significance of free ATP in non-chlorinated drinking water, it was concluded that:

- Total and microbial ATP concentrations in Danish non-chlorinated, groundwater-based drinking water were in the same order of magnitude as observed in the Netherlands and Switzerland.
- Microbial ATP concentrations in non-chlorinated drinking water samples at waterworks and the distribution network were generally <5 pg/mL in 75% of the samples, whereas the highest average concentration of 7.9 pg/mL was observed for tap water after overnight stagnation, most likely due to aftergrowth.
- The microbial ATP fraction was more significant at the waterworks (25-75%-quartile: 56-88%) compared to distributed drinking water (25-75%-quartile: 6-70%).
- Free ATP was >1 pg/mL in 82% of the drinking water samples, and the free ATP fraction was >50% in 35% of the samples, i.e. present in significant amounts relative to microbial ATP in numerous samples. The presence of free ATP was ubiquitous, with an inconsistent ratio between microbial ATP and free ATP.
- Free ATP constitutes a larger fraction of the total ATP in drinking water (47%) than in other environmental samples such as surface water (19%) and wastewater (10%).
- Measuring microbial ATP provides a more accurate description of microbial activity in drinking water, and can make it easier to detect small changes in drinking water, characterised by fairly low and stable microbial ATP concentrations. This promotes microbial ATP as a parameter for rapid assessment of microbial drinking water quality.

Acknowledgements

This work was supported by the Danish Council for Strategic Research through the project SENSOWAQ – Sensors for Monitoring and Control of Water Quality. Thanks also go to the Urban Water Technology Research School. Thanks to laboratory technicians Mona Refstrup and Sabrina Nedell for their assistance in the laboratory. Torben Dolin is acknowledged for his assistance with the graphics for this article. Andreas Henriques is acknowledged for carrying out a laboratory experiment included in this paper. Thanks are also extended to Professor Karsten Arnbjerg and Associate Professor Henrik Andersen, for valuable discussions and input related to statistics and data treatment, as well as to Hans Dalsgaard Jensen for his contribution. A much appreciated thanks goes to all the water supplies – HOFOR, VandCenter Syd, Nordvand A/S and LyngbyTaarbæk Forsyning – for their involvement in the different research projects and for their assistance during the different sampling campaigns at their waterworks and distribution networks.

References

Azam, F., Hodson, R.E. (1977) Dissolved ATP in the sea and its utilization by marine bacteria. Nature, 267, 696-697.

Berney, M., Vital, M., Hülshoff, I., Weilenmann, H.-U., Egli, T., Hammes, F. (2008) Rapid, cultivation-independent assessment of microbial viability in drinking water. Water Research, 42, 4010-4018.

Björkman, K.M., Karl, D.M. (2001) A novel method for the measurement of dissolved adenosine and guanosine triphosphate in aquatic habitats: applications to marine microbial ecology. Journal of Microbiological Methods, 47, 2, 159-167.

Cowan, D.A., Casanueva, A. (2007) Stability of ATP in Antarctic mineral soils. Polar Biology, 30, 1599-1603.

Cowan, D.A., Russel, N.J., Mamais, A., Sheppard, D.M. (2002) Antarctic dry valley mineral soils contain unexpectedly high levels of microbial biomass. Extremophiles, 6, 431-436.

Delahaye, E., Welté, B., Levi, Y., Leblon, G., Montiel, A. (2003) An ATP-based method for monitoring the microbiological drinking water quality in a distribution network. Water Research, 37, 3689-3696.

Eydal, H.S., Pedersen, K. (2007) Use of an ATP assay to determine viable microbial biomass in Fennoscandian Shield groundwater from depths of 3-1000 m. Journal of Microbiological Methods, 70, 363-373.

Hammes, F., Berney, M., Wang, Y., Vital, M., Köster, O., Egli, T. (2008) Flow-cytometric total bacterial cell counts as a descriptive microbiological parameter for drinking water treatment processes. Water Research, 42, 269-277.

Hammes, F., Goldschmidt, F., Vital, M., Wang, Y., Egli, T. (2010) Measurement and interpretation of microbial adenosine tri-phosphate (ATP) in aquatic environments. Water Research, 44, 3915-3923.

Hobbie, J.E., Daley, R.J., Jasper, S. (1977) Use of Nuclepore filters for counting bacteria by fluorescence microscopy. Applied and Environmental Microbiology , 33, 5, 1225-1228.

Hodson, R.E., Maccubbin, A.E., Pomeroy, L.R. (1981) Dissolved adenosine triphosphate utilization by free-living and attached bacterioplankton. Marine Biology, 64, 43-51.

Jewson, D.H., Dokulil, M. (1982) Adenylate energy charge measurements in freshwater microbial studies. Journal of Ecology, 70, 595-606.

Jones, J.G., Simon, B.M. (1977) Increased sensitivity in the measurement of ATP in freshwater samples with a comment on the adverse effect of membrane filtration. Freshwater Biology, 7, 253-260.

Jørgensen, P.E., Eriksen, T., Jensen, B.K. (1992) Estimation of viable biomass in wastewater and activated sludge by determination of ATP, oxygen utilization rate and FDA hydrolysis. Water Research, 26, 11, 1495-1501.

Karl, D.M. (1980) Cellular nucleotide measurements and applications in microbial ecology. Microbiological Reviews, 44, 4, 739-796.

Karl, D.M., Holm-Hansen, O. (1978) Methodology and measurement of adenylate energy charge ratios in environmental samples, Marine Biology, 48, 185-197.

Lautenschlager, K., Boon, N., Wang, Y., Egli, T., Hammes, F. (2010) Overnight stagnation of drinking water in household taps induces microbial growth and changes in community, Water Research, 44, 4868-4877.

Lautenschlager, K., Hwang, C., Liu, W.-T., Boon, N., Köster, O., Vrouwenvelder, H., Egli, T., Hammes, F. (2013) A microbiology-based multi-parameter approach towards assessing biological stability in drinking water distribution networks. Water Research, 47, 3015-3025.

LeChevallier, M.W., Shaw, N.E., Kaplan, L.A., Bott, T.L. (1993) Development of a rapid assimilable organic carbon method for water. Applied and Environmental Microbiology, 59,5, 1526-1531.

Lee, J., Deininger, R.A. (1999) A rapid method for detecting bacteria in drinking water. Journal of Rapid Method and Automation in Microbiology, 7, 135-145.

Liu, G., van der Mark, E.J., Verberk, J.Q.J.C., van Dijk, J.C. (2013) Flow cytometry total cell counts: A field study assessing microbiological water quality and growth in unchlorinated drinking water distribution systems. BioMed Research International, Vol. 2013, Article ID 595872, p 1-10.

Marriott, N.J., Barnes, S.P., Smith, R.N. (1992) A comparative evaluation of three commercially available luminometers and two ATP reagent kits. International Biodeterioration & Biodegradation, 30, 339-352.

Riemann, B. (1979) The occurrence and ecological importance of dissolved ATP in fresh water, Freshwater Biology, 9, 481-490.

Roser, D.J., Seppelt, R.D., Ashbolt, N. (1993) Microbiology of ornithogenic soils from the Windmill Islands, Budd Coast, Continental Antarctica; microbial biomass distribution. Soil Biology and Biochemistry, Vol. 25, 2, 165-175.

Siebel, E., Wang, Y., Egli, T., Hammes, F. (2008) Correlations between total cell concentration, adenosine tri-phosphate concentration and heterotrophic plate counts during microbiological monitoring of drinking water. Drinking Water Engineering and Science, 1, 1-6.

Stanley, P.E. (1986) Extraction of adenosine triphosphate from microbial and somatic cells. Methods in Enzymology, 133, 14-22.

Stanley, P.E. (1989) A Concise Beginner's Guide to Rapid Microbiology Using Adenosine Triphosphate (ATP) and Luminescence. Eds. P.E Stanley, B.J. McCarthy, R. Smither. Society for Applied Bacteriology. Technical Series 26. Blackwell scientific publications. Oxford, UK.

Tuovila, B.J., LaRock, P.A. (1987) Occurrence and preservation of ATP in Antarctic Rocks and its implications in biomass determinations. Geomicrobiology Journal, Vol 5, 3, 105-117.

van der Kooij, D. (1992) Assimilable Organic Carbon as an indicator of Bacterial Regrowth. American Water Works Association (AWWA) Journal Research and Technology, 57-65.

van der Kooij (2003) Managing regrowth in drinking-water distribution systems. In: Emerging Issues in Water and Infectious Diseases Series. Heterotrophic plate counts and drinking-water safety: The significance of HPC's for water quality and human health, 199-232.

van der Wielen, P.W.J.J., van der Kooij, D. (2010) Effect of water composition, distance and season on the adenosine triphosphate concentration in unchlorinated drinking water in the Netherlands. Water Research 44, 17, 4860-4867.

Vang, Ó.K., Corfitzen, C.B., Smith, C., Albrechtsen, H.-J. (I) Evaluation of ATP measurements to detect microbial ingress in drinking water by waste water and surface water. Submitted.

Vang, Ó.K., Corfitzen, C.B., Smith, C., Albrechtsen, H.-J. (III) Levels of microbial ATP and free ATP in non-chlorinated drinking water - assessing microbial drinking water quality with ATP measurements. Manuscript in preparation.

Vital, M; Dignum, M; Magic-Knezev, A; Ross, P; rietveld, L; Hammes, F (2012) Flow cytometry and adenosine tri-phosphate analysis: Alternative possibilities to evaluate major bacteriological changes in drinking water treatment and distribution systems. Water Research, 46, 4665-4676.

Wang, Y., Hammes, F., Bood, N., Egli, T. (2007) Quantification of the filterability if freshwater bacteria through 0.45, 0.22 and 0.1 μm pore size filters and shape-dependent enrichment of filterable bacterial communities. Environmental Science and Technology, 41, 7080-7086.

Supplementary information

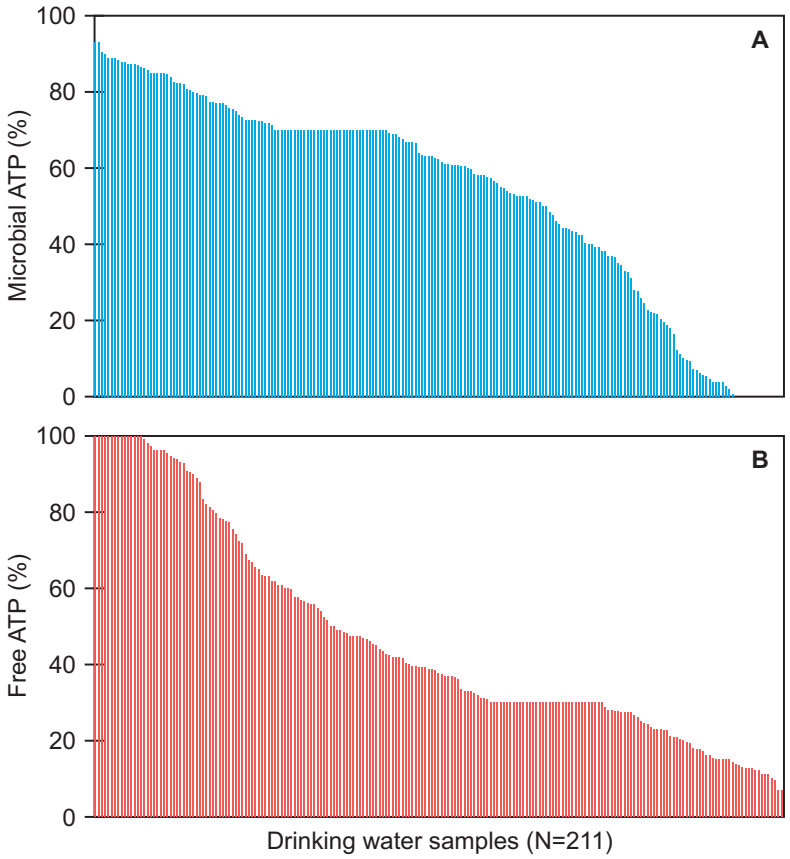

Figure S1: Fraction (%) of microbial ATP (A) and free ATP (B) in non-chlorinated drinking water in decreasing order (N=211).

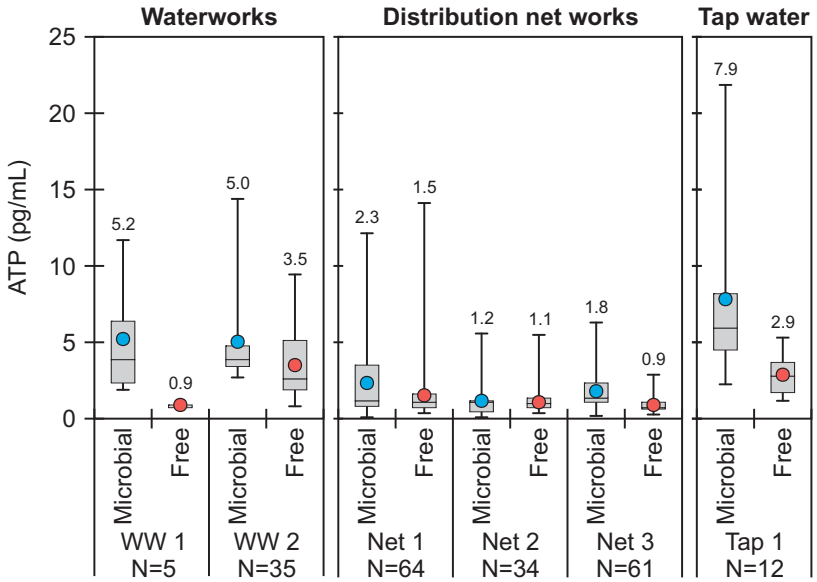

Figure S2: Microbial and free ATP concentrations in non-chlorinated drinking water samples (total N=211) from the various locations showing the 25%-quartile, median and 75%-quartile in a Box-Whisker plot. Whiskers denote minimum and maximum concentrations/percentages. Blue/red circles represent arithmetic mean concentrations of microbial and free ATP. Number of samples at each location is specified at the bottom of the diagram.

III

An evaluation of reagents for the ATP assay for assessing ATP microbial drinking water quality

Vang, Ó.K., Corfitzen, C.B., Smith, C., Albrechtsen H.-J.

Manuscript in preparation

An evaluation of reagents for the ATP assay for assessing microbial drinking water quality

Óluva K. Vang[1,*], Charlotte B. Corfitzen[1], Chrsitian Smith[2], Hans-Jørgen Albrechtsen[1]

[1]Department of Environmental Engineering, Technical University of Denmark, Miljoevej, Building 113, DK-2800 Kgs. Lyngby.

[2]GRUNDFOS Holding A/S, Poul Due Jensens Vej 7, DK-8850 Bjerringbro.

*Corresponding author: okva@env.dtu.dk

Abstract

The ATP assay is used as a parameter for monitoring microbial drinking water quality, where a variety of reagent types are used for measuring ATP in drinking water. Twelve different reagent combinations of enzyme and ATP releasing agents (six commercial reagent kits and six R&D reagents) for the ATP assay were investigated on their inherent properties by measuring ATP standard dilutions and ATP concentrations in non-chlorinated drinking water. The study included reagents of different kinetic properties, i.e. flash reagents and stable light reagents, where parameters of sensitivity, limit of detection, quenching by extractant, extraction efficiency of ATP, enzyme stability, repeatability were investigated. For the commercial reagents for the ATP assay the limit of detection (LOD) was lower for the flash reagents (<1 pg/mL) than for the stable light reagents (CLS), where one the CLS reagents (RoCLS) was considered not applicable with a LOD of 9.2 pg/mL for direct measurement of the relatively low concentrations of ATP usually found in drinking water. For the R&D reagents (Promicol), the Pro1 reagent did not exhibit the required sensitivity for direct drinking water analysis; also the ATP releasing agent Ex4 was not efficient at extracting cellular ATP within the 10 seconds protocol given here. In an overall comparison of the reagents based on results on the investigated parameters, it was not possible to identify an optimum reagent for measuring ATP in drinking

water, since many of the reagents achieve a very similar total rating, due to the fact that they perform well on some parameters and less good on other parameters. However, extraction efficiency is considered one of the most important parameters for a reliable and accurate ATP measurement in drinking water.

Key words: adenosine triphosphate, luciferase assay, reagents, bacteria, drinking water

1 Introduction

The bioluminescent system of the Luc assay has over recent years been used in several investigations for determination of ATP as a parameter for microbial drinking water quality (Delahaye, 2003; Frundzhyan and Ugarova, 2007; Hammes et al. 2010; van der Wielen & van der Kooij, 2010; Vital et al., 2012). The ATP assay is sensitive (<1 pg/mL) and availability of results is considerably faster (minutes) than culture based standard methods e.g. heterotrophic plate counts (HPC) (days). Also, availability of the many different commercial reagents and automatic luminometers for the ATP assay has contributed to the fairly uncomplicated assay design and protocol.

Essentially, three main aspects influence the ATP assay and its applicability to measure microbial activity in environmental samples, i.e. sensitivity of the enzyme/substrate reagent, which depends and enzyme kinetics (flash versus stable light reagents) and their purity, the efficiency to extract ATP and the influence of the extraction method/reagent on the enzyme reagent (i.e. inhibition and quenching) (Karl, 1980; Stanley, 1989; Lundin, 2000).

The first step of the ATP bioluminescence assay is to extract ATP from the cells by destroying the cell membrane, and consequently making cellular ATP available for the reaction with the substrate-enzyme reagent. In addition to extracting cellular ATP, the extraction reagent often also has the role to inactivate any potential endogenous ATP hydrolysing enzymes. Also, the extraction reagent should not inhibit the bioluminescence reaction of luciferin-luciferase with ATP. The extraction step is crucial and has in several ways a direct impact on the reliability and performance of the ATP assay (Stanley, 1986).

Current standard methods for the ATP assay are based on extraction of ATP in boiling Tris buffer (Eaton et al., 2005; ASTM D4012-81, 2009). Also trichloroacetic acid (TCA) is recommended as a reference method for other ATP releasing agents (Lundin, 2000). When investigating ATP in drinking water samples, the ATP is most often extracted by using commercial ATP releasing agents (cold extractants) (e.g. Delahaye, 2003; Eydal and Pedersen, 2007; Hammes et al., 2010; van der Wielen and van der Kooij, 2010, Vang et al., II), i.e. the existing standard methods for extraction of ATP are generally not used. Commercial reagents have for many years been used for extracting ATP, because of their ease of use and efficiency for sample processing e.g. high output screening (HTS) products. The use of cationic reagents, which usually are

quaternary ammonium salts, for the extraction of bacterial ATP has become a routine procedure already in the late 80'ties (Schram and Witzenburg, 1989). Their advantage compared with the use of perchloric (PCA) and trichloroacetic (TCA) acids or boiling buffer is that dilution of samples can be reduced to a minimum (Schram and Witzenburg, 1989).

Some manufacturers state the efficiency of the commercial extractant compared to the mentioned standard/reference methods. This comparison of extraction efficiency is usually done for pure cultures - presumably in high concentrations. Commercial ATP releasing agents are relatively easy to use compared to the more complicated standard protocol for extraction of ATP. Consequently, the different methodologies, various enzyme reagents and ATP releasing agents to measure and quantify ATP in drinking water samples might induce differences in ATP concentrations between studies.

Marriott et al. (1992) did a comparative study of commercial luminometers as well as two ATP reagents kits. However, the effect of applying various enzyme reagents and ATP releasing agents for measuring and quantifying ATP in drinking water has to our knowledge not been assessed recently. It is important to investigate potential discrepancies, and to which extent results achieved with the ATP assay can be compared across studies whit different methodologies and different reagents.

This paper addresses some of the fundamental as well as practical aspects of the ATP assay with special focus on its application for monitoring microbial drinking water quality. A number of different commercial reagents used for quantifying ATP in drinking water for the past years were identified and investigated to clarify the significance of applying different reagents for determining ATP in drinking water, i.e. if ATP concentrations measured with different reagents are comparable. More specifically, here we report the results of different reagents for the ATP assay with identification and quantification a number of inherent properties such as sensitivity, limit of detection, repeatability, stability as well as their applicability for determining ATP in drinking water samples with regard to extraction efficiency and protocol design.

2 Materials and methods

2.1 ATP reagents

ATP reagents included in this study were identified and selected based on a literature review on commercial ATP reagents recently used for drinking water purposes (Table 1). Besides the six commercial reagents investigated, three research and development (R&D) enzyme reagents (denoted Pro1, Pro2 and Pro3) of various sensitivity and two different ATP releasing agents (denoted as EX4 and EX5) were provided by Promicol (The Netherlands). All six combinations of enzyme reagent and ATP releasing agents were investigated (Table S1, supplementary information).

The list of reagents in Table 1 is not intended to be exhaustive. Reagents with both flash (denoted HS, i.e. high sensitivity) and stable (denoted CLS, i.e. constant light signal) light kinetics were included in the study for comparison. Some reagents used for aquatic ATP measurements (e.g. Filtravette/Profile-1 by New Horizons Diagnostics, Quench-Gone by LuminUltra, CheckLite HS Set by Kikkoman) were not included because of no immediate provider, incompatibility with luminometer used in the present study, assay design/recommended protocol by manufacturer etc.

Table 1: Investigated reagents for the ATP assay – six commercial reagents and three R&D reagents. CLS: stable light reagent, HS: flash light reagent.

Abbrev. name	Reagent kit	Cat. No.	Reagent type	Manufacturer	Assay price[a)] (EUR/assay)	Reference
CeCLS	LuminATE	92687	CLS[b)]	Celsis	1.61	van der Wielen and van der Kooij (2010); DTU Environment
CeHS	RapiScreen Health	1230839	HS[c)]	Celsis	3.49	DTU Environment
RoCLS	ATP Bioluminescence Assay Kit CLS II[d)]	1699695	CLS	Roche	0.93	DTU Environment
RoHS	ATP Bioluminescence Assay Kit HS II	1699709	HS	Roche	0.91	DTU Environment
BioHS	ATP Biomass Kit HS	266-311	HS	BioThema	2.21	Eydal and Pedersen (2007)
PromCLS	BacTiter-Glo™ Microbial Cell Viability Assay[e)]	G8231	CLS[f)]	Promega	0.40	Hammes et al. (2010)
Pro1	Promicol Enzyme 1	-	-	Promicol	-[g)]	Bukh et al. (2012)
Pro2	Promicol Enzyme 2	-	-	Promicol	-[g)]	
Pro3	Promicol Enzyme 3	-	-	Promicol	-[g)]	

[a)]Prices by March 2011 - except for Roche CLS II (May 2013).
[b)]Constant light signal, i.e. stable light reagent.
[c)]High sensitivity, i.e. flash light reagent.
[d)]No ATP releasing agent included in the kit.
[e)]Combined enzyme and ATP releasing agent.
[f)]This reagent is considered to be a CLS reagent (i.e. a decay rate of <1%/min) based of the definition on stable light reagents given by Lundin (2000) and information on luminescence given in the protocol for the reagent (Promega, 2011).
[g)]R&D reagents are not priced.

The ATP releasing agent included in the RoHS kit was also used together with the RoCLS kit, since no releasing reagent was included in this kit. Special for the PromCLS reagent kit was that the enzyme and ATP releasing reagent are one reagent mix and not separate reagents as the other reagent kits investigated. Hence, a filtration (0.1 µm, PVDF membrane, Millipore) step is required in order to measure free ATP. The reagents were investigated on two different days, and to compare the results obtained with all reagents, the CeCLS reagent was measured on both days as a reference.

2.2 ATP assay protocols

Total ATP and free ATP were measured in all samples. Total ATP was measured by adding an ATP releasing reagent (cell lysis) and then luciferin/luciferase reagent. Free ATP was measured without cell lysis by adding only luciferin/luciferase reagent to the sample. The microbial ATP was calculated as the difference between the total ATP and the free ATP (microbial ATP = total ATP - free ATP).

For all reagents the manufacturer protocol was followed regarding volume of sample, extraction reagent and enzyme reagent (Table S1, supplementary information). For all reagents - besides the Promega reagent - the time for extraction of ATP from cells was set to 10 seconds and a reaction time of 2 seconds with the enzyme reagent. For the Promega reagent a reaction time of 1 minute is required for ATP standard dilutions and an extraction/reaction time of 5 minutes for samples. Light signal in relative light units (RLUs) was measured over a 10 seconds integral period for all reagents.

Samples were measured with an Advance Coupe luminometer (Celsis, the Netherlands). Protocols were programmed in the software (Celsis Advance.im, ver. 3) for the luminometer. In order to simplify the measurement protocol the same reaction time and integral period was applied for all reagents.

Water samples and ATP standard dilutions were added manually to a cuvette; while the ATP releasing agents and enzyme reagents were added automatically with the dispensing system on the luminometer. The protocol for PromCLS was performed manually, since it was not possible to program in the software. Consequently, both sample and the combined enzyme and ATP releasing agent were added manually to the cuvettes. Polypropylene cuvettes (Celsis 1280139) were used for all measurements. All enzyme reagents, ATP releasing reagents, buffers and standards were brought to room temperature before reconstituted and

used for assay measurements. Unless otherwise stated, drinking water samples were measured in duplicates and ATP standard dilutions for calibration curves were measured in single replicates.

The investigation was done on two different days. On the first day of the experiment following reagents were investigated: CeCLS, CeHS, RoCLS, RoHS, Pro1/Ex4, Pro1/Ex5, Pro2/Ex4, Pro2/Ex5, Pro3/Ex4 and Pro3/Ex5. On the second day the reagents CeCLS, PromCLS and BioHS were investigated. ATP standard dilutions and samples were measured with the CeCLS reagent on both days to compare results achieved with all reagents.

2.3 ATP standard dilutions

Light emission (RLUs) was converted into ATP concentrations from a calibration curve of ATP standard dilutions measured on the same day as the experiment. Two different calibration curves were made - one for total ATP and one for free ATP - for all reagent kits. ATP standard dilutions were prepared with ATP standard salt (92638, Celsis) which was reconstituted in Lumin(PM) buffer (92678, Celsis) to a concentration of $1x10^6$ pg ATP/mL, and dilution series in the range 0-1,000 pg ATP/mL were made in autoclaved sterile filtrated (0.20 µm cellulose acetate membrane, Sartorius Minisart, 16534-K) tap water as base for an ATP-standard curve. The same batch of prepared ATP standard dilutions was measured with all investigated reagents in order to minimize variation when comparing the reagents.

2.4 Drinking water samples

Six drinking water samples from in-house installations in the same area, i.e. tap water, and one sample from a local water works (Lyngby Waterworks) were measured with all reagent kits. All tap water samples were sampled as a first flush of approx. 250 mL. A drinking water sample (tap water) with the addition of yeast extract (2.0 mg yeast/mL, incubated 2 days at 20°C) was measured with all reagent kits in order to have a drinking water sample with a high ATP concentration. Samples were collected on the day of the experiment. Samples were kept cool at 10°C throughout the day of the experiment in order to avoid significant changes in ATP concentration.

2.5 General working procedures

All glassware used for collecting water and for the experiments was acid washed and heated to 540°C for 6 h before use. All laboratory equipment and pipetting tips used were ATP-free. All samples were handled under aseptic conditions. All experiments were performed at room temperature.

2.6 Evaluated parameters

The performance of each reagent for the ATP assay was evaluated on a number of their inherent properties such as sensitivity, limit of detection, extraction efficiency etc. A brief overview on which parameters were investigated and how they were evaluated is given in Table 2. These are explained in detail in the results section.

Table 2: Overview of investigated parameters of reagents for the ATP assay.

Parameter	**How?**	**Quantification**
Sensitivity	Calibration curve	Slope
Linearity	Calibration curve	Coefficient of determination (R^2)
Quenching of extractant	Calibration curves measured with and without extractant	Difference between slopes (%)
Limit of detection	Calibration curve	Standard error of the estimate
Repeatability	Replicate measurements (N=5) of two ATP standards and two drinking water samples	Coefficient of variation – CV (%)
Extraction efficiency	Comparison of drinking water samples measured with reagents for the ATP assay	Concentration (pg/mL)
Stability of enzyme reagent	Difference between measured ATP standards in beginning and end of experiment	Relative light units
Protocol **Price**	Ease of use Price of reagent kit	- EUR/assay measurement

3 Results

3.1 Sensitivity and linearity

Each reagent kit combination was evaluated on their sensitivity (response) and linearity when measuring ATP standard dilutions with and without ATP releasing agents, except for the Promega reagent, where the ATP releasing agent and enzyme reagent is combined in one reagent. Sensitivity was assessed based on slope and y-axis intercept of the calibration curve (Miller & Miller, 1993). The steeper the slope or the stronger the response to a concentration change the more sensitive the reagent for the ATP assay. Also, the lower the blank measurement (response from instrument and reagents, i.e. no sample matrix or analyte present) the more sensitive is the assay.

The various reagents investigated in this study demonstrated different sensitivities (Figure 1). Measuring ATP standard dilutions with the addition of enzyme reagent resulted in the following order of sensitivity arranged from highest to the lowest sensitivity - based on the slope of the calibration curves (Figure 1; Table S2 supplementary information):

CeHS > RoHS > Pro3 > PromCLS > Pro2 > BioHS > Pro1 > RoCLS > CeCLS

As expected, two of the flash reagents (CeHS and RoHS) had the highest sensitivities, while the two stable light reagents - RoCLS and CeCLS - had the lowest sensitivities. It is important to highlight that the sensitivity of the PromCLS reagent is a combined reagent with both enzyme reagent and ATP releasing agent, i.e. any quenching from the ATP releasing agent is included when evaluating the sensitivity of this specific reagent. Somewhat surprising was that PromCLS, which we classified as stable light reagent according to Guardigli et al. (2011), had a significantly higher sensitivity than the other two commercial CLS reagents, and also had a higher sensitivity than the flash reagent BioHS. The BioHS reagent actually had a considerably lower sensitivity than the other two commercial flash reagents (Figure 1; Table S2, supplementary information).

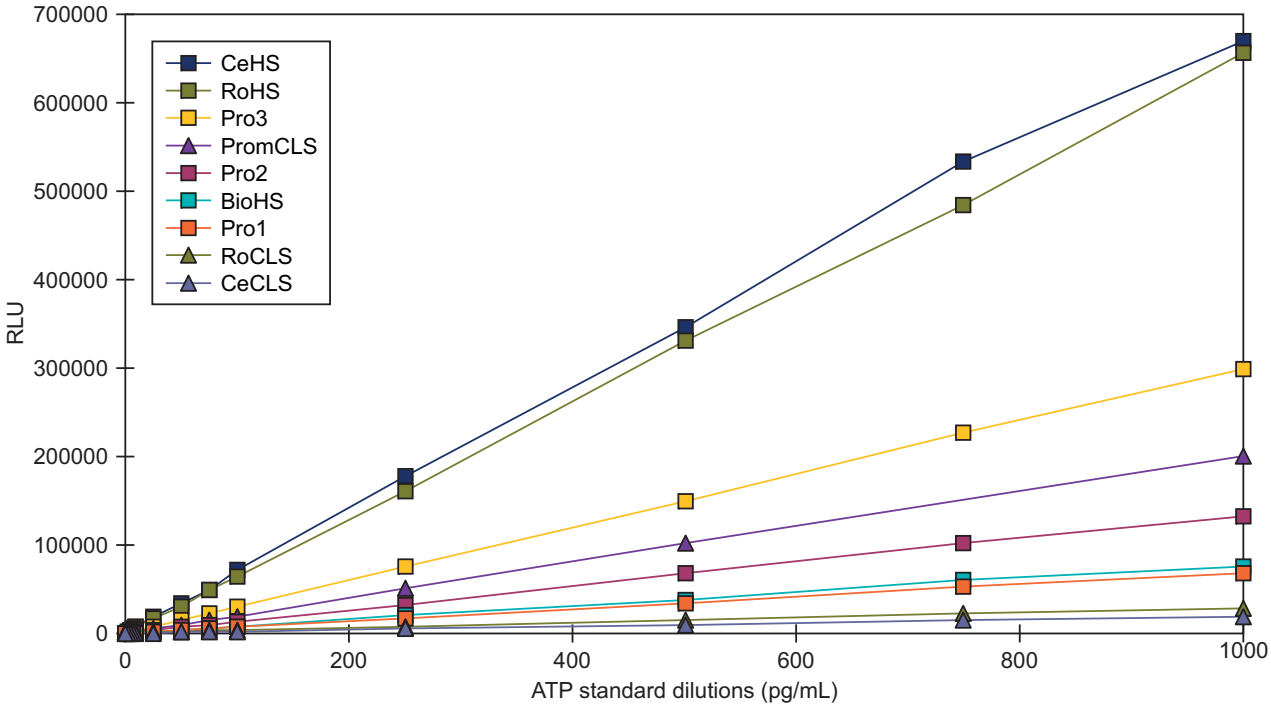

Figure 1: ATP calibration curves of ATP standard dilution in the range of 0-1000 pg ATP/ml) for nine investigated enzyme reagents (six commercial and three R&D enzyme reagents).

The advantage of flash reagents is their high sensitivity and thus can detect very low amounts of ATP (<0.5 cells, i.e. <1 amol) (Lundin, 2000). Since the decay rate of the light emission for the flash reagent is fast (235% min^{-1}) it is necessary with an automated protocol to achieve the same reaction rates between sample and reagents and also to have the same integration time and thus keeping measurement variability to a minimum. The inherent properties of the constant light reagent (decay rate of light emission of 0.5% min^{-1}) allow a longer reaction time with sample and a longer integration period of the light emission, and are therefore well suited for investigations of e.g. enzyme kinetics. The trade-off for a constant light emission is a lower sensitivity (>500 bacterial cell, i.e. >1000 amol). The application of flash reagents is obviously essential for sterility testing, such as pharmaceuticals and personal care products.

Considering that drinking water contains approximately 10^5 cells/mL (Vital et al., 2012), and based on the definitions on reagent types given by Lundin (2000), a constant light reagent should be sufficient for analysing ATP in drinking water samples, though both flash and stable light reagents can be applied.

Linearity of calibration curves was examined in terms of coefficient of determination (R^2) for three different concentration intervals of 0-10 pg/mL, 0-100 pg/mL and 0-1000 pg/mL, where R^2 is a measure for how well the measured values fit the modelled straight line and equation:

$$R^2 = \frac{ss_{reg}}{ss_{total}}$$

ss_{reg} is the regression sum of squares and ss_{total} is the total sum of squares (i.e. $ss_{reg} + ss_{res}$). This means the smaller the residual sum of squares (ss_{res}) the larger the coefficient of determination. Linearity of the calibration curves was also established by plotting the curve and inspecting them visually. The criteria for linearity was set to a variation of <5% for between slopes of the three different concentrations ranges. All linear regression statistics were computed by Excel.

Working range for ATP concentrations in drinking water is reported to be of <1 to 10 pg ATP/mL, with a few values above 10 pg/mL (van der Kooij, 1992; Velten et al., 2007; Berney et al., 2008; Hammes et al., 2008; Siebel et al., 2008; van der Wielen & van der Kooij, 2010; Hammes et al., 2010; Vital et al., 2012). Therefore, the low range of the calibration curves (Figure 1) is relevant when converting light emission into an ATP concentration. Any curving of the calibration curve of measured ATP standard dilutions will result in an erroneous ATP concentration, i.e. linearity of the calibration curve is essential. When examining linearity by the slope of the calibration curves covering several orders of magnitude (0-10 pg/mL, 0-100 pg/mL and 0-1000 pg/mL) the two flash reagents, CeHS and RoHS, had a variation >5% (though <10%), while RoCLS had a variation in slope significantly higher than 5%, i.e. ATP standard dilutions measured with the RoCLS did not yield linearity over the three concentration ranges (Table S1, supplementary information). Linearity was achieved for all other calibration curves examined by the coefficient of determination (R^2), visual inspection of calibration curves and investigating the variation in slope with a criteria of <5%).

The response of the blank measurement, i.e. RLU signal from the enzyme reagents alone, were consistently low for nearly all reagents, i.e. meaning that the enzyme reagents alone did not add significantly to the signal noise from the instrument (approx. 40-50 RLU) (Table S1, supplementary information). One of the commercial enzyme reagents – RoCLS – had a significantly higher blank response than the other reagents. The blank measurement of the enzyme reagent

gives an indication of the sensitivity, and thus this specific enzyme has a loss of sensitivity compared to the other enzyme reagents.

Results with investigated reagents are arranged according to sensitivity (measured with only enzyme reagent) in tables and figures in following sections.

3.2 Quenching by the ATP releasing agent

The reagents used for extracting cellular ATP interfered with the assay by quenching/inhibition of the luciferin-luciferase reagent. This has a direct influence on the sensitivity of the assay. The sensitivity of the ATP assay resulted in the following order when ATP standard dilutions where measured with both enzyme reagent and ATP releasing agent (Figure S1 and Table S3, supplementary information):

CeHS > RoHS > Pro3/Ex4 > PromCLS > Pro2/Ex4 > Pro3/Ex5 > BioHS > Pro2/Ex5 > Pro1/Ex4 > Pro1/Ex5 > CeCLS > RoCLS

Reduction in light signal due to quenching by the ATP releasing agents was calculated as the difference in slope of ATP calibration curves measured with and without ATP releasing agent for the three different concentration intervals (0-10, 0-100 and 0-1000 pg/mL) and given as a percentage. Since the Promega kit has enzyme and extraction reagent, it was not included in this part of the investigation.

The ATP releasing agents reduced (quenched) the light emission to different extent and demonstrated that the ATP releasing agent can have a significant impact on the sensitivity of the assay (Table 3).

Table 3: Quenching (%) of extraction reagent on RLU signal - calculated from the differences in slopes of ATP calibration curves measured with and without ATP releasing agent for three different concentration ranges.

Reagent	Quenching of ATP releasing agent on signal (%)			
	Concentration range (pg/mL)			Average
	0-10	0-100	0-1000	
CeHS	-	9	9	9
RoHS	64	65	65	65
Pro3/Ex5	67	67	67	67
Pro3/Ex4	19	26	24	23
PromCLS	-	-	-	-
Pro2/Ex4	27	30	29	29
Pro2/Ex5	59	62	61	61
BioHS	2	-1	3	2
Pro1/Ex5	26	67	63	52
Pro1/Ex4	32	30	27	30
RoCLS	83	76	73	77
CeCLS	21	27	24	24

The ATP releasing agent for the BioHS reagent gave the lowest quenching of the light emission (2%), while the ATP releasing agent used together with RoHS and RoCLS together with Promicols ATP releasing agent - Ex5 - gave a significant quenching of the light signal (>60%) (Table 3). Quenching of the light emission by the ATP releasing agent is not an issue, as long as the sensitivity of the enzyme reagent remains high enough for the specific application and linearity of the calibration curve is maintained. Sensitivity for the Pro1 R&D enzyme reagent and the commercial RoCLS reagent was compromised when measuring ATP standard dilutions with both enzyme and ATP releasing, since linearity in the low range of the calibration curve was not maintained (Table S2, supplementary information). Thus, these reagents do not have the required sensitivity for measuring the relatively low ATP concentrations in drinking water. This is also reflected in the limit of detection for these reagents in the section 3.3.

3.3 Limit of detection of reagents

The limit of detection (LOD) is the lowest concentration of the analyte which can be detected with the specific instrument, the specific reagents applied and the given sample matrix.

The limit of detection (LOD) for total ATP and free ATP was determined by the linear regression analysis of the calibration curve for ATP standard dilutions, which were measured with both enzyme reagent and ATP releasing reagent (total ATP) and enzyme reagent alone (free ATP) (Miller and Miller, 1993):

$$y_{LOD} = a + 3s_{y/x}$$

The calibration curve is defined as y=bx+a, thus a is the y-axis intercept and $s_{y/x}$ is the standard error of the estimate of y on x, which is calculated from the residual sum of squares (ss_{res}), as the difference between each measured y-value (y_i) and that calculated from the calibration curve ($\hat{y}_i$):

$$s_{y/x} = \sqrt{\frac{\sum(y_i - \hat{y}_i)^2}{n-2}}$$

In linear regression calculations the degrees of freedom is (n-2). The limit of detection was calculated for the section of the calibration curve close to the origin (i.e. 0-10 pg/mL). Linear regression statistics were computed in Excel. The limit of detection for microbial ATP was calculated as (Jensen, 2013):

$$LOD_{microbial\ ATP} = \sqrt{(LOD_{total\ ATP})^2 + (LOD_{free\ ATP})^2}$$

The LOD of all three commercial flash (HS) reagents was in the range of 0.2 to 0.6 pg/mL for total ATP. Based on several studies on ATP measurements in Danish drinking water, we evaluate a LOD of 1 pg/mL or less to be satisfactory for quantifying total ATP in drinking water (Vang et al., II). The two commercial stable light reagents - PromCLS and CeCLS - with LOD of 1.8 and 2.2 pg/mL, are not considered adequate, since total ATP often is less than 1.5 pg/mL in drinking water (Table 4) (Vang et al., II).

Table 4: Limit of detection (LOD) for total ATP and free ATP calculated from the linear regression statistics for the ATP calibration curves in the range of 0-10 pg/mL; and the theoretical LOD for microbial ATP.

	Total ATP			Free ATP			Microbial ATP
	Slope (RLU)	$s_{y/x}$ (RLU)	LOD (pg/mL)	Slope (RLU)	$s_{y/x}$ (RLU)	LOD (pg/mL)	LOD (pg/mL)
CeHS[a)]	531	43	0.3	698	105	0.5	0.6
RoHS	238	16	0.2	662	104	0.3	0.4
Pro3/Ex5	103	37	1.3	307	45	0.5	1.4
Pro3/Ex4	224	24	0.3	307	45	0.5	0.6
PromCLS	205	113	1.8	-	-	-	1.8
Pro2/Ex4	101	17	0.6	136	12.1	0.3	0.7
Pro2/Ex5	55	21	1.3	136	12.1	0.3	1.4
BioHS	77	17	0.6	79	38.2	1.4	1.5
Pro1/Ex5	52	111	5.2	70	11	0.6	5.2
Pro1/Ex4	43	57	3.0	70	11	0.6	3.0
RoCLS	6	19	8.9	37	21	1.1	9.0
CeCLS	15	11	2.2	19	1.9	0.3	2.3

[a)]Measurements from another experiment with this specific reagent were used for calculating LOD, since measurements in this specific experiment were erroneous when measuring ATP standard dilutions with both enzyme and ATP releasing reagent.

The protocol for the PromCLS reagent can be optimised with regard to volume, reaction time and temperature in order to get the highest signal output and thereby achieving a lower LOD (0.0001 nM ATP, i.e. approx. 0.05 pg/mL) (Hammes et al., 2010). Many studies with the CeCLS reagent in our own group have demonstrated that LOD for this reagent varies from experiment to experiment - in average approx. 1 pg/mL for total ATP. This variation in LOD can be caused by surroundings which are not thermostatically controlled, and it is well known that the substrate-enzyme reagent for the ATP analysis is very temperature sensitive with an optimum at approximately 18-23°C for e.g. CeCLS and Pro1, 2 and 3 reagents (Guardigli et al., 2011). Higher or lower temperature will influence reaction rates and thereby also influence the sensitivity and LOD of the assay. The RoCLS reagent together with the specific ATP releasing agent, which had an LOD of 8.9 pg/mL, was not considered applicable for direct ATP analysis of drinking water samples. In order to use a reagent with a high LOD, the sample needs to been concentrated to above LOD of the specific reagent.

The R&D reagents – Pro2 and Pro3 – had similar LOD's, despite Pro3 was more sensitive than Pro2. LOD might be expected to decrease with increasing sensitivity, though this was not always the case. The residual ($s_{y/x}$) for the Pro3 calibration curves was relatively high compared to Pro2 calibration curve, which has a direct impact on the calculated value of LOD. The LOD for Pro3 and Pro2 were evaluated to be adequate for drinking water analysis, while Pro1 was not considered satisfactory, since its LOD was 3.0 and 5.2 pg/mL when combined with Ex4 and Ex5, respectively. Common for all three R&D enzyme reagents was, that they had considerably lower LOD when combined with the ATP releasing reagent Ex4 compared to Ex5, due to a larger quenching effect of the light emission by the Ex5 ATP releasing agent than Ex4 (Table 3).

LOD was somewhat lower for free ATP with nearly all reagents, since the sensitivity of the assay was higher when ATP standard dilutions were measured with only enzyme reagent. The BioHS and RoCLS reagents had an LOD above 1 pg/mL, which again relates to the relatively high value of $s_{y/x}$. The other reagents had an LOD <1 pg/mL. Free ATP concentrations can vary significantly (7-100% of total ATP) in drinking water (Vang et al., II; Hammes et al., 2010; Vital et al., 2012). However, the concentration of free ATP is usually lower than for total ATP, i.e. a lower LOD is also required for the quantification of free ATP.

3.5 Extraction of ATP

Extraction of cellular ATP is essential for the ATP assay. The standard boiling method with buffers has to our knowledge not been used for extracting ATP in any recent studies on drinking water. Instead commercial ATP releasing agents – so called cold extraction reagents – are used for extraction of cellular ATP (e.g. Vang et al., I; Hammes et al., 2010; Liu et al., 2013). The extraction reagents are often included in the kit when purchasing the substrate-enzyme reagent and are presumably optimised to go together with the specific substrate-enzyme reagent in terms of optimum pH and temperature range as well as stabilising chemicals.

The spiked water sample had a significantly higher ATP concentration than the drinking water samples, and was well above the LOD of RoCLS, Pro1/Ex4 and Pro1/Ex5 reagents. In fact, one of the highest concentrations measured was with RoCLS (1068 pg/mL) and was equivalent to that of the RoHS reagent (1080 pg/mL) (Figure 2). The highest concentration was achieved with the CeHS reagent (1093 pg/mL). All three R&D enzyme reagents combined with the Ex4 extraction reagent had significantly lower ATP concentrations that for the other investigated reagents (Figure 2). Overall, there were large variations in measured

total ATP concentrations with the different reagents, demonstrating that some of the reagents were performing better, i.e. were better at extracting ATP, than others with the specific protocol design.

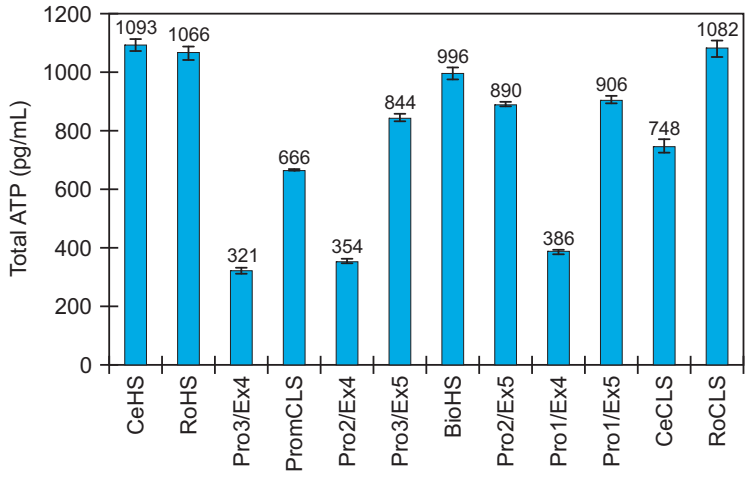

Figure 2: Total ATP concentrations in a drinking water sample spiked with yeast, of which ATP was assayed with six commercial reagents and six R&D reagents.

All reagents were used for direct measurement of total and free ATP in drinking water samples to evaluate potential differences in lysis efficiency and variation in ATP concentrations measured with different reagents. As demonstrated previously the RoCLS, Pro1/Ex4 and Pro1/Ex5 were not sensitive enough for the specific assay design used in this study, i.e. direct measurements of small sample volumes with relatively low ATP concentrations. Also, the calibration curve in the low to medium range for the CeHS reagent was erroneous due to instrument instability, i.e. it was not possible to determine the total ATP concentration in the drinking water samples.

As for the spiked waster sample, the total ATP concentrations in the six drinking water samples were all lower when extracted with Ex4 compared to e.g. Ex5 (Figure 3). The Pro3/Ex5 and PromCLS also measured slightly lower total ATP concentrations compared to CeCLS (which was used as a reference, since experiments were done on different days). The RoHS, BioHS and Pro2/Ex5 were higher for samples with concentrations >10 pg/mL and similar concentrations to CeCLS for samples with approximately <10 pg/mL. Hence, there was a tendency to higher concentrations, i.e. higher extraction efficiency with extraction reagents included in kits with reagents of high sensitivity (flash reagents).

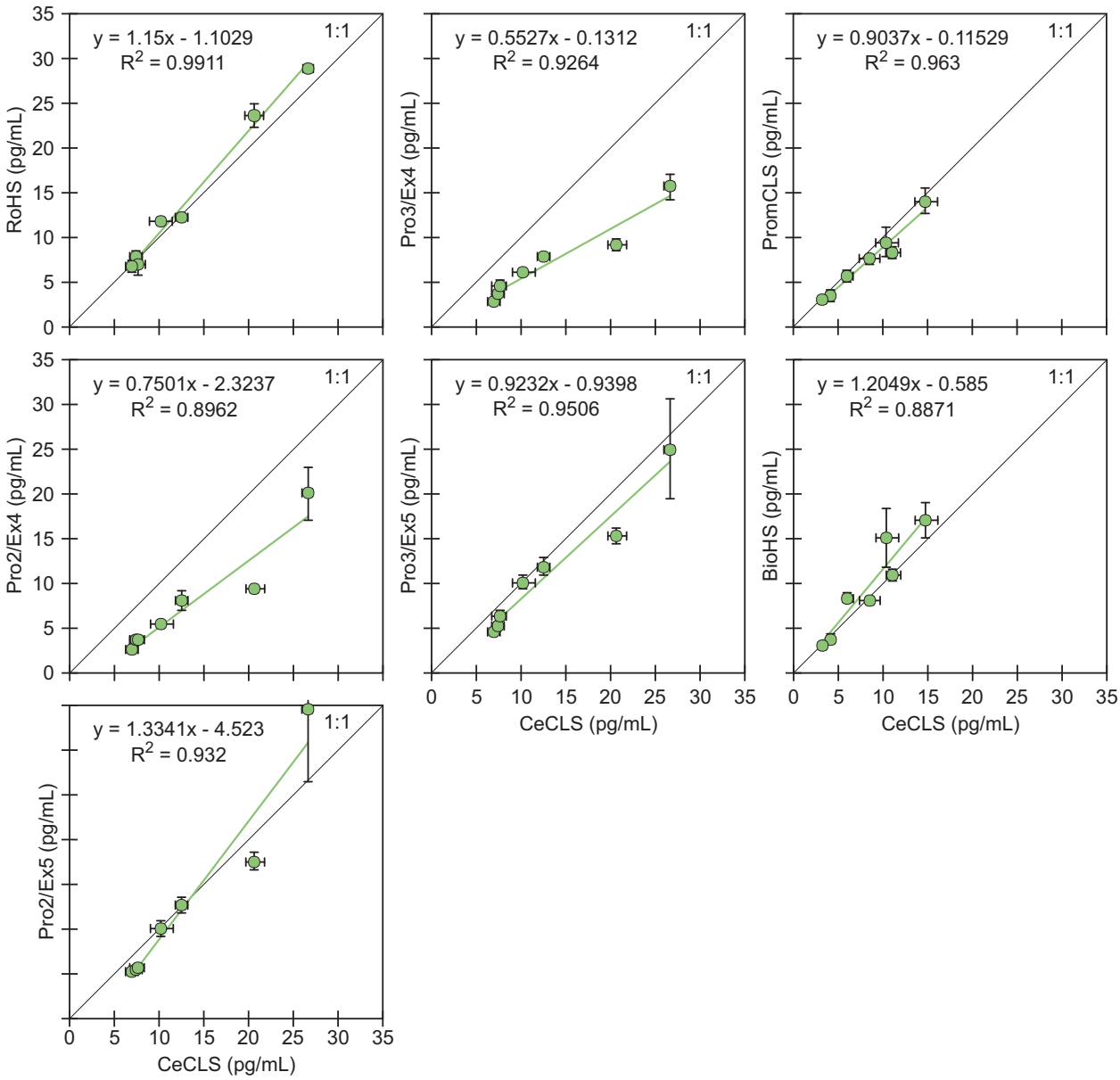

Figure 3: Total ATP concentrations in drinking water samples measured with different enzyme and ATP releasing reagents versus the CeCLS reagent.

However, the measured free ATP concentrations in drinking water samples were generally lower for nearly all reagents compared to the CeCLS reagent, though often with large measurement variation (Figure S2, supplementary information), i.e. a larger uncertainty was associated with the free ATP concentrations, most likely since concentrations were low and close to the limit of detection.

The correlation between microbial ATP concentrations of the various reagents versus CeCLS did not change significantly, when calculated as the difference between total ATP and free ATP (Figure S3, supplementary information).

This experiment demonstrated that not all of the investigated reagents are suited for rapid (10 seconds) extraction of ATP in biological samples such as drinking water, and might require a longer extraction time.

3.6 Repeatability

Five replicates for three different ATP standard concentrations - 2.5, 10 and 100 pg ATP/mL – were measured. Repeatability of standards was evaluated based on calculation of the coefficient of variation (CV). Four reagents - CeCLS, CeHS, RoCLS and RoHS - were not evaluated on this parameter due to luminometer instability, which resulted in insufficient reagent for the additional measurements of the ATP standard dilutions. The repeatability (CV) for the ATP standard concentration of 2.5 pg/mL ranged between 2.3% to 9.7% and was in general highest for this ATP standard compared to the two other ATP standards of 10 and 100 pg/mL (Table S4, supplementary information). The CV for the ATP standard of 10 pg/mL and 100 pg/mL was <5%, with the exception of ATP standard of 10 pg/mL for reagent Pro1/Ex5. Repeatability seemed not related to the sensitivity of the reagents. Overall, the repeatability was similar for the reagents investigated on this parameter.

Five replicates for two different drinking water samples (sample 208 and 421) were measured with all reagent kits. Repeatability of sample measurements was evaluated based on the coefficient of variation (CV) of the five measurements in RLUs. The repeatability determined by the coefficient of variation for two drinking water samples varied between 2.0% to 7.8% with two CV above this (12% and 11%) (Table S5, supplementary information). Drinking water sample 421 had a higher ATP concentration than sample 208, i.e. higher RLU values, though this was not reflected in a lower CV. As for the repeatability of ATP standard dilutions there was no correlation between type or sensitivity of the reagent and the repeatability.

3.7 Stability of enzyme reagent

ATP standards of 75 or 100 pg/mL were measured in the beginning and in the end of the experiment with each specific enzyme reagent, i.e. without ATP releasing agent. The stability of the enzyme reagent throughout the experiment was evaluated as the difference in RLU signal (%). The enzyme reagents RoHS and Pro1 were not evaluated on this parameter.

The most stable enzyme reagents were the CeCLS and Pro2 reagents, with a decrease of 0.2 $\%\cdot h^{-1}$. Also the Pro3 reagent was fairly stable despite it had a relatively high sensitivity (Table 5). The highest decrease of 71 $\%\cdot h^{-1}$ was observed for the flash reagent CeHS. Even though the BioHS reagent is classified as a flash reagent it was a significantly more stable reagent than the CeHS reagent. This may relate to the fact that its sensitivity was not as high as for CeHS reagent; despite both are classified as flash reagents by their manufacturers. Overall the stability of the enzyme reagent was not consistent with the sensitivity of the enzyme reagent.

Table 5: Stability of reconstituted enzyme reagents.

Reagent kit	ATP standard (pg/mL)	Start (RLU)	End (RLU)	Duration h	Reduced signal ($\%\cdot h^{-1}$)
CeHS	100	72373	65526	0.1	71
RoHS	-	-	-	-	-
Pro3	100	29723	29570	0.4	1.5
PromCLS	75	14653	12384	2.4	6.4
Pro2	100	13496	13487	0.4	0.2
BioHS	75	5643	5170	0.9	9.7
Pro1	-	-	-	-	-
RoCLS	100	3959	3505	1.1	10
CeCLS	100	2037	2028	2.5	0.2

For drinking water any type of reagent can be applied, as long as requirements for sensitivity and LOD are fulfilled. Though when using a flash reagent it is necessary to account for loss in enzyme activity for the reconstituted enzyme reagent. This can be done by measuring ATP standard dilutions throughout the experiment. Alternatively, standard addition for every sample measurement can be applied, which is considered more correct, when using a flash reagent. Also, it is more suitable to use standard addition for e.g. continuous monitoring of ATP in drinking water, i.e. many measurements over time.

3.8 Evaluation of measurement protocols and costs

The protocol for each reagent kit was evaluated in terms of ease of use, required manual activity and potential for automation. Finally, the price, i.e. expenses of reagent per ATP measurement, was calculated and considered against benefits and drawbacks of the various reagents.

All investigated reagents, except the PromCLS reagent, had separate ATP releasing agent and substrate-enzyme reagent. A combined enzyme and ATP releasing agent reduces the number of measuring steps – especially if only total ATP is of interest. For determination of the free ATP and microbial ATP, the combined reagent type complicates the assay protocol, since a filtration step has to be included in order to separate the free ATP and microbial ATP fractions. One disadvantage of the PromCLS reagent was that a longer reaction time (5 minutes) was required compared to the other five commercial reagents and R&D reagents. The obvious advantage of the ATP assay is the rapid availability of results.

An automated protocol for the ATP assay is preferable in order to achieve the same reaction rates between sample and reagents, minimizing pipetting errors, and thus keeping measurement variability to a minimum. In this study all protocols were set up automatically except for the PromCLS reagent. There was significantly more manual handling of sample and reagent required with the PromCLS reagent. Firstly, it was not possible to program a 5 minute reaction time with the luminometer applied in this study. Secondly, a filtration step was required in order quantify free ATP.

The cost per assay measurement by the commercially available reagents was based on the total volume of enzyme reagent of a kit and volume of enzyme required per assay measurement (based on recommended protocol by manufacturer). Assay costs did not comprise costs for cuvettes, ATP free pipette tips or other expenses related to the ATP assay. To some extent the assay price reflected sensitivity of the reagents, i.e. a flash reagent had the highest assay measurement costs (Table 1). The PromCLS reagent had the lowest assay cost, despite it had a higher sensitivity than three of the other commercial reagents (BioHS, RoCLS and CeCLS). Measuring free ATP concentrations with the PromCLS reagent, additional costs have to be added due to membrane filtration. This is not needed for reagents, which consist of separate substrate-enzyme and ATP releasing reagents, e.g. CeCLS or RoHS.

4 Discussion

To make an overall comparison of reagents the results of each investigated parameter were normalised according to:

$$x_{new} = \frac{x - x_{min}}{|x_{max} - x_{min}|}$$

Parameters were rated from 0 to 10, 10 being the best performance (Table 6).

Generally, the flash reagents got a high rating in sensitivity, limit of detection and extraction efficiency, while for example repeatability and stability appear to be similar or poorer than for the other reagents. It was not possible to identify one optimum reagent based on these investigations, since all reagent types perform well and poor, depending on the parameter in question. If extraction is not as complete as possible, as with e.g. the Ex4 ATP releasing agent (Promicol), a part of the sensitivity of the assay measurement is lost when analysing low ATP concentrations. Hence, it is recommended to choose reagents, which have high extraction efficiencies, since an efficient lysis procedure is a prerequisite for accurate ATP determinations in biological samples (Stanley, 1986).

The three reagents which have the lowest overall rating - RoCLS, Pro1/Ex4 and Pro1/Ex5 reagents – were already identified on beforehand as unsuitable for drinking water analysis based on their limit of detection. Though, this can be improved by changing or adapting the assay to the specific application (e.g. LeChevallier et al., 1992; Hammes et al., 2010). Limit of detection for some of the reagents could possibly also be improved by using another luminometer (Marriott et al., 1992; Jago et al., 1989).

A ring-test was performed by six laboratories in an EU project (van der Kooij et al., 2003), where ATP results achieved with different luminometers and different reagents to measure ATP in the same set of samples were compared. The ring test showed both similarities and relatively large differences between ATP concentrations. The main source of error was attributed to differences in slopes of calibration curves due to preparation of ATP standard dilutions in different water types, i.e. buffer, demineralised water, distilled water or tap water. In this study the same set of ATP standard dilutions was used for all reagents, and all assay measurements were done with the same luminometer by the same person. Thus, the only difference in ATP concentrations can be ascribed to the differences between reagent properties. The result of the study demonstrated differences in ATP concentrations despite eliminating some of the assay differences which were identified the EU project. Thus, variations observed in

ATP concentrations of the same sample are in this study are mainly ascribed to different extraction efficiencies between reagents.

.

Table 6: Overall rating of reagents for the ATP assay based on normalised results for the various parameters investigated.

	Range		CeHS	RoHS	Pro3/Ex5	Pro3/Ex4	PromCLS	Pro2/Ex4	Pro2/Ex5	BioHS	Pro1/Ex5	Pro1/Ex4	RoCLS	CeCLS
Sensitivity (enzyme reagent)	10-698	RLU	**10**	9.5	4.2	4.2	2.7	1.7	1.7	0.9	0.8	0.8	0.3	0
Sensitivity (enzyme and ATP releasing agent)	6-531	RLU	**10**	4.4	1.8	4.1	3.8	1.8	0.9	1.3	0.9	0.7	0	1.0
Quenching	2-77	**%**	9.1	1.6	1.3	7.2	-	6.4	2.1	**10**	3.3	6.3	0	7.1
LOD total ATP	0.2-8.9	pg/mL	9.9	**10**	8.7	9.8	8.1	9.5	8.6	9.5	4.3	6.8	0	7.6
LOD free ATP	0.3-1.4	pg/mL	8.4	9.8	8.2	8.2	-	**10**	**10**	0	7.7	7.7	2.5	9.7
LOD microbial ATP	0.6-9.0	pg/mL	9.8	**10**	8.9	9.7	8.3	9.7	8.8	8.7	4.4	6.9	0	7.8
Repeatability (ATP standards)	2.2-9.5	CV	-	-	7.4	**10**	**10**	9.6	7.2	8.1	0	6.0	-	8.6
Repeatability (DW samples)	3.0-8.8	CV	5.0	0	6.9	5.7	7.0	**10**	9.0	0.9	6.0	4.3	8.1	9.5
Extraction efficiency (DW)	50-105	pg/mL	-	**10**	5.9	0.0	6.5	0.6	8.6	**10**	-	-	-	8.7
Extraction efficiency (>100pg/mL)	321-1093	pg/mL	**10**	9.6	6.8	0	3.3	0.4	7.4	7.0	7.6	0.8	9.9	5.5
Stability of enzyme reagent	0.2-71	$\%\cdot h^{-1}$	0	-	9.8	9.8	9.1	**10**	**10**	8.7	-	-	8.6	**10**
Costs	0.4-3.5	EUR/assay	0	8.3	-	-	**10**	-	-	4.1	-	-	8.3	6.1
Total score			**72**	**73**	**70**	**69**	**69**	**70**	**74**	**69**	**35**	**40**	**38**	**82**
Total score/no. evaluated parameters			**7.2**	**7.3**	**6.4**	**6.3**	**6.9**	**6.3**	**6.8**	**5.8**	**3.9**	**4.5**	**3.8**	**6.8**

4 Conclusions

Various types of reagents for the ATP assay were investigated on their inherent properties for the identification of optimum criteria for reagents used for determination of ATP in drinking water.

The study demonstrated:

- the enzyme reagents had various sensitivities and limit of detection; where the flash reagents in general had the highest sensitivities and also had the lowest limit of detection (<1 pg/mL).
- some of the reagents with the required limit of detection for analysing ATP in drinking water were flash reagents which are not particularly stable. Hence, these lose sensitivity and the required limit of detection required for drinking water analysis is compromised.
- quenching of the light emission caused by the ATP releasing agent was in the range of 2-70%, thus in some cases the extraction reagent had a significant impact on the sensitivity due to quenching/inhibition of the light signal.
- the repeatability for ATP standard dilutions and drinking water samples was similar for all reagents despite differences in type of enzyme reagent and sensitivity.
- There were significant differences in ATP concentrations in a drinking water with high ATP concentration (spiked sample) when measured with the different reagents, demonstrating different extraction efficiencies of the extraction reagents. ATP concentrations in drinking water samples varied when measured with different reagents - most likely due to different extraction efficiencies.
- an overall rating of investigated parameters did not elucidate the most applicable reagent for drinking water purposes, since the reagents performed well on some parameters and less good on others. Though it is recommended to choose reagents which are efficient at extracting cellular ATP in terms of amount and speed (seconds).

Acknowledgements

This work was supported by the Danish Council for Strategic Research, the project SENZSOWAQ – Sensors for Monitoring and Control of Water Quality. The Urban Water Technology Research School is acknowledged. Thanks to Jos Rijkx, CEO of Promicol, The Netherlands for their collaboration and supply of R&D reagents for the ATP assay. Thanks to Professor Karsten Arnbjerg Nielsen and Associate Professor Henrik Andersen on discussions and input related to statistics and data treatment; also thanks Hans Dalsgaard Jensen for his contribution. Thanks to laboratory technician Sabrina Nedell for assisting in the laboratory. Torben Dolin is acknowledged for his assistance with the graphics for this article. Thanks to Lyngby waterworks for allowing access to drinking water from the clean water tank.

References

ASTM D4012-81 (2009) Standard Test Method for Adenosine Triphosphate (ATP) Content of Microorganisms in Water, American Society for Testing and Materials (ASTM), standard designation: D4012 – 81 of 1997, reapproved in 2009.

Delahaye, E., Welté, B., Levi, Y., Leblon, G., Montiel, A. (2003) An ATP-based method for monitoring the microbiological drinking water quality in a distribution network. Water Research, 37, 3689-3696.

Eaton, A.D., Clesceri, L.S., Rice, E.W., Greenber, A.E. (2005) Standard methods for the examination of water and waste water. American Health Association, 21st edition, USA.

Eydal, H.S., Pedersen, K., (2007) Use of an ATP assay to determine viable microbial biomass in Fennoscandian Shield groundwater from depths of 3-1000 m. Journal of Microbiological Methods 70, 363-373.

Frundzhyan, V., Ugarova, N. (2007) Bioluminescent assay of total bacterial contamination of drinking water. Luminescence, 22, 241-244.

Guardigli, M., Lundin, A. and Roda, A. (2011) "Classical" applications of chemiluminescence and bioluminescence. In: Chemiluminescence and Bioluminescence: Past, present and future. ED. Roda, A.. pp. 162-190, Royal Society of Chemistry, Cambridge, UK.

Hammes, F., Goldschmidt, F., Vital, M., Wang, Y., Egli, T. (2010) Measurement and interpretation of microbial adenosine tri-phosphate (ATP) in aquatic environments. Water Research, 44, 3915-3923.

Jago, P.H., Simpson, W.J., Denyer, S.P., Evans, A.W., Griffiths, M.W., Hammond, J.R.M., Ingram, T.P., Lacey, R.F., Macey, N.W., McCarthy, B.J., Salusbury, T.T., Senior, P.S., Sidorowics, S., Smither, R., Stanfield, G., Stanley, P.E. (1989) An evaluation of the performance of ten commercial luminometers, Journal of Bioluminescence and Chemiluminescence, 3, 131-145.

Jensen, H.D. (2013) Personal comment. Dansk Fundamental Metrologi A/S (Danish National Metrology Institute), Matematiktorvet 307, 2800 Kgs. Lyngby, Denmark.

Karl, D.M. (1980) Cellular nucleotide measurements and applications in microbial ecology. Microbiological Reviews, 44, 4, 739-796.

Liu, G., Van der Mark, E.J., Verberk, J.Q.J.C., van Dijk, J.C. (2013) Flow cytometry total cell counts: A field study assessing microbiological water quality and growth in unchlorinated drinking water distribution systems. BioMed Research International, Vol. 2013, Article ID 595872, 10 pages.

Lundin, A. (2000) Use of firefly luciferase in ATP-related assays of biomass, enzymes and metabolites. Methods in Enzymology 305, 346-370.

Marriott, N.J., Barnes, S.P., Smith, R.N. (1992) A comparative evaluation of three commercially available luminometers and two ATP reagent kits. International Biodeterioration & Biodegradation, 30, 339-352.

Miller, J.C., Miller, J.N. (1993) Statistics for analytical chemistry. Ellis Horwood Limited, 3rd edition, England.

Stanley, P.E. (1986) Extraction of adenosine triphosphate from microbial and somatic cells. Methods in Enzymology, 133, 14-22.

Stanley, P.E., (1989) A concise beginner's guide to rapid microbiology using Adenosine Triphosphat (ATP) and luminescence. In: ATP Luminescence – rapid methods in microbiology, P.E. Stanley; B.J. McCarthy; R. Smither (Eds.), Blackwell Scientific Publications, Oxford, 1-10.

Vang, Ó.K., Corfitzen, C.B., Smith, C., Albrechtsen, H.-J. (I) Evaluation of ATP measurements to detect microbial ingress in drinking water by waste water and surface water. Submitted manuscript.

Vang, Ó.K., Corfitzen, C.B., Spliid, H., Albrechtsen, H.-J. (II) Levels of microbial ATP and free ATP in non-chlorinated drinking water - assessing microbial drinking water quality with ATP measurements. *Submitted manuscript*.

van der Kooij, D., Albrechtsen, H.J., Corfitzen, C.B., Ashworth, J., Parry, I., Enkiri, F., Hambsch, B., Hametner, C., Kloiber, R., Veenendaal, H.R., Verhamme, D., Hoekstra, E.J. (2003) Assessment of the microbial growth support potential of products in contact with drinking water. CPDW-Project. Report EUR 20832.

van der Wielen, P.W.J.J., van der Kooij, D. (2010) Effect of water composition, distance and season on the adenosine triphosphate concentration in unchlorinated drinking water in the Netherlands. Water Research 44, 17, 4860-4867.

Vital, M; Dignum, M; Magic-Knezev, A; Ross, P; rietveld, L; Hammes, F (2012) Flow cytometry and adenosine tri-phosphate analysis: Alternative possibilities to evaluate major bacteriological changes in drinking water treatment and distribution systems. Water Research, 46, 4665-4676

Supplementary information

Table S1: ATP assay protocols (guidelines by manufacturers) - volumes of sample, ATP releasing agent and enzyme reagent and reaction times for the various regents.

	Sample	ATP releasing agent[a)]		Enzyme reagent	
Reagent	**volume (µL)**	**volume (µL)**	**reaction time**	**volume (µL)**	**reaction time**
CeCLS	100	100	10 sec	100	2 sec
CeHS	50	200	10 sec	100	2 sec
RoCLS	100	100	10 sec	100	2 sec
RoHS	100	100	10 sec	100	2 sec
BioHS	50	50	10 sec	400	10 sec
PromCLS[a)]	100	-	-	100	1min/5 min
Pro1/EX4	100	100	10 sec	100	2 sec
Pro1/EX5	100	100	10 sec	100	2 sec
Pro2/EX4	100	100	10 sec	100	2 sec
Pro2/EX5	100	100	10 sec	100	2 sec
Pro3/EX4	100	100	10 sec	100	2 sec
Pro3/EX5	100	100	10 sec	100	2 sec

[a)]The ATP releasing reagent was excluded from the protocol when measuring free ATP in the sample and the ATP standard calibration curve for free ATP.

Table S2: Slope and y-intercept for calibration curves for determination of free ATP in three different intervals (0-10, 0-100 and 0-1000 pg ATP/mL) measured with different enzyme reagents.

	ATP calibration curve data (with enzyme reagent)											
		0-10 pg/mL				0-100 pg/mL				0-1000 pg/mL			
	Blank[a)]	Slope	y-intercept			Slope	y-intercept			Slope	y-intercept		
Reagent	**RLU**	**RLU**	**RLU**	**N**	**R^2**	**RLU**	**RLU**	**N**	**R^2**	**RLU**	**RLU**	**N**	**R^2**
CeHS[b)]	49	698 ±13	70 ±81	5	1.00	650 ±9	595 ±409	9	1.00	707 ±6	-1490 ±2055	13	1.00
RoHS	61	662 ±13	-18 ±81	5	1.00	640 ±5	61 ±213	9	1.00	654 ±2	-357 ± 813	13	1.00
Pro3	36	307 ± 6	62 ±34	5	1.00	296 ±3	165 ±141	9	1.00	301 ±0.8	31 ± 292	13	1.00
PromCLS	40	205 ±14	178 ±87	5	0.99	193 ±2	155 ±100	9	1.00	202 ±0	-45 ± 129	13	1.00
Pro2	45	136 ± 2	42 ± 9	5	1.00	134 ±0.6	38 ± 30	9	1.00	134 ±0.8	96 ± 322	13	1.00
BioHS	46	79 ± 5	41 ±30	5	0.99	75 ±0.6	73 ± 29	9	1.00	77 ±0.7	97 ± 277	13	1.00
Pro1	45	70 ± 1	45 ± 8	5	1.00	69 ±0.5	62 ± 25	9	1.00	70 ±0.4	77 ± 169	13	1.00
RoCLS	863	37 ± 3	961 ±16	5	0.98	30 ±0.3	995 ± 12	9	1.00	29 ±0.3	1046 ± 123	13	1.00
CeCLS	53	19 ± 0	58 ± 1	5	1.00	20 ±0.3	60 ± 13	9	1.00	20 ±0.1	82 ± 23	13	1.00

[a)]Blank is a measurement luminescence from reagents alone, i.e. no analyte and sample matrix present.

[b)]Measurements from another experiment with this specific reagent were used for calculating LOD, since measurements in this specific experiment were erroneous when measuring ATP standard dilutions with both enzyme and ATP releasing reagent.

Table S3: Slope and y-intercept for calibration curves for determination of total ATP in three different intervals (0-10, 0-100 and 0-1000 pg ATP/mL) measured with different enzyme reagents and the corresponding ATP releasing agents.

	ATP calibration curve data (with enzyme reagent and ATP releasing reagent)												
		0-10 pg/mL				0-100 pg/mL				0-1000 pg/mL			
	Blank[a)]	Slope	y-intercept			Slope	y-intercept			Slope	y-intercept		
Reagent	RLU	RLU	RLU	N	R^2	RLU	RLU	N	R^2	RLU	RLU	N	R^2
CeHS[b)]	47	531 ±5	69 ±34	5	1.00	540 ±2.3	81 ±106	9	1.00	529 ±1.6	248 ±549	13	1.00
RoHS	169	238 ±2	207 ±12	5	1.00	224 ±4.4	421 ±201	9	1.00	230 ±2.5	809 ±936	13	1.00
Pro3/Ex5	66	103 ±5	84 ±29	5	0.99	98 ±0.5	123 ± 21	9	1.00	98 ±0.1	119 ± 34	13	1.00
Pro3/Ex4	88	224 ±3	86 ±18	5	1.00	216 ±1.7	165 ± 80	9	1.00	223 ±0.6	-101 ±213	13	1.00
PromCLS	40	205 ±14	178 ±87	5	0.99	194 ±2.2	155 ±100	9	1.00	201 ±0.4	-45 ±129	13	1.00
Pro2/Ex4	127	101 ±2	120 ±13	5	1.00	96 ±0.8	161 ± 35	9	1.00	99 ±0.5	-7 ±204	13	1.00
Pro2/Ex5	71	55 ±3	83 ±16	5	0.99	52 ±0.3	107 ± 12	9	1.00	53 ±0.2	69 ± 68	13	1.00
BioHS	50	77 ±2	42 ±13	5	1.00	76 ±0.9	50 ± 43	9	1.00	74 ±0.9	253 ±328	13	1.00
Pro1/Ex5	614	52 ±15	508 ±90	3	0.92	23 ±1.2	621 ± 62	7	0.99	26 ±0.2	485 ± 66	11	1.00
Pro1/Ex4	549	43 ±7	495 ±44	5	0.92	43 ±0.4	495 ± 19	9	0.92	51 ±0.2	363 ± 65	13	1.00
RoCLS	242	6 ±2	317 ±15	5	0.69	7.2 ±0.3	320 ± 12	9	0.99	7.6 ±0.1	325 ± 35	13	0.99
CeCLS	53	15 ±1	61 ± 9	5	0.97	15 ±0.1	63 ± 4	9	1.00	15 ±0.1	56 ± 48	13	1.00

[a)]Blank is a measurement luminescence from reagents alone, i.e. no analyte and sample matrix present.

[b)]Measurements from another experiment with this specific reagent were used for calculating LOD, since measurements in this specific experiment were erroneous when measuring ATP standard dilutions with both enzyme and ATP releasing reagent.

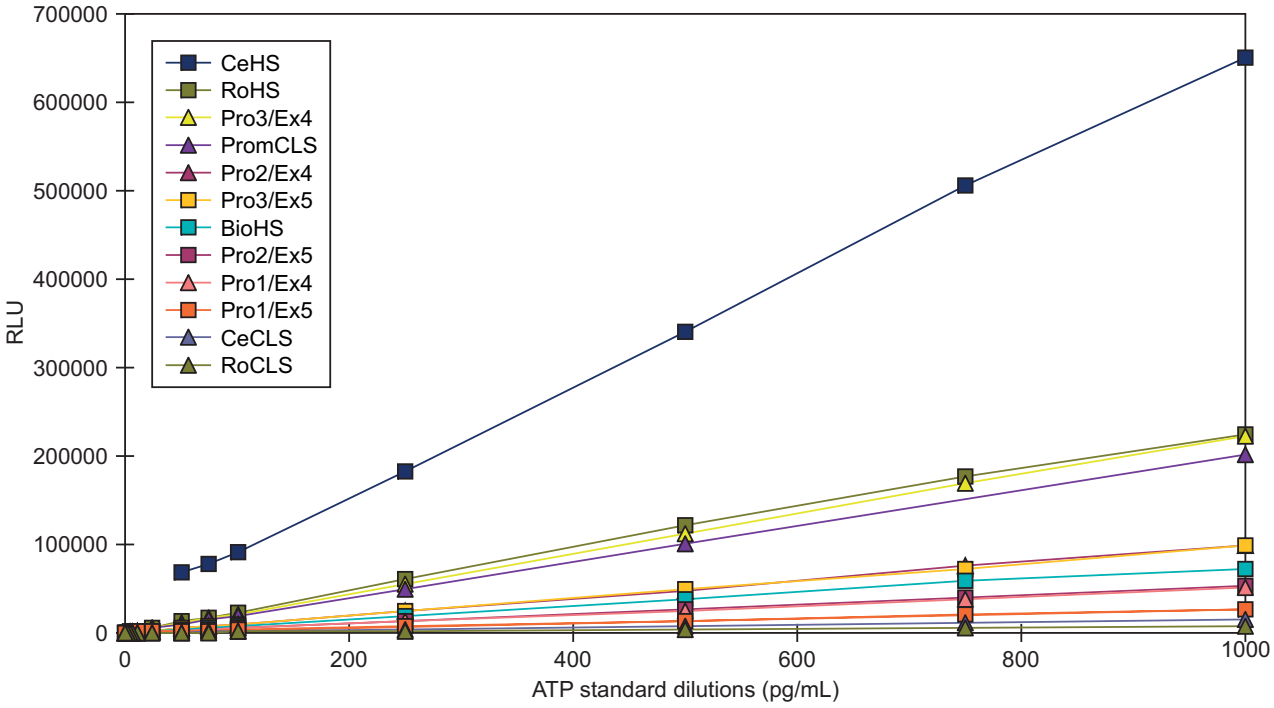

Figure S1: ATP calibration curves for ATP standard dilutions in the range of 0-1000 pg ATP/ml) measured for twelve combinations of enzyme reagents and ATP releasing agents, i.e. calibration curves for total ATP.

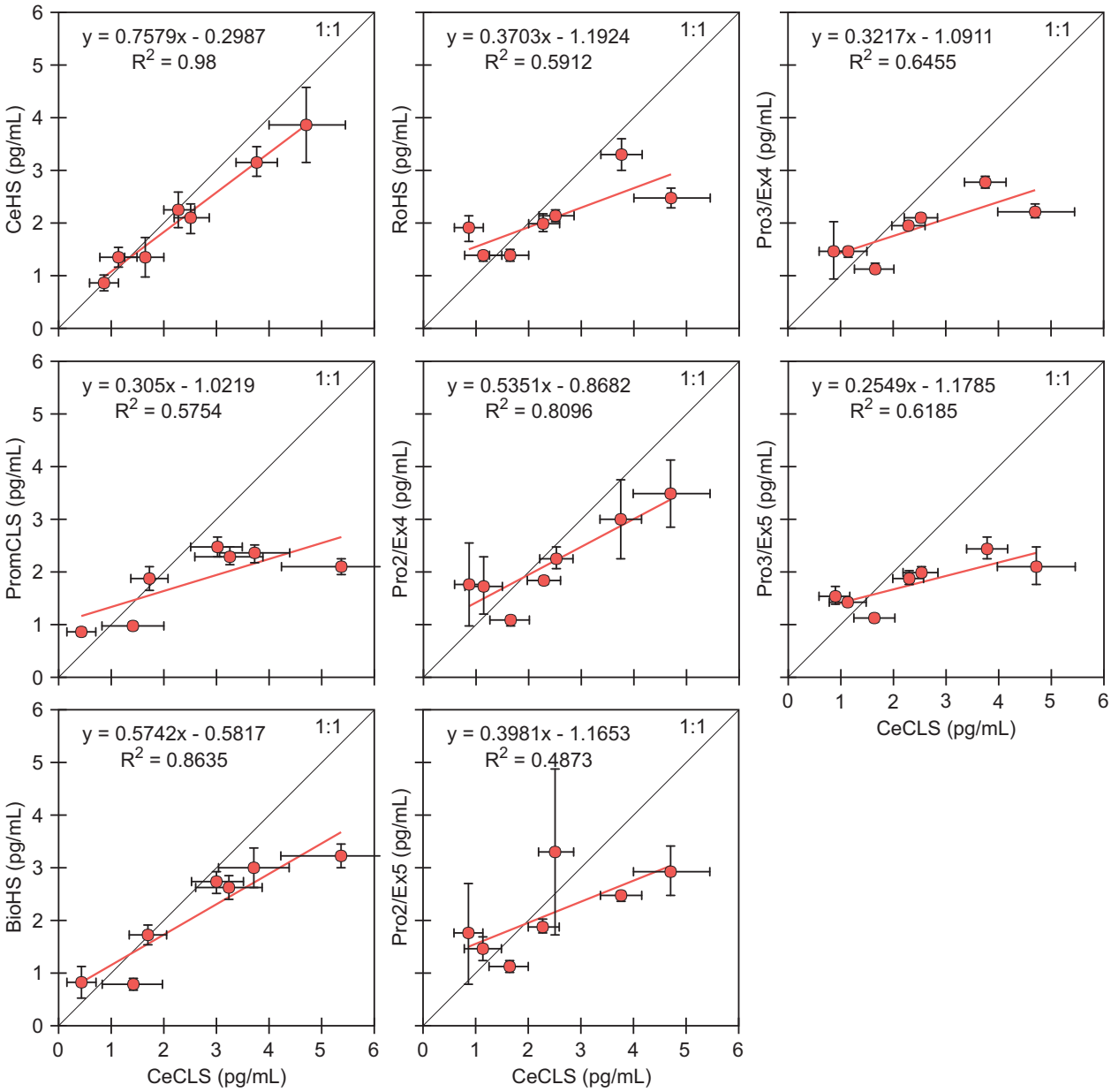

Figure S2: Free ATP concentrations in drinking water samples measured with different enzyme and ATP releasing reagents versus the CeCLS reagent.

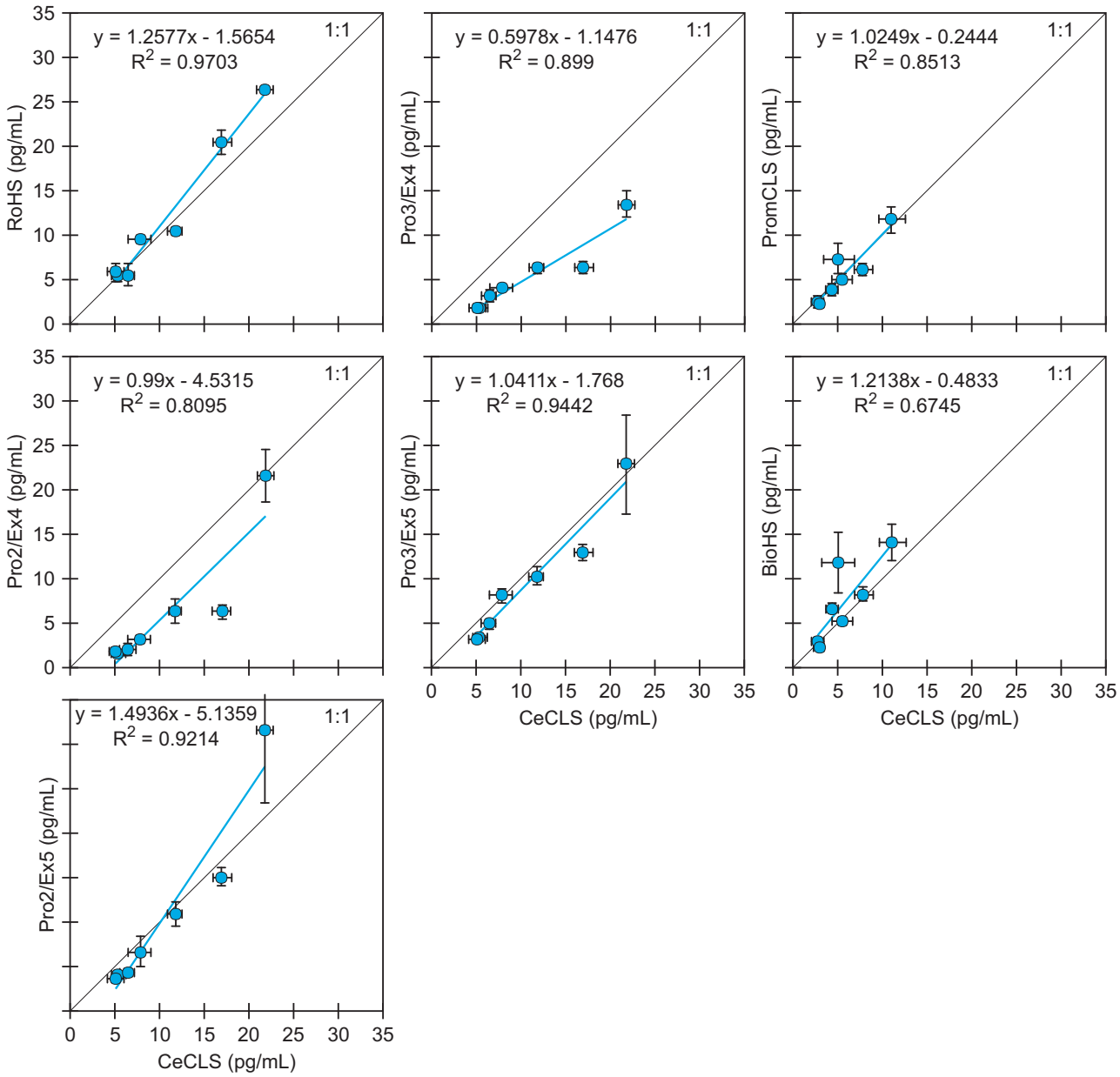

Figure S3: Microbial ATP concentrations in drinking water samples measured with different enzyme and ATP releasing reagents versus the CeCLS reagent.

Table S4: Repeatability of standards 2.5, 10 and 100 pg ATP/mL (N=5). Average relative light units (rlu) with standard deviation, calculated coefficient of variation (CV), and average of CV for the three ATP standards.

Reagent kit	2.5 pg/ml		10 pg/ml		100 pg/ml		Average CV
	Average (RLU)	CV	Average (rlu)	CV	Average (RLU)	CV	
CeHS	-	-	-	-	-	-	-
RoHS	-	-	-	-	-	-	-
Pro3/Ex5	329 ±26	8.1	1075 ± 25	2.4	9845 ±174	1.8	4.1
Pro3/Ex4	637 ±18	2.8	2297 ± 55	2.4	21431 ±297	1.4	2.2
PromCLS	666 ±15	2.3	2109 ± 52	2.4	19841 ±388	2.0	2.2
Pro2/Ex4	375 ±12	3.1	1113 ± 35	3.1	9731 ±115	1.2	2.5
Pro2/Ex5	215 ±20	9.4	616 ± 12	2.0	5220 ± 69	1.3	4.2
BioHS	226 ± 7	3.0	804 ± 30	3.7	7545 ±309	4.1	3.6
Pro1/Ex5	550 ±24	4.3	1046 ±223	21	2889 ± 88	3.0	9.5
Pro1/Ex4	523 ±51	9.7	914 ± 44	4.8	5207 ± 46	0.9	5.1
RoCLS	-	-	-	-	-	-	-
CeCLS	83 ± 3	3.9	168 ± 6	3.3	1210 ± 31	2.5	3.2

Table S5: Repeatability (CV) of RLU values for two drinking water samples measured with all reagents (N=5).

Reagent	Sample 208		Sample 421	
	(RLU)	CV %	(RLU)	CV %
CeHS	14540 ±776	5.3	7834 ±498	6.4
RoHS	2029 ±252	12	5467 ±279	5.1
Pro3/Ex5	598 ± 35	5.8	1642 ± 62	3.8
Pro3/Ex4	910 ± 37	4.0	2135 ±148	6.9
PromCLS	1752 ± 76	4.3	2919 ±150	5.1
Pro2/Ex4	495 ± 20	4.0	1069 ± 22	2.0
Pro2/Ex5	378 ± 13	3.4	975 ± 37	3.7
BioHS	888 ± 48	5.5	1364 ±150	11
Pro1/Ex4	449 ± 18	4.1	719 ± 47	6.5
Pro1/Ex5	465 ± 36	7.8	664 ± 32	4.8
RoCLS	302 ± 8	2.5	405 ± 23	5.7
CeCLS	165 ± 5	2.7	360 ± 14	3.9

The Department of Environmental Engineering (DTU Environment) conducts science-based engineering research within four sections:
Water Resources Engineering, Urban Water Engineering,
Residual Resource Engineering and Environmental Chemistry & Microbiology.

The department dates back to 1865, when Ludvig August Colding, the founder of the department, gave the first lecture on sanitary engineering as response to the cholera epidemics in Copenhagen in the late 1800s.

DTU Environment
Department of Environmental Engineering
Technical University of Denmark

Miljoevej, building 113
2800 Kgs. Lyngby
Denmark

Phone: +45 4525 1600
Fax: +45 4593 2850
e-mail: reception@env.dtu.dk
www.env.dtu.dk