ATP as an indicator of microbiological activity in tap water

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INTRODUCTION

The DWD [7] requires from Member States to take all measures to ensure that water intended for human consumption is free from any micro-organisms, parasites and from any substances, which in numbers or concentrations, constitute a potential danger to human health. Traditional methods to evaluate the presence of bacteria in drinking water samples, such as Heterotrophic Plate Count (HPC), need a few days of incubation and require selection of appropriate temperature and medium. Another important disadvantage is that a small fraction of microorganisms is able to cultivate on artificial mediums [1].

Among others, ATP is a general indicator for the presence of living cells. ATP can be measured in a very sensitive way, using firefly extracted from Photinus pyralis. The light emission is in the range between 500 to 700 nm wavelength [16] and the assay requires the presence of the luciferase, luciferin, magnesium and oxygen (Figure 1). The measured amount of light is proportional to the ATP in the sample. In optimum conditions 1 photon of light is produced by 1 molecule of ATP [22].

None of the other nucleotide triphosphates are active as substrates and therefore ATP can be measured in biological samples without interference from other naturally occurring compounds [10]. Adenosine triphosphate plays essential role in cell metabolism, is present in high concentrations compared with other metabolites and is uniformly distributed in the protoplasm of microorganisms from where it may be readily extracted. Due to its high rate of turnover ATP could be a good index of cell viability [13]. A number of studies have indicated that bacterial levels of ATP are correlated with cell numbers [5,8,9,12,13,14]. Wide range of bacterial ATP levels has been reported in the literature [5,8,12,13,21]. The levels will vary not only between different species of bacteria, but also within any particular species depending on the constituents of the growth medium [2,6,11] growth phase of the organism at the time of sampling [2], oxygen tension [6].

The goal of this report is to explore the use of ATP as an indicator of microbiological activity in tap water.
METHODS AND MATERIALS

Reagents

_Celsius LuminATE_ is the light generating reagent containing luciferin and luciferase. It is supplied as a freeze-dried pellet and stored at 5° C. It is reconstituted with 7 ml of _Celsius LuminATE Buffer_ and is stable for 24 h at room temperature [4].

_Celsius LuminATE Buffer_ is used for _LuminATE_ and _ATP Standard_ reconstitution. It is stored at 5° C [4].

_Celsius LuminEX_ is used for the destruction of the microbial cell membrane. It is stored at 5° C [4].

_ATP Standard_ is supplied by Celsius in freeze-dried form. Each vial of adenosine-5’-triphosphate contains 10 µg of disodium salt, 0.025 M Hepes buffer, MgSO₄, EDTA, sodium azide and 0.02 mg bovine serum albumin [4]. Small aliquots containing 100 µl of 2 mg/l ATP solutions after reconstitution with _LuminATE Buffer_ were immediately frozen and stored at -80° C in capped tubes. During the performance of the analyses reagents were maintained at room temperature.

_Milli-Q water_ was obtained from a combined Elix-Element system (Millipore). Tap water initially passes through a Progard™ pretreatment pack. It is designed to remove free particles and free chlorine from the water. The water is pressurized with a pump and than is purified by reverse osmosis (RO). Afterwards the RO product water passes through an electrodeionisation (E.D.I) module, where organic and mineral contaminants levels are reduced. Pre-treated water is exposed a 185/254 nm UV lamp to ensure the destruction of organisms, including those with trapped metals. The released elements can than be retained by the ion exchange resins. Afterwards water goes through the G-Gard polishing packs, which contains high quality ion-exchange mixed bed resin in a pure natural polypropylene housing selected for its low leaching characteristics. Final filtration is ensured through a 0.1 µm filter containing ultra high molecular weight polyethylene membrane able to remove trace ions and oxidation by-products produced by the action of the UV light [18].

_Sterile water_ produced by Monico SPA is bought in glass bottles containing 500 ml. It is prepared by reverse osmosis followed by distillation and sterilization at 120° C.

_ATP analysis_

ATP was analyzed using a luminometer (Celsius Advance™ Coupe). 100 µl of the sample was put in the ATP-free disposable polystyrene tube and put in the autosampler. For the determination of free ATP, i.e. the fraction not present inside the cells, 100 µl of the mixture of luciferin-luciferase (Celsius, _LuminATE_) was injected into the sample by the automatic dispenser. For the determination of total ATP, i.e. free and cellular ATP, first 100 µl of a reagent that destroys the microbial cell membrane (Celsius, _LuminEX_) was added followed by the mixture of 100 µl of luciferin-luciferase (Celsius, _LuminATE_).

The background measuring time and sample measuring time were set at 10 s. The background measuring time is the measuring time prior to the actual injection of the reagents. The sample measuring time is the time that the instrument reads the sample tube, where the bioluminescent reaction is taking place.

To enable proper dissolution and reaction of LuminEX in the sample, the injection of LuminATE was delayed by 30 sec. A delay of 2 sec. was used between the injection of LuminATE and the measuring
time. The luminometer is calibrated to measure optimally at 560 nm wavelength of light emitted by the luminescence reaction [3].

Following technical specifications the start-up and shut-down procedure by rinsing and washing the reagents out of the tubing has been applied every measuring day. Prior to calibration and analyzing samples, blank empty tubes have been analyzed to check the background response [3].

**Heterotrophic Plate Count**

Water samples have been cultivated on two different mediums: R2A and PCA [19] for total counts of heterotrophic bacteria. 1 ml of the sample was placed in the Petri dish on both mediums. The plates were incubated at 22°C for 68 hours and at 37°C for 44 hours. Additionally for each medium and temperature blank mediums have been prepared.

**Samples**

Samples have been collected from tap water on the premises of JRC in Ispra, Italy and in surrounding villages. The volume of the samples was 50 or 500 ml. Sampling bottles before sampling have been thoroughly washed: firstly in an automatic washing machine, subsequently washed with 1% nitric acid solution and followed by three times with Milli-Q water.

The tap sampling procedure was consisted of flushing the tap for one minute and taking directly a sample first followed by a known stagnation period and sampling two or more successive samples.

Additionally samples have been obtained using the Dynamic Test Facility. The Dynamic Test Facility is a device that simulates consumer behaviour enabling to measure automatically temperature, pH, conductivity and dissolved oxygen of the incoming water. The facility is used to study corrosion and the potential of materials to form a biofilm. Currently 4 lines with different pipe materials are in use: copper, stainless steel, galvanized, polypropylene. The DTF has been programmed to take samples automatically after stagnation times of 0.5, 1, 2, 4, 8 and 16 h. Since 0.5 and 1 h stagnation (0.5 HS and 1 HS) occur twice in the measuring procedure, the nomenclature 1st and 2nd stagnation have been used. The volume of all DTF samples is 200 ml.
CALIBRATION CURVE

On each measuring day a new ATP stock solution containing 100 µl of 2 mg/l ATP has been used. After thawing for half an hour at a room temperature, the stock solution was 1000-fold diluted followed by steps of 10-fold dilutions down to 2 ng ATP/l. This latter solution was diluted 2-fold (1 ng ATP/l). On each day, a calibration curve (Figure 2) for total and free ATP concentration has been prepared. Annex 2 gives the details for all curves.

As a solvent for ATP dilutions firstly Milli-Q water and sterile water have been used. However, considering the fact that this may lead to underestimation of ATP concentrations in samples, due to the composition of the tap water [22] further dilutions have been prepared using water from water production point in JRC-Ispra, Italy.

$$y = 15.666x + 45.283$$
$$R^2 = 1$$

$$y = 24.89x + 32.523$$
$$R^2 = 1$$

Figure 2  Calibration curves for total and free ATP

Slopes obtained through linear regression of all calibration curves (prepared on each measuring day) are illustrated in Figure 3 for total ATP and Figure 4 for free ATP. The average slope of calibration curve for total ATP is 15±2.29 and for free ATP 25±2.60.
Figure 3  Slopes of calibration curves for total ATP obtained with different water types

Figure 4  Slopes of calibration curves for free ATP obtained with different water types
DETECTION LIMIT OF ATP

To estimate the limit of detection that is significantly different from the background, the comparison of the RLU average of two concentrations was performed. It was assumed that the standard deviations of the two means are not significantly different [17]:

\[ S^2 = \frac{((n_1-1)s_1^2+(n_2-1)s_2^2)}{(n_1+n_2-2)} \]

- \( n_1 \): amount of water samples (milli-Q water samples)
- \( n_2 \): amount of samples with 0.2 ng/l ATP concentration
- \( s_1 \): standard deviation of water samples (milli-Q water samples)
- \( s_2 \): standard deviation of samples with 0.2 ng/l ATP concentration

\[ t = \frac{(x_1-x_2)}{s\sqrt{1/n_1+1/n_2}} \]

- \( x_1 \): mean of water samples (milli-Q water samples)
- \( x_2 \): mean of samples with 0.2 ng/l ATP concentration
- \( t \): has \( f = (n_1+n_2)-2 \) degrees of freedom

Annex 1 gives the data of milli-Q water, 0.2, 1, 2, 20, 200 ng/l of ATP. As an example the hypothesis was tested that the two RLU means obtained for Milli-Q water samples and 0.2 ng/l ATP samples are equal. There are 10 degrees of freedom for comparison of Milli-Q water samples and 0.2 ng/l ATP samples. The calculated \( |t| \) value is 1.42. The critical value of \( |t| \) (P=0.05) is 2.23. Since the critical \( |t| \) (P=0.05) value is higher than the calculated value, the difference between RLU values of Milli-Q water samples and 0.2 ng/l ATP samples is not significant.

Using the same statistical test RLU values of the following averages have been compared:

a. Milli-Q water and 1 ng/l ATP
b. 0.2 ng/l ATP and 1 ng/l ATP
c. 1 ng/l ATP and 2 ng/l ATP

The test show that the critical values of \( |t| \) (P=0.05) for the above samples are lower than the calculated \( |t| \) values, therefore the differences between RLU means for these cases are significant. The concentration of 1 ng/l ATP is a good estimate of the limit of detection since it is significantly different from milli-Q water and 0.2 ng/l ATP.
CALCULATION OF ATP CONCENTRATION IN THE SAMPLES

The method for calculating the concentration of ATP in a sample has been two times modified due to the fact that initial calculations gave negative values of ATP concentration in the samples (Eq. 3 and Eq. 4). It happened especially when “b” value in Eq. 3 was very high, what suggested that water used for ATP dilutions was not ATP free. In the next equation this situation occurred when RLU value of the sample was lower than background value of the sample, i.e. the value measured just before adding the reagents. Considering above problems a third modification has been adopted (Eq. 5). In new method “b” values and background values of particular samples have been replaced by the mean value of the RLU values measured for empty tubes without reagents.

The average of RLU values measured for empty tubes as well as the average of background response measured for empty tubes almost overlap. Both means were obtained for the same amount of samples (60 samples). However, RLU values of empty tubes were much more homogeneous and therefore the standard deviation is smaller, i.e. 37±4 RLU (See Fig.5), in comparison to the standard deviation of the background response for empty tubes, i.e. 38±9 RLU.

Both, average of background responses of all samples as well as average of background responses of samples excluding blanks presents a value of 42.75±12.39 and 43.07±12.51, respectively. Stdev is quite high, what could be due to the fact that amount of samples is very big (876-936 samples). See Table 1.

All data and calculations are presented in Annex 4.

Eq.3 \[ X = \frac{(y-b)}{a} \]

Eq.4 \[ X = \frac{(y-\text{background})}{a} \]

Eq.5 \[ X = \frac{(y-\text{factor})}{a} \]

where:

- \( y \): RLU value of a particular sample
- \( b \): constant value received from the linear regression of the calibration curve (\( y = ax+b \))
- \( \text{background} \): background value as measured for a particular sample just before adding the reagents
- \( a \): coefficient corresponding to each x-value received from the linear regression of a calibration curve (\( y = ax+b \))
- \( \text{factor} \): mean value of RLU values measured for empty tubes without reagents. The value of the factor is 40 RLU.
Figure 5  Shewart chart for RLU values of empty tubes

Table 1  Averages and standard deviations of background and RLU values in the samples and blanks

<table>
<thead>
<tr>
<th>Samples</th>
<th>mean</th>
<th>stdev</th>
<th>amount of samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>RLU values of empty tubes</td>
<td>37.31</td>
<td>4.08</td>
<td>60</td>
</tr>
<tr>
<td>background values of empty tubes</td>
<td>38.16</td>
<td>9.47</td>
<td>60</td>
</tr>
<tr>
<td>background values of all samples</td>
<td>42.75</td>
<td>12.39</td>
<td>936</td>
</tr>
<tr>
<td>background values of samples</td>
<td>43.07</td>
<td>12.51</td>
<td>876</td>
</tr>
<tr>
<td>excluding blanks</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
RLU RESPONSE

The assay was performed on samples containing only milli-Q water in order to check how different variable would modify the response. Different volume of milli-Q water samples seems not to have a significant influence. RLU values were fluctuating in the range 35-45 RLU. Sunlight exposure was another parameter of interest. Although some samples gave elevated RLU values after sunlight exposure has been applied others didn’t change the response. Additionally, since all tubes used in experiments were polystyrene, an “antistatic test”, to see if material of tubes have any influence on the response, have been included.

Effect of sample volume

An experiment has been conducted to check if volume of the sample has an influence on the response. Samples with different volumes of Milli-Q water have been prepared and measured immediately without adding reagents.

RLU values between 6 samples with 100 µl milli-Q water, 6 samples with 200 µl milli-Q water and 6 samples with 300 µl milli-Q water don’t differ significantly. The conclusion is that the volume of water sample is not important. However, repetition of samples containing 100 µl milli-Q water showed higher RLU values (see Fig.6 and Annex 3).

![Figure 6 Influence of volume in water samples on RLU values](image)

Effect of ambient light

Purpose of this experiment was to see if samples exposed to sunlight will give different response from samples that were not exposed. Tubes have been filled with 100 µl of Milli-Q water and exposed to sunlight for different periods of time.
RLU values between samples with water exposed on sunlight vary between 40-80 RLU. One can notice that after 2 min on sunlight exposure a significant increase in RLU values can be observed. RLU values of 2 min, 5 min and 10 min samples are very similar and significantly different from 0 min samples. Subsequently samples kept for 2 minutes on sunlight and afterwards during 2 minutes in the dark chamber of the luminometer prior to measurement, show a significant reduction in RLU values. After 40 min, 80 min and 160 min of sunlight exposure even higher RLU values can be observed (significantly different from 0 min samples). However samples exposed through 20 min and 320 min show lower RLU values. 20 min samples and 0 min samples don’t show any significant difference in contrast to 320 min samples that are significantly different from 0 min samples. Results are presented on Fig. 7 and in Annex 3.

![Graph showing RLU values over exposure time](image)

**Figure 7** Sunlight exposure of water samples.

Experiment on sunlight exposure has been repeated after few days. Samples have been exposed on sunlight but additionally tubes prior to measuring were made antistatic with the ethanol. All samples had the same volume 100 µl of Milli-Q water. Samples in the last set have been rubbed with a synthetic blouse to check if polyethylene tubes containing water samples would change RLU values being initially antistatic. For better comparison results of this experiment as well as the previous one are presented together in Fig. 8 (see also Annex 3).

RLU values of 0 min-ethanol samples are significantly higher comparing to those obtained in previous experiment 0 min samples. On the contrary RLU values of sunlight exposed 2 min-ethanol samples are rather low comparing to previous results (2 min). However RLU values of 2 min light-2 min dark-ethanol samples and 2 min light-5 min dark-ethanol samples are comparable to RLU values of 2 min light-2 min dark samples from the previous experiment. RLU values of these samples are similar to non-exposed samples (0 min). Tubes charged with static electricity by rubbing with the synthetic blouse and measured just after the preparation show low RLU values. This suggests that the luminometer is able to discharge the static electricity and that there is no need to make the tubes...
antistatic. However, the control of analyzing empty tubes serves to control if the luminometer as a whole is not charged and thus influencing the measurements.

![Graph showing RLU values over exposure time](image)

**Figure 8  Sunlight exposure and antistatic test of water samples**

**Effect of LuminEX on response**

Samples with the same ATP concentrations have been measured for total ATP and for free ATP. The purpose of the experiment was to verify the influence of LuminEX on the RLU values.

Samples with 100 µl of 200 ng ATP/l have been separated for two batches. In one batch 100 µl of Milli-Q water and 100 µl of LuminATE has been added, while in the second batch 100 µl of LuminEX and 100 µl of LuminATE have been added. The overall volume of samples in both batches was therefore 300 µl. ATP concentrations have been prepared accordingly to Calibration curve procedure, as a solvent for ATP Standards Milli-Q water have been used.

The average RLU value of the samples without LuminEX addition (3125.67±105.3) compared to average value of the samples with addition of LuminEX (2774.33±155.5) is significantly different. The probable explanation is that LuminEX is absorbing the light that is produced in the luciferin-luciferase reaction. Consequently RLU values obtained for samples where both reagents were added are lower than those where only LuminATE was added (See Annex 3).

**Effect of LuminATE volume on response**

The purpose of this experiment was to check if the RLU value obtained from the samples with the same ATP concentration (200 ng/l ATP) is incident to the concentration of LuminATE. Therefore samples have been made with variable volumes of LuminATE and Milli-Q water, while keeping the overall volume constant, thus 300µl.
The measurement shows that the volume of LuminATE has influence on the RLU values. The higher the concentration of LuminATE the higher is the response. Results are presented on Fig. 9 and in Annex 3.

![Figure 9 Influence of LuminATE on the RLU values](image-url)
ATP STABILITY AND DEGRADATION

To assess ATP stability and degradation the following experiment has been conducted. 100µl of 2mg/l ATP solutions have been diluted in water from the water production point and in Milli-Q water to see if degradation depends on the type of water. All dilutions were prepared in successive dilution using first a 1000 time step followed by 10 times dilution down to 2 ng/l. The latter was diluted to 1 ng/l.

Also blank water samples (without ATP addition) of water from the water production point and milli-Q water were included in the experiment. Due to fact that it was difficult to take the whole ATP aliquot from the vial to prepare dilutions, real ATP concentrations in samples prepared on different days could vary significantly giving diverse RLU values.

Samples have been kept for 4 days in 4°C and 24°C environment. All the samples during storage have been capped. On the measuring day 20th of September also new ATP dilutions for calibration curve have been prepared and directly afterwards analyzed (see Annex 2).

In order to have more data and trace more in detail ATP degradation, the experiment on ATP degradation has been modified and repeated. ATP dilutions have been prepared in the same way as previously but stagnation time has been changed. Dilutions of ATP Standards have been prepared and stored in 4°C and 24°C environment for 1 day, 4 days, 8 days, 9 days and 10 days. As previously, on the day of analysis also new ATP dilutions for calibration curve have been prepared (see Annex 2).

**Calculation of ATP concentration in samples prepared for ATP degradation**

ATP concentrations in the samples prepared for ATP degradation have been firstly calculated using X=(y-factor)/a method. However calculated in this way ATP concentrations especially in samples with low initial ATP concentrations (1 ng/l ATP and 2 ng/l ATP) gave always too elevated calculated ATP concentrations in proportion to initial ATP concentrations in those samples. That could be due to the fact that RLU values of blank tap water samples were very similar to RLU values received from samples containing 1 ng/l ATP and 2 ng/l ATP. Therefore the responses of the samples have been corrected by the RLU response of the blank before the calculation method was applied. This new method has also given negative concentrations: e.g.: all ATP samples prepared with tap water after 10 days of stagnation in 24°C environment gave lower RLU values than blank tap water sample also stored for 10 days at 24°C (See Annex 5).

It appears that ATP stability and degradation depends on the type of water used for the dilutions. ATP is rather stable when diluted in Milli-Q water regardless the temperature and time. On the other hand ATP diluted in tap water show gradual degradation with time especially at 24°C. 4-5 days seem to be a half-life of ATP especially for 200 ng/l ATP and 20 ng/l ATP concentrations. Samples with lower concentrations: 2 ng/l ATP and 1 ng/l ATP show slower ATP degradation and their half-life can be estimated as 8-9 days. After 10 days only trace amounts of ATP remained in the samples (Fig.10).

ATP concentrations prepared with tap water and kept in 4°C environment show much slower ATP degradation comparing to samples prepared with the same water and kept in 24°C environment. Half-life for all ATP concentrations in this temperature can be assessed as 8-10 days (Fig. 11).

ATP concentrations prepared with milli-Q water and kept at 4°C and 24°C seem rather stable with time (Fig.12 and Fig.13). Unexpectedly the initial concentration of 200 ng/l seems to increase with time. However it might be explained by the precision of the analysis and the way the samples have been prepared.
Figure 10  Tap water samples stored in 24°C

Figure 11  Tap water samples stored in 4°C
Figure 12 Milli-Q water samples stored in 24°C

Figure 13 Milli-Q water samples stored in 4°C
INFLUENCE OF STAGNATION TIME ON ATP CONCENTRATION IN TAP WATER SAMPLES

500 ml samples at consumers’ tap in Leggiuno and Laveno

The aim of the experiment was to scrutinize if stagnation time has an influence on ATP concentrations at the consumers’ tap. 15 samples with defined stagnation times have been taken from two private houses in Laveno and in Leggiuno and measured for total and free ATP. The sample procedure was as follows. The tap in the kitchen was flushed with the tap fully open for 1 min. A FF sample of 500 ml was taken and the tap was closed for stagnation. After 0.5 h (about 9.30-10.00), 1 h (about 10.00-11.00), 2 h (about 11.00-13.00), 4 h (about 13.45-17.45) and 8 h (about 23.00-7.00) stagnation, two successive samples of 500 ml were taken. Experiment has been conducted on 23/08/05 and 20/09/05 (see Annex 6.1).

Fig. 14 and Fig. 15 present ATP concentrations in tap water samples from Leggiuno. Samples contain concentrations of total ATP in the range of 15-52 ng/l. FF samples and short stagnation time samples of 0.5, 1 and 2 h, contain the highest ATP concentrations. With longer stagnation times of 4 and 8 h the concentration gradually decreases. Free ATP is mostly below 10 ng/l ATP, what suggests that microbial ATP constitutes the major part of total ATP.

Figure 14  Dependence of ATP concentration on the stagnation time in tap water samples from Leggiuno (23/08/05)
Figure 15  Dependence of ATP concentration on the stagnation time in tap water samples from Leggiuno (20/09/05)

Tap water samples from Laveno show ATP concentrations in the range of 0.5-9 ng/l ATP. In contrast to the samples from Leggiuno ATP concentrations in samples from Laveno show a completely reverse distribution. ATP concentrations increase very slowly with time (especially in the 1st 500 ml total samples). Free ATP concentrations are below 2 ng/l ATP. See Fig. 16 and Fig. 17.

It can be concluded that regardless the sampling place almost all 1st 500 ml samples (of 2 successive samples with defined stagnation time) contain higher ATP concentrations (except 8HS total samples from Leggiuno). One can also notice that the disparity between 1st and 2nd 500 ml free ATP samples is much lower comparing to the disparity between 1st and 2nd 500 ml total ATP samples.

The overall deduction is also that ATP concentrations of 23rd August samples are higher than those from 20th September. The effect of temperature may play a role here.
Figure 16  Dependence of ATP concentration on the stagnation time in tap water samples from Laveno (23/08/05)

Figure 17  Dependence of ATP concentration on the stagnation time in tap water samples from Laveno (20/09/05)
**ATP concentrations in 20 successive 50 ml tap water samples from Leggiuno and Laveno**

In order to trace in detail the distribution of total and free ATP concentration in 1 litre volume, 20 successive 50 ml tap water samples from Laveno and Leggiuno have been taken (see Annex 6.2). Prior to stagnation of 8 h and sampling a fully flushed sample has been taken. Samples have been gathered on 16/08/05.

Samples from Laveno show much higher total ATP concentrations in the first 10 successive samples, what confirms our observation from the previous experiment where ATP concentration in the 1st 500ml total samples after 8HS was much higher. Three first samples and 9th sample from Laveno present a little elevated ATP concentration (12-21 ng/l ATP). Other samples including FF sample are below 10 ng/l ATP concentration (Fig.18). The free ATP concentration doesn’t change much in the samples, regardless the increase of total ATP in some samples.

![Figure 18 ATP concentration in 20 successive tap water samples from Laveno after 8 HS (16/08/05)](image)

Samples collected in the same way in Leggiuno (Fig.19) present a completely different distribution of total ATP concentration. There isn’t much difference between the samples (15-20 ng/l), although significant lower ATP concentration in the first two samples can be observed (10 ng/l). These results confirm the results from the 500 ml sampling, where the 1st 500ml total samples after 8HS had a similar ATP concentration as the 2nd 500ml sample. Free ATP concentration constitutes around 30% of total one and don’t exceed 6 ng/l ATP.
The Dynamic Test Facility is programmable and simulates the water consumption by consumers. The instrument uses tap water provided by water production point (JRC, Ispra, Italy). Water before distribution is chlorinated by the addition of chlorine dioxide. Four different pipes are installed in the DTF: copper, stainless steel, galvanized and polypropylene. The DTF has been programmed to take samples after defined stagnation times, as described in experimental part. The aim of the experiment was to trace the distribution of total and free ATP concentrations depending on stagnation time in four different lines. The same experiment has been conducted on 01/07/05 and 20/09/05 (see Annex 6.3).

It can be noticed on the charts presented below that in general the ATP concentration gradually decreases with longer stagnation time for all materials. There is one exception for the galvanized pipe, where a gradual increase of the total ATP concentration is observed in samples obtained on 20th of September 2005. In addition, the ATP concentration in the first stagnation is mostly higher than in the second stagnation of the same period on the same day, with 2 exceptions for copper and polypropene on 20/09/05.

Generally ATP concentrations do not only differ between materials, but also within the same line one can notice variations in samples taken on two different measuring days.

On the 1st of July the ATP concentration was gradually decreasing in the copper pipe. On the other hand on the 20th of September 2005 an unusual distribution of total and free ATP concentration has been observed. All the samples from this day have a higher free ATP concentration then a total ATP concentration. The explanation to this phenomenon has not been found so far (see Fig.20 and Fig.21).
Figure 20 Dependence of ATP concentration on the stagnation time in copper pipe (01/07/05)

Figure 21 Dependence of ATP concentration on the stagnation time in copper pipe (20/09/05)
In the stainless steel pipe the ATP concentrations were quite elevated, up to 23 ng/l ATP for 0.5 and 1 h stagnation, on the 20th of September 2005 in comparison to results from 1st of July 2005. The difference is visible also in the distribution of total and free ATP concentration- in samples from 01/07/05 total and free ATP almost overlap, whereas on 20/09/05 they are separated with 8-20 ng/l ATP difference (see Fig.22 and Fig.23).

On the 01/07/05 a significant decrease of ATP concentration with longer stagnation time have been observed in the galvanized pipe, whereas on 20/09/05 reverse correlation was noticed. Total ATP concentration was gradually increasing with time and after 16 HS reached its maximum, thus 18.67 ng/l ATP. The difference between total and free ATP concentration was also increasing with longer stagnation times in samples from 20/09/05 (see Fig. 24 and Fig.25).

On the 01/07/05 ATP concentrations were decreasing with stagnation time in the polypropylene pipe and total and free ATP concentrations almost overlap. Similarly on the 20/09/05 total and free ATP concentrations were slowly decreasing with time, but unexpectedly total ATP concentration after 8HS slowly started to increase (see Fig.26 and Fig.27).

The overall conclusion for samples from all materials can be that total and free ATP concentrations are very close to each other (sometimes overlap) in the samples of 01/07/05, whereas the difference is larger in the samples of 20/09/05. The explanation can be that on 20/09/05 significant amounts of bacterial ATP has been detected whereas on 01/07/05 there have been mostly free ATP.

Figure 22 Dependence of ATP concentration on the stagnation time in stainless steel pipe (01/07/05)
Figure 23 Dependence of ATP concentration on the stagnation time in stainless steel pipe (20/09/05)

Figure 24 Dependence of ATP concentration on the stagnation time in galvanized pipe (01/07/05)
Figure 25 Dependence of ATP concentration on the stagnation time in galvanized pipe (20/09/05)

Figure 26 Dependence of ATP concentration on the stagnation time in polypropylene pipe (01/07/05)
Figure 27 Dependence of ATP concentration on the stagnation time in polypropylene pipe (20/09/05)
Overview of ATP concentrations in tap water samples from different distribution systems

On the 22nd –23rd of August 2005 samples from 6 different places have been collected. The purpose of this survey was to examine microbial activity of tap waters delivered by different distribution systems (see Fig.28). All samples contained 500 ml. FF samples have been taken on 22nd of August after 1 minute of flushing, xHS-1 samples have been taken after 4:40-9:30 hour’s stagnation on 23rd of August, xHS-2 samples have been taken directly after xHS-1 samples (see Annex 6.4).

None of the collected samples exceed 10 ng/l ATP, thus the microbial water activity was rather low. In all samples free ATP consisted less than 50% of total ATP. All xHS-1 samples, except the one in Foresteria, contained higher ATP concentrations than FF samples.

![Figure 28](image-url)  
**Figure 28** Total ATP concentration in tap water samples
HETEROPTROPHIC PLATE COUNTS OF TAP WATER SAMPLES

On the 24th of May 2005 samples have been collected on JRC premises (see list of samples below) and analyzed for total colony count. The sampling procedure started by taking 2 successive 500 ml samples (RDT – random day time), followed by 1 minute of flushing and taking a fully flushed sample (FF). From each numerated sample 1 ml has been put on a petri plate. PCA or R2A medium, kept in a water bath at 45°C, was added and carefully mixed. The dishes were incubated either at 22°C or 37°C. (see Annex 7). A control plate has been prepared for each medium and temperature. The samples on PCA and R2A [19] mediums incubated at 37°C were counted after 44 hours. Petri plates with mediums and samples have been put to incubator on 24/05/05 at 19:00 and counted on 26/05/05 at 15:00. The samples on PCA and R2A mediums incubated in 22°C were counted after 68 hours. Mediums with samples have been inserted to incubator on 24/05/05 at 19:00 and counted on 27/05/05 at 15:00.

List of samples collected on the JRC premises:

ED 30, room 004 - Milli-Q water (001)
ED 8, room 006 (old mensa):
   a. RDT 1 (002)
   b. RDT 2 (003)
   c. FF (004)
ED 8A, room 003 (new mensa):
   a. RDT 1 (005)
   b. RDT 2 (006)
   c. FF (007)
ED 15, room 004 (the water production point)- FF (008)
ED 30 A, room E15 (the men’s toilet):
   a. RDT 1 (009)
   b. RDT 2 (010)
   c. FF (011)
ED 30, room 012 (the valve used for Dynamic Test Facility):
   a. RDT 1 (012)
   b. RDT 2 (013)
   c. FF (014)

Experiment with total colony counts has been repeated on 31st of May 2005. Newly collected samples (see list of samples below) as previously have been analyzed as above (see Annex 7). The samples on PCA and R2A mediums incubated in 37°C have been inserted to incubator on 01/06/05 at 13:30 and counted on 03/06/05 at 15:00. The samples on PCA and R2A mediums incubated in 22°C have been inserted to incubator on 01/06/05 at 13:30 and counted on 06/06/05 at 10:00.

List of samples collected in Laveno, Leggiunio and on the JRC premises:

private house in Laveno, kitchen:
   a. RDT 1 (001)
   b. RDT 2 (002)
   c. FF (003)
private house in Leggiuno, kitchen:
   a. RDT 1 (004)
   b. RDT 2 (005)
   c. FF (006)
Comparison between HPC and ATP assay

An attempt has been made to compare the numbers of microbial cells counted on the mediums and those calculated from the concentration of ATP. All samples cultivated on mediums have been also analyzed for total and free ATP concentrations. ATP concentrations in the samples have been calculated using method for ATP calculation in the samples. ATP values were calculated in ng/l ATP.

In order to estimate the amount of bacteria from the ATP concentration, the concentration of ATP in the cell was assumed to be 1 fg ATP/cell [9,21].

Calculated numbers of cells should represent only living bacteria and therefore free ATP concentrations have been subtracted from the total ATP concentrations to receive the microbial ATP concentrations in the samples. Since on the PCA medium microbial growth was rather limited, the comparison has been made for R2A medium at both temperatures.

Results of the comparison between microbial numbers cultivated on R2A mediums and calculated using the ATP assay show that the cell concentration calculated from the ATP concentrations are higher that the real measured colonies (Fig.29, Fig.30, Fig.31, Fig.32). Differences can be due to the presence of non-culturable bacteria. There is also a unknown uncertainty in the assumption of 1 fg ATP per cell.

Detection limit for number of microbial cells counted on mediums has been assessed as $10^3$ cells/litre. On R2A medium only one colony have been observed in 1ml in 22°C, but since the number of colonies had to be recalculated and presented in litres the value has been multiplied. Therefore the detection limit went up to $10^3$ cells/litre.
Figure 29  Comparison between number of microbial cells counted on R2A medium in 22\(^\circ\) C and calculated using ATP assay (24/05/05)

Figure 30  Comparison between number of microbial cells counted on R2A medium in 37\(^\circ\) C and calculated using ATP assay (24/05/05)
Figure 31  Comparison between number of microbial cells counted on R2A medium in 22° C and calculated using ATP assay (01/06/05)

Figure 32  Comparison between number of microbial cells counted on R2A medium in 37° C and calculated using ATP assay (01/06/05)
CONCLUSIONS

1) The detection limit of ATP is determined as 1 ng/l.

2) Polyethylene cuvettes used in all experiments gave a background level of about 40 RLU. The instrument discharged the cuvettes properly even if they were made static. The volume of water sample doesn’t have an influence on the background level. Sunlight exposure has an ambiguous influence on RLU values. The background level may vary significantly from batch to batch and it seems better to keep samples in the dark prior to measurement.

3) RLU values of the samples with the same ATP concentrations and the same overall volume are dependent on the concentration of the reagents:
   a) Samples treated with only light generating reagent LuminATE give higher response comparing to samples treated additionally with LuminEX (quenching effect)
   b) The higher the volume of LuminATE used during the measurement the higher will be the RLU value

4) ATP concentrations prepared with tap water show gradual degradation when stored at 24°C. The half-life is 4-5 days and 8-9 days for 20-200 ng/l ATP and 1-20 ng/l ATP concentration, respectively. Half-life for all ATP concentrations prepared with tap water and keep at 4°C is in the range of 8-10 days. ATP concentrations prepared with milli-Q water and kept at 4°C and 24°C are rather stable with time.

5) Tap water samples collected in Leggiuno gradually decrease with longer stagnation times, fully flushed samples and short stagnation time samples contain the highest ATP concentrations. Tap water samples from Laveno show completely reverse distribution, ATP concentrations increase very slowly with stagnation time.

6) Almost all first 500 ml samples (of totally 1 litre of 2 successive RDT samples with defined stagnation time) contain higher ATP concentrations. Leggiuno samples had very similar ATP concentrations.

7) 20 successive 50 ml samples collected in Laveno and Leggiuno confirmed the results of the 500 ml samples. In addition the 50 ml samples show an ATP production in the distribution system near the tap of the house in Laveno. The house in Leggiuno seems to be supplied with drinking water which is rather biologically active.

8) ATP concentrations in all tubes in the Dynamic Test Facility were decreasing with increasing stagnation time. One exception was observed for galvanized steel with a reverse ATP stagnation curve. The first 0.5 and 1 hour stagnation times on a day gave always higher values than the second ones. ATP concentration was also slowly decreasing with time in Leggiuno samples.

9) It is not possible to compare results of tap water samples with results of DTF samples. Samples probably contained different physical and chemical parameters, but real values were not measured. In future experiments they should be taken into account (e.g. it is essential to check chlorine levels in tap water, prior to sampling).

10) Incoming water samples in DTF should be also included in sampling protocol of future experiments (water from the main valve).

11) Control plates (blank) show that mediums with tap water samples have been prepared properly – there weren’t any colonies forming.
12) Comparison between numbers of microbial cells counted on R2A mediums and calculated using ATP assay show that results doesn’t fit very well. Whereas for some samples the data are comparable, for others the difference in number of microbial cells is within three orders of magnitude higher. The number of microbial cells calculated using ATP concentrations is higher most probably due to the fact that not all bacterial cells were culturable on mediums.

13) On the PCA medium in both temperatures microbiological growth was lower than on the R2A medium, therefore R2A was a better medium for microbiological growth in our samples. The highest bacterial growth was on R2A medium in 22°C, than on R2A medium in 37°C. On PCA medium growth was mostly better in 37°C than in 22°C.

14) ATP can be a good, fast and sensitive indicator of microbiological activity in tap water samples.
REFERENCES


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20. Somberg R., Pferdehirt B., Kupcho K., Promega Corporation: A Universal Kinase Assay for a World of Kinases”, Introducing the Kinase-Glo<sup>™</sup> Luminescent Kinase Assay, Number 83 2003


## ANNEX 1 DETECTION LIMIT OF ATP

### Table 1 detection limit for total ATP

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ANNEX 2 CALIBRATION DATA

Table 2  Calibration of total ATP on 4 May 2005 in milliQ water

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Table 4  Calibration of free and total ATP on 31 May 2005 in milliQ water

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### Table 5  Calibration of free and total ATP on 9 June 2005 in milliQ water, sterile water and water of water supply

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Table 6  Calibration of free and total ATP on 1 July 2005 in sterile water and water of water supply

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Table 7  Calibration of free and total ATP on 16 August 2005 in water of water supply

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Table 8  Calibration of free and total ATP on 23 August 2005 in water of water supply

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Table 9  Calibration of free and total ATP on 20 September 2005 in water of water supply

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<th>b</th>
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▲  these ATP concentrations have been inserted and measured between samples for control.
Table 10  Calibration of total ATP on 14 October 2005 in milliQ water and water of water supply

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▲ these ATP concentrations have been inserted and measured between samples for control.
## ANNEX 3 RLU RESPONSE

### Table 11  Effect of the volume of water in the cuvette on the background response

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<th>stdev RLU</th>
<th>rstdev %</th>
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Table 12  effect of sunlight on the background response of cuvettes filled with 100 µl milliQ water

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<td>44</td>
<td>40</td>
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<td></td>
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<td></td>
</tr>
<tr>
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<td>49</td>
<td>60</td>
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<td></td>
</tr>
</tbody>
</table>

Table 13  Effect of sunlight on the background response of cuvettes filled with 100 µl milliQ water made anti-static by ethanol

<table>
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<tr>
<th>Exposure time</th>
<th>Response RLU</th>
<th>Background RLU</th>
<th>average RLU</th>
<th>stdev RLU</th>
<th>rstdev %</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 min</td>
<td>52</td>
<td>70</td>
<td>50.33</td>
<td>4.27</td>
<td>8.49</td>
<td>6</td>
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<tr>
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<td>49</td>
<td>50</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 min</td>
<td>51</td>
<td>40</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 min</td>
<td>49</td>
<td>60</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 min</td>
<td>57</td>
<td>40</td>
<td></td>
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<td></td>
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<td></td>
<td></td>
</tr>
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<td>50</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
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<td>43</td>
<td>50</td>
<td></td>
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</tr>
<tr>
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<td>46</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>2 min</td>
<td>49</td>
<td>30</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 min light-2 min dark</td>
<td>40</td>
<td>20</td>
<td>42.17</td>
<td>2.23</td>
<td>5.29</td>
<td>6</td>
</tr>
<tr>
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<td>43</td>
<td>50</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 min light-2 min dark</td>
<td>42</td>
<td>40</td>
<td></td>
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</tr>
<tr>
<td>2 min light-2 min dark</td>
<td>46</td>
<td>50</td>
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</tr>
<tr>
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<td>40</td>
<td>60</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>2 min light-5 min dark</td>
<td>43</td>
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<td>45.33</td>
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<td>5.70</td>
<td>6</td>
</tr>
<tr>
<td>2 min light-5 min dark</td>
<td>46</td>
<td>30</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 min light-5 min dark</td>
<td>43</td>
<td>40</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
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<td>43</td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>2 min light-5 min dark</td>
<td>49</td>
<td>40</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 min light-5 min dark</td>
<td>47</td>
<td>40</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 min light-5 min dark</td>
<td>42</td>
<td>40</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>46.00</td>
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<td>7.01</td>
<td>6</td>
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<td>40</td>
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<td>60</td>
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<td></td>
</tr>
<tr>
<td>0 min made static</td>
<td>43</td>
<td>40</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>0 min made static</td>
<td>48</td>
<td>40</td>
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</table>

Table 14  Effect of LuminEx on response of a 200 ng/l solution of ATP

<table>
<thead>
<tr>
<th>Sample</th>
<th>Response RLU</th>
<th>Background RLU</th>
<th>average RLU</th>
<th>stdev RLU</th>
<th>rstdev %</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>100µl LuminEX</td>
<td>2833</td>
<td>50</td>
<td>2774.33</td>
<td>155.5</td>
<td>5.61</td>
<td>3</td>
</tr>
<tr>
<td>100µl LuminEX</td>
<td>2892</td>
<td>50</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100µl LuminEX</td>
<td>2598</td>
<td>50</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100µl H2O</td>
<td>3010</td>
<td>70</td>
<td>3125.67</td>
<td>105.3</td>
<td>3.37</td>
<td>3</td>
</tr>
<tr>
<td>100µl H2O</td>
<td>3151</td>
<td>50</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100µl H2O</td>
<td>3216</td>
<td>40</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 15  Effect of concentration of LuminATE on the response of mixture of 100 µl of 200 ng/l ATP, while the total volume remains constant

<table>
<thead>
<tr>
<th>Volume</th>
<th>Response RLU</th>
<th>Background RLU</th>
</tr>
</thead>
<tbody>
<tr>
<td>150 µl H2O + 50 µl LuminATE</td>
<td>1150</td>
<td>60</td>
</tr>
<tr>
<td>100 µl H2O + 100 µl LuminATE</td>
<td>3189</td>
<td>70</td>
</tr>
<tr>
<td>50 µl H2O + 150 µl LuminATE</td>
<td>2004*</td>
<td>40</td>
</tr>
<tr>
<td>150 µl H2O + 50 µl LuminATE</td>
<td>1135</td>
<td>40</td>
</tr>
<tr>
<td>100 µl H2O + 100 µl LuminATE</td>
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<td>40</td>
</tr>
<tr>
<td>50 µl H2O + 150 µl LuminATE</td>
<td>4708</td>
<td>40</td>
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</table>

* the response in this sample is not correct, since a wrong method had been used
## ANNEX 4 METHODS FOR ATP CALCULATION IN THE SAMPLES

### Table 16  Samples of 09/06/05 calculated from calibration curve X = (y-b)/a

<table>
<thead>
<tr>
<th></th>
<th>Response</th>
<th>Background</th>
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<th>0-2000 ng/l</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>RLU</td>
<td>RLU</td>
<td>supply ng/l</td>
<td>sterile ng/l</td>
</tr>
<tr>
<td>Total ATP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>001</td>
<td>98</td>
<td>40</td>
<td>-4.64</td>
<td>4.92</td>
</tr>
<tr>
<td>002</td>
<td>87</td>
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<td>-5.23</td>
<td>4.35</td>
</tr>
<tr>
<td>003</td>
<td>78</td>
<td>40</td>
<td>-5.71</td>
<td>3.89</td>
</tr>
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<td>86</td>
<td>40</td>
<td>-5.29</td>
<td>4.30</td>
</tr>
<tr>
<td>005</td>
<td>148</td>
<td>50</td>
<td>-1.97</td>
<td>7.50</td>
</tr>
<tr>
<td>MilliQ</td>
<td>49</td>
<td>30</td>
<td>-7.27</td>
<td>2.39</td>
</tr>
<tr>
<td>Free ATP</td>
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<td></td>
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<tr>
<td>001</td>
<td>101</td>
<td>50</td>
<td>-5.89</td>
<td>6.55</td>
</tr>
<tr>
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<td>94</td>
<td>30</td>
<td>-6.16</td>
<td>6.31</td>
</tr>
<tr>
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<td>88</td>
<td>30</td>
<td>-6.38</td>
<td>6.11</td>
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<td>82</td>
<td>40</td>
<td>-6.61</td>
<td>5.91</td>
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<tr>
<td>005</td>
<td>57</td>
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<td>-7.55</td>
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<table>
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<th>1-2000 ng/l</th>
<th>1-2000 ng/l</th>
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</thead>
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<td>40</td>
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<td>-5.87</td>
</tr>
<tr>
<td>003</td>
<td>78</td>
<td>40</td>
<td>-6.35</td>
</tr>
<tr>
<td>004</td>
<td>86</td>
<td>40</td>
<td>-5.92</td>
</tr>
<tr>
<td>005</td>
<td>148</td>
<td>50</td>
<td>-2.60</td>
</tr>
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<td>-7.90</td>
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<td>101</td>
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<td>94</td>
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<td>-6.92</td>
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<td>82</td>
<td>40</td>
<td>-7.38</td>
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<td>70</td>
<td>-8.54</td>
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</table>
Table 17  Samples of 09/06/05 calculated from slope calibration curve “a” and the background measured: \( X = \frac{y \text{-background}}{a} \)

<table>
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<tr>
<th>Response</th>
<th>Background</th>
<th>1-200 ng/l</th>
<th>ng/l</th>
<th>ng/l</th>
<th>ng/l</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RLU</td>
<td>RLU</td>
<td>supply</td>
<td>sterile</td>
<td>Milli-Q</td>
</tr>
<tr>
<td>Total ATP</td>
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<td>40</td>
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<td>5.42</td>
</tr>
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<td>4.81</td>
</tr>
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<td>40</td>
<td>3.97</td>
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<td>1.92</td>
<td>1.65</td>
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</table>

Table 18  Samples of 09/06/05 calculated from slope calibration curve “a” and the average value of the empty cuvettes: \( X = \frac{y \text{-factor}}{a} \)

<table>
<thead>
<tr>
<th>Response</th>
<th>Background</th>
<th>1-200 ng/l</th>
<th>ng/l</th>
<th>ng/l</th>
<th>ng/l</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RLU</td>
<td>RLU</td>
<td>supply</td>
<td>sterile</td>
<td>Milli-Q</td>
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<td>40</td>
<td>2.95</td>
<td>3.21</td>
</tr>
<tr>
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<td>2.39</td>
<td>2.60</td>
</tr>
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<td>1.94</td>
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<tr>
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<td>5.50</td>
<td>5.97</td>
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<td>0.50</td>
<td>0.46</td>
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<tr>
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<td>50</td>
<td>2.17</td>
<td>2.29</td>
</tr>
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<td>0.41</td>
<td>0.36</td>
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</table>
## ANNEX 5 ATP STABILITY AND DEGRADATION

### Table 19 ATP degradation for four days (16-20/09/05)

<table>
<thead>
<tr>
<th></th>
<th>Response RLU</th>
<th>Bkgnd RLU</th>
<th>Corrected concentration* RLU</th>
<th>Corrected concentration* ng/l</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total ATP, 24°C</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>wat.prod.point</td>
<td>110</td>
<td>40</td>
<td>4.68</td>
<td></td>
</tr>
<tr>
<td>1 ng/l water.prod.point</td>
<td>109</td>
<td>40</td>
<td>4.62</td>
<td>-0.07</td>
</tr>
<tr>
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<td>5.15</td>
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<td>12.98</td>
<td>8.30</td>
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<td>108.18</td>
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<td>milli-Q water</td>
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<td>40</td>
<td>0.40</td>
<td></td>
</tr>
<tr>
<td>1 ng/l milli-Q water</td>
<td>61</td>
<td>20</td>
<td>1.40</td>
<td>1.00</td>
</tr>
<tr>
<td>2 ng/l milli-Q water</td>
<td>79</td>
<td>50</td>
<td>2.61</td>
<td>2.21</td>
</tr>
<tr>
<td>20 ng/l milli-Q water</td>
<td>350</td>
<td>40</td>
<td>20.74</td>
<td>20.34</td>
</tr>
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<td>200 ng/l milli-Q water</td>
<td>3568</td>
<td>40</td>
<td>236.04</td>
<td>235.64</td>
</tr>
<tr>
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<td>6690458.13</td>
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</tr>
<tr>
<td><strong>Free ATP, 24°C</strong></td>
<td></td>
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<td></td>
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Table 20 ATP degradation experiments in the period of 4-14/10/05

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* correction for the ATP content of the water of the water supply or milliQ water
### ANNEX 6 INFLUENCE OF STAGNATION TIME ON ATP CONCENTRATION IN TAP WATER SAMPLES

**Table 21a** Fully Flushed and two successive stagnation samples of 500 ml from Leggiuno on 23 August 2005

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<th>Stagnation time h</th>
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<th>Concentration ng/l</th>
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Table 21b Fully Flushed and two successive stagnation samples of 500 ml from Laveno on 23 August 2005

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<th>Bkgnd RLU</th>
<th>Concentration ng/l</th>
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Table 22  Fully Flushed and two successive stagnation samples of 500 ml from Laveno on 20/09/05

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## ANNEX 7 HETEROTROPHIC PLATE COUNTS OF TAP WATER SAMPLES

### Table 29  HPC of samples taken on 24/05/05

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a  small colonies  
b  white colonies  
c  yellow colonies  
d  1 colony 0.5 cm Ø  
e  circa 400 colonies in one quarter  
f  medium not transparent  
g  medium was 33-50% dry  
h  solid particles  
i  near the number of counted colonies means that the calculation was very tentative, due to fact that it was difficult to decide whether there have been colonies or solid particles
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<th>Note</th>
<th>PCA 37°C cfu/ml</th>
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- **a** small colonies
- **b** white colonies
- **c** yellow colonies
- **d** fungi
- **d** medium is opaque
- **e** brown colonies
- **f** orange colonies
Mission of the JRC

The mission of the JRC is to provide customer-driven scientific and technical support for the conception, development, implementation and monitoring of EU policies. As a service of the European Commission, the JRC functions as a reference centre of science and technology for the Union. Close to the policy-making process, it serves the common interest of the Member States, while being independent of special interests, whether private or national.