CPDW project

Assessment of the microbial growth support potential of products in contact with drinking water

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Assessment of the microbial growth support potential of construction products in contact with drinking water (CPDW)

Development of a harmonised test to be used in the European Acceptance Scheme concerning CPDW

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Preface

The investigations described in this report were conducted as part of the European Project Development of harmonised tests to be used in the European Approval Scheme (EAS) concerning Construction Products in Contact with Drinking water (CPDW), under Contract no. EVK1-CT2000-00052. This project is financially supported by the European Commission, the national authorities of Austria, Denmark, France, Germany, the Netherlands and the United Kingdom and the material suppliers in these countries and Europe, respectively. Work Package 1 of this project concerned the microbial-growth promoting properties of materials. The institutes participating in the investigations and discussions in WP1 are listed below.

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This report describes the results as obtained in WP1 of the project. The reports of the participants about their observations in the various project stages have been collected in a separate appendices report.
### Abbreviations

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<th>Abbreviation</th>
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<tr>
<td>AOC</td>
<td>Assimilable Organic Carbon (µg C/l)</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate (ng/l; pg/cm²)</td>
</tr>
<tr>
<td>BP</td>
<td>Biomass Production (ng ATP/l; pg ATP/cm²)</td>
</tr>
<tr>
<td>BPP</td>
<td>Biomass Production Potential (ng ATP/l; pg ATP/cm²)</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony Forming Units (bacteria)</td>
</tr>
<tr>
<td>CPDW</td>
<td>Construction Products in Contact with Drinking Water</td>
</tr>
<tr>
<td>DOC</td>
<td>Dissolved Organic Carbon (mg/l)</td>
</tr>
<tr>
<td>EAS</td>
<td>European Acceptance Scheme</td>
</tr>
<tr>
<td>EPDM</td>
<td>Ethylene Propylene Diene Monomer</td>
</tr>
<tr>
<td>NBR</td>
<td>Nitrile Rubber</td>
</tr>
<tr>
<td>PE</td>
<td>Polyethylene</td>
</tr>
<tr>
<td>HDPE</td>
<td>High Density PE</td>
</tr>
<tr>
<td>HPC</td>
<td>Heterotrophic Plate Count (CFU/ml; CFU/cm²)</td>
</tr>
<tr>
<td>PVC</td>
<td>Polyvinylchloride</td>
</tr>
<tr>
<td>PVC-C</td>
<td>Chlorinated PVC</td>
</tr>
<tr>
<td>PVC-P</td>
<td>Plasticized PVC</td>
</tr>
<tr>
<td>PVC-U</td>
<td>Unplasticized PVC</td>
</tr>
<tr>
<td>Q₀</td>
<td>Quotient of reaction rates at a 10°C temperature difference</td>
</tr>
<tr>
<td>RSD</td>
<td>Relative Standard Deviation (%)</td>
</tr>
<tr>
<td>SILR</td>
<td>Silicone Rubber</td>
</tr>
<tr>
<td>SS</td>
<td>Stainless Steel</td>
</tr>
<tr>
<td>S/V</td>
<td>Surface area to volume contact ratio (cm⁻¹)</td>
</tr>
<tr>
<td>VCᵣ</td>
<td>Coefficient of variation of reproducibility (%)</td>
</tr>
<tr>
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Summary

A harmonised test method is needed for determining the microbial growth promoting properties of construction products in contact with drinking water (CPDW). To achieve this goal, the characteristics of the existing methods have been evaluated in discussions. The methods considered included the Mean Dissolved Oxygen Difference (MDOD) test as applied in the UK, the German W270 method, the Austrian test and the Biomass Production Potential (BPP) test developed in The Netherlands. Criteria for the quality of the test water were defined based on the criteria for tap water used for human consumption and criteria as defined for the existing tests. Also it was decided to design a test based on semi-static conditions, i.e. a batch test with regular water replacement, and to maintain the surface area to volume (S/V) ratio (0.16 cm⁻¹) as used in the MDOD and the BPP tests. An incubation temperature of 30 °C was considered most appropriate, but initial experiments were conducted at 25 °C. A limited variety of CPDW, viz. Glass, HDPE, Nitrile Rubber, PVC-C, PVC-P, Silicone Rubber and Stainless Steel, was selected to determine the microbial growth support potential at different test conditions.

Adenosine triphosphate (ATP) was used in the investigations as the parameter for microbial biomass. As a first step, a standardised method for ATP analysis was introduced in the laboratories of the participants and Round Robin tests (‘ring tests’) were organised to further improve the performance of the method. The use of different diluents (buffer, tap water, or demineralised water) for preparing the calibration solutions was the main cause of the relatively large variations in the ATP concentrations reported in these ring tests. Light production in the assay decreases with increasing salt concentration, with calcium as a main inhibiting compound. Identical water types must be used for the calibration solutions to achieve similar results in ring tests. To obtain the real ATP value in a test (with a product), calibration should either be conducted in the test water or a conversion factor for the ATP yield in the test water and the calibration solution should be known. Addition of a known amount of ATP to the sample to be analysed is another option.

Each participant selected an appropriate (tap) water type for use in the investigations with a test determining the growth potential of the water. Water types with a maximum Biomass Production (BP) below 10 ng ATP/l were considered suitable. A 1 % river water inoculum had no impact on the BP value of the selected tap water. The BP value in the presence of certain products was higher with inoculum than with only the indigenous bacteria of the test water. For these reasons it was decided to use of 1% river water inoculum in the test. Thoroughly-cleaned borosilicate flasks with a plastic screw cap were found suited for the investigations.

The effect of water replacement on the biomass production of selected products was tested, with frequencies ranging from 0 (as used in the original BPP test), once in two weeks, once a week and twice a week (as used in the
MDOD test). BP values with a number of products were slightly, but significantly, elevated in the test without water replacement in comparison to values in tests with water replacement. Products with relatively strong growth-promoting properties (Nitrile Rubber, PVC-P) however, had higher BP values with water replacement, suggesting that a lack of oxygen and/or an inorganic nutrient may limit growth with such products when the water is not replaced. A water replacement frequency of once a week was considered most appropriate.

The reproducibility of the BPP test with water replacement was tested in jars with a wide neck, each containing 900 ml of test water and three product pieces of about 50 cm² each. Tests were conducted with selected products at 10°C (one laboratory), 22°C (one laboratory), 25°C (five laboratories) and at 30°C (three laboratories). The BPP value, which is the mean of the BP values observed on days 56, 84 and 112, of the selected products tested at 25°C ranged from about 25 pg ATP/cm² (glass) to more than 40,000 pg ATP/cm² (PVC-P). The relative standard deviation (RSD) of the mean of the BP values ranged from 3% to 73% with a median value of 33%. Differences in average RSD values between laboratories indicated that further improvement of the test is achievable. The coefficient of variation of reproducibility (VCv) ranged from 34 to 107% for the tests conducted at 25°C (six products in five laboratories) and from 20 to 34% for the tests conducted at 30°C (three products in three laboratories). Critical factors to improve the performance of the method are the quality (biostability) of the test water and the quality of the ATP analysis (calibration).

Temperature had a distinct impact on the BPP values, with lower values at higher temperature for most tested products. This effect is probably caused by the increase of endogenous respiration at increasing temperature, resulting in less biomass production. With some products temperature increase may accelerate biodegradability and/or the release of biodegradable compounds. To account for these effects it is concluded that an incubation temperature of 30°C is preferred to 25°C.

To obtain information about the interpretation of the BPP values, selected products were tested with the existing methods and in test systems with a continuous flow, simulating practical conditions. Apart from a relationship obtained between MDOD values and BPP values, no relations could be derived from the comparisons with the W270 test and the Austrian method, respectively. The number of products tested was too limited and/or test conditions differed from those described in the original methods. Biomass production in the test rig (DTU) was limited on most products after a test period of 40 days. Limitation of inorganic nutrients and/or oxygen may have delayed growth on PVC-P in this system. Biofilm concentrations on PVC-C, Silicone Rubber, and PVC-P in the biofilm monitor with a continuous flow (0.2 m/s) of tap water were in good agreement with the observations in the BPP test.

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In some experiments it was observed that application of high-energy sonication (HES) after the series of low energy sonication (LES) treatments resulted in additional biomass detachment. A project extension was obtained and further experiments were conducted to elucidate the impact of the nature and duration of sonication on biomass yield. A significant increase in yield after the application of an additional HES treatment was observed only with the soft material PVC-P. Results obtained with one HES treatment were similar to values obtained with six LES treatments in sequence. Two HES treatments in sequence or one HES treatment after six LES treatments gave high biomass recoveries (>90%) for all products tested. Both LES and HES treatments are relatively laborious and alternative methods for biomass detachment should be investigated.

The collected data demonstrated that the BPP test with water replacement once a week and a duration of 16 weeks is a promising method for determining the growth-promoting properties of CPDW. The test covers BPP values ranging from less than 50 pg ATP/cm² (as observed for glass, stainless steel and PVC-C) to more than 40,000 pg ATP/cm² (PVC-P). The use of ATP for determining biomass concentrations in drinking water and on surfaces in contact with drinking water in distribution systems facilitates the interpretation of the BPP values of CPDW.

A number of aspects needs further standardisation, including:
- test water quality (biostability, inorganic nutrients),
- ATP analysis
- biomass detachment.

Further comparison with existing methods and studies aiming at determining the effects of biofilm concentrations on the microbiological quality of water intended for human consumption are needed to establish pass-fail criteria for CPDW, based on BPP testing.
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1 Introduction

1.1 Water quality deterioration caused by regrowth
Maintaining the quality of drinking water during transportation, storage and distribution is a main objective in water supply. Measures are taken to protect treated water against external contamination as may be caused by cross-connections, back siphonage and other types of ingress. Biomass formation ('regrowth') in drinking water distribution systems and in plumbing systems may also cause a deterioration of the microbiological quality of water used for consumption and other domestic purposes. Multiplication of microorganisms in these systems depends on a combination of conditions ('risk factors'), viz. (i) the concentration of biodegradable compounds, (ii) elevated temperature and (iii) residence time. Water temperature and residence time are difficult to control and limitation of regrowth therefore generally is achieved by maintaining a disinfectant residual in drinking water during distribution and/or by limiting the concentration of biodegradable compounds. Biodegradable compounds present in treated water may originate from the raw water or from certain water treatment processes (e.g. oxidation). Accumulated sediments, interactions between corroding iron and humic compounds as well as the release of biodegradable compounds from synthetic or natural construction products in contact with water may also enhance microbial processes during distribution. This report will focus on the growth-promoting properties of construction products.

Frequently observed water quality problems caused by the multiplication of microorganisms in distributions and plumbing systems are:

- High heterotrophic plate counts (HPC). Elevated HPC values, exceeding criteria as defined in national legislation, is the most commonly reported signal of a deterioration of the microbiological water quality in water supply systems. In many situations coatings releasing biodegradable solvents have been identified as the cause of an increase of HPC values in storage tanks, reservoirs and pipes. Increased HPC values also have been reported with other materials including PE, PVC and cementitious materials with organic constituents (Burman and Colbourne, 1977; Schoenen and Schöler, 1983; Frensch et al. 1987; Bernhardt and Liesen, 1988);

- Presence of coliforms. Coliform ron compliance has frequently been reported, also in water supply systems with a disinfectant residual (Wieringa, 1985; Smith et al. 1990). Sediments and interactions between organic compounds in the water and the surface of corroding pipes may enhance coliform growth (Baylis 1930; Camper et al. 1999). Certain materials e.g. wood, jarn, leather, catings, lubricants, have been found to enhance the growth of coliform bacteria (Burman and Colbourne, 1977; Seidler et al. 1977; Ellgas and Lee, 1980; Schoenen und Schöler, 1983);

- Presence of opportunistic pathogens including Legionella pneumophila, Pseudomonas aeruginosa and Mycobacterium spp. Exposure to elevated levels
of these bacteria in water poses a potential health threat, especially for immunocompromised persons. In 1980, Legionella present in warm tap water was identified as the etiological agent for waterborne cases of pneumonia (Tobin et al. 1980; Cordes et al. 1981). The maximum number of annually reported cases of legionellosis (pneumonia) approached 20 per million persons in some countries in Europe in 1999 (WHO, 2000). However, not all cases were diagnosed and/or reported and not all reported cases were related to Legionella in tap water systems. Legionella multiplies in certain protozoa grazing on biofilm bacteria (Rowbotham, 1980; Abu Kwaik, 1998). Certain rubber components, other elastomers and plastic materials may promote the multiplication of Legionella by enhancing biofilm formation (Colbourne et al. 1984; Hengesbach et al. 1993; Niedeveld et al. 1986; Rogers et al. 1994; Schoenen et al. 1988; Schofield and Locci 1984). P. aeruginosa also is an etiological agent for waterborne infections, mainly nosocomial pneumonia (Anaissie et al. 2002). Mycobacterium spp. (avium complex) multiplying in tap water systems can infect AIDS patients (Von Reyn et al. 1994). Mycobacteria have been observed in biofilms on material surfaces (Falkinham et al. 2001; Schultze-Röckecke and Fischeder, 1989).

Aesthetic problems. Microbial growth can cause aesthetic problems, e.g. the presence of flocs, turbidity, invertebrates or impair the flavour and odour of the water (Burman and Colbourne, 1977; Schoenen und Schöler, 1983; Van Lieverloo et al. 1994).

Maintaining a disinfectant residual in drinking water during distribution is a commonly applied measure to control regrowth. However, the formation of undesirable byproducts and complaints about flavour and odour in relation to the presence of disinfectants stimulate limitation of the growth potential of water and construction products as alternative or additional control measure. In the past two decades methods have been developed for determining the microbial-growth promoting properties of drinking water, e.g. assimilable organic carbon (AOC), biodegradable dissolved organic carbon (BDOC) (van der Kooij et al. 1982; Joret et al. 1985). Present European and national legislation demands that materials in contact with tap water should not reduce the protection of human health as provided for in the Council Directive 98/83/EC (European Union, 1998). Consequently, given the potential of certain construction products to enhance the growth of microorganisms and the steadily increasing number (and application) of synthetic materials, a generally accepted method and pass-fail criteria are needed to enable selection of construction products on the basis of their growth-promoting properties.

1.2 Existing methods for determining the microbial growth potential of construction products

Methods for determining the growth-promoting properties of construction products have been developed in several European countries. The Mean Oxygen Difference (MDOD) test as described by Colbourne and Brown (1979) has become the standard method in the UK (BS 6920-2.4; British Standard, 2000). The Slime Production (SP) test as developed by Schoenen (Schoenen
and Schölfer, 1983) is the standard method in Germany (DVGW, 1998). In the Netherlands, the BPP test has been developed (van der Kooij and Veenendaal, 1994; Van der Kooij and Veenendaal, 2001). More recently also in Austria a method has been introduced to determine the growth promoting properties of pipe materials in contact with drinking water (Önorm B5018, 2002 a,b). Table 1.1 gives an overview of the main characteristics of these methods. Brief descriptions are given below.

Table 1.1 Characteristics of methods for determining the growth potential of materials in contact with drinking water

<table>
<thead>
<tr>
<th>Condition/parameter</th>
<th>MDOD (UK)</th>
<th>W270 (Germany)</th>
<th>BPP (NL)</th>
<th>Önorm B5018 (Austria)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature (°C)</td>
<td>30 ± 1</td>
<td>Ambient (&gt; 6°C)</td>
<td>25 ± 1</td>
<td>22 ± 2</td>
</tr>
<tr>
<td>Surface (S) of material (cm²)</td>
<td>150</td>
<td>800</td>
<td>12 x 8</td>
<td>1256 – 3140*</td>
</tr>
<tr>
<td>Volume (V) of water (cm³)</td>
<td>1000</td>
<td>&gt;100,000**</td>
<td>600</td>
<td>1256 – 7850*</td>
</tr>
<tr>
<td>S/V (cm⁻¹)</td>
<td>0.15</td>
<td>n.a.**</td>
<td>0.16</td>
<td>1</td>
</tr>
<tr>
<td>Replacement</td>
<td>Twice a week</td>
<td>Continuously</td>
<td>none</td>
<td>Once a week</td>
</tr>
<tr>
<td>Water type</td>
<td>Tap water#</td>
<td>Tap water#</td>
<td>SSF##</td>
<td>Tap water</td>
</tr>
<tr>
<td>Duration (weeks)</td>
<td>7.5</td>
<td>26</td>
<td>16</td>
<td>12</td>
</tr>
<tr>
<td>Microbiological activity</td>
<td>Oxygen</td>
<td>Slime volume</td>
<td>ATP</td>
<td>ATP/HPC</td>
</tr>
</tbody>
</table>

*, depending on pipe diameter (4 cm or 10 cm respectively); **, continuous flow (2l/1/h); n.a., not applicable; #, dechlorinated (by applying GAC filtration or addition of thiosulfate) when needed; **, SSF, slow sand filtrate.

1.2.1 Mean Oxygen Difference (MDOD) test
In the MDOD test the additional oxygen consumption in the presence of the material to be tested is used as the parameter for microbiological activity. This method has been used for several decades and a large number of materials has been tested (Colbourne, 1985). Typical MDOD values range from about 0.5 mg/l (blank with glass) to values of about 8 mg/l for paraffin wax (positive control). Materials with an MDOD value > 2.3 mg/l are considered not to be suited for use in contact with water intended for human consumption (Colbourne, 1985).

1.2.2 Slime Production (SP) test
The Slime Production (SP) test as developed in Germany has also been used for a long time (Schoenen and Schölfer, 1983). This method is applied in a flow through (dynamic) system, with sheets (800 cm²) of materials in contact with continuously flowing tap water. The volume of slime on the surface of the tested material is used as the biomass parameter. Typical SP values range from less than 0.1 ml (Stainless Steel blank) to more than 15 ml on solvent-containing bitumen or plasticized PVC (PVC-P). Materials with an SP value > 0.1 ml are considered unsuitable for use in contact with drinking water (DVGW, 1998).
The MDOD and SP tests have been compared on a number of PVC materials (Schoenen and Colbourne, 1987). Apart from one material, the test results in combination with the defined criterion gave the same conclusion regarding pass/failure of the tested materials. Subsequent tests using a wider range of material types did not show the same degree of agreement.

1.2.3 The Biomass Production Potential (BPP) Test

The BPP test is derived from the Biofilm Formation Potential test (van der Kooij and Veenendaal, 1994). In these tests Adenosinetriphosphate (ATP) is used as parameter for active biomass. ATP is an energy-rich compound, which is present in all living (= active) organisms. ATP analysis enables the detection of very low concentrations of microorganisms. The detection limit of the test when applied to (tap) water is 1 ng/l. Furthermore, the analysis can be conducted within a few minutes.

In the BPP test the production of active biomass (ATP) as a function of time in the presence of the material to be tested is determined. The BPP test is carried out as a static test without replacement of the water. In this situation the concentration of active biomass, as measured with ATP, is in balance with the supply of biodegradable compounds originating from the material. The Biomass Production (BP, pg ATP/cm²) is calculated from the concentration of attached biomass (biofilm) and the concentration of planktonic (suspended) biomass (SB) using the SV⁻¹ ratio. The maximum biomass production (BP_{max}) is the maximum BP value which usually is observed within 2 weeks of incubation. The BPP value is the average of the BP-values obtained on day 56, 84 and 112. This BPP value is composed of the Biofilm Formation Potential (BFP), which represents the attached biomass and the potential to form suspended biomass (SBP). Hence, BPP = BFP + SBP. These parameters are all expressed as the quantity of active biomass per surface unit of the tested material (pg ATP cm⁻²).

Legionella bacteria (or other selected bacteria) can be added to the test water at the start of the test. In this way also information is obtained on the degree to which a particular material is able to enhance the growth of Legionella (or other bacteria) under the test conditions (Van der Kooij and Veenendaal, 2001).

ATP analysis is also used for determining the biofilm concentration on walls of cistern distribution system pipes and in the biofilm monitor for determining the Biofilm Formation Rate (BFR) values of drinking water (Van der Kooij et al. 1999). A database of biomass concentrations in water, in biofilms and on materials facilitates the interpretation of individual measurements (Unifying Biofilm Analysis) (Van der Kooij et al. 2003).

1.2.4 Austrian method

An alternative method for testing of pipes materials in contact with drinking water has recently been published in Austria (Önorm B 5018, 2002). The recommended inner diameter of test samples is 4 cm, which gives an SV⁻¹ ratio of 1 cm⁻¹. The length of the pipe sample is 100 cm. The pipes are fixed in a vertical position and their bottom ends are closed by means of foamed PE-
stoppers. These pipes are filled with tap water and the top ends are covered with sterile petri dishes. The incubation of the pipes is performed at 22 ± 2°C with weekly water replacement. The water is aerated from below with a pressure of 0.3 bar. The HPC values of the water in the pipes are determined according to EN ISO 6222 at 22°C after one, two and three months of incubation. At the end of the test, after a three months incubation period, the biofilm grown on the inner pipe wall is assessed using the ATP-method. The test samples are rings of 1 cm height, which are cut out 20 cm above the bottom end of the tested pipes under sterile conditions. Biomass is detached with low energy sonication.

The evaluation is performed in relation to a negative control (glass pipe) and a positive control (PVC-P tube). Pipes are not recommended for use in contact with drinking water if the HPC value of the test water in contact with the material is 10 times higher than in the glass control or when the biofilm on the pipe specimen is 5 times higher than in the glass control. The results are only valid if the HPC value of the PVC-P control is at minimum 100 times higher than the HPC value of the glass control and the amount of biofilm grown on PVC-P is at minimum 10 times higher than the amount grown on glass.

1.3 Need for harmonisation
Different methods for testing of products hamper the development of the European market. Therefore, European standard methods are defined in the framework of CEN. Workpackage 1 (WP1) 'Microbial Growth' of the EU Project 'Development of Harmonised Tests to be used in the European Approval Scheme concerning Construction Products in Contact with Drinking Water (CPDW)' has the objective to develop a generally accepted test method for determining the microbial growth promoting properties of CPDW. Initially, it was intended that this method should be based on the methods as developed in Germany (W270), the UK (MDOD test) and the Netherlands (BPP test), respectively. These methods have been described in prENV 'Methods for determining the potential of materials to influence the microbiological quality of drinking water', produced by Ad Hoc Group 3 of CEN WG 164 WG3. An important starting point was the decision of the involved legislators that ATP should serve as the biomass parameter. At the beginning of the project it was decided also to include the Austrian method in the investigations.

The first stage of WP1 included the selection of test parameters by evaluating the present methods and by considering the test conditions accepted in CEN TC 164. These conditions have been described in EN13652, part 1 and prEN12873 part 1 (European Norm, 2001; 2003). Microbiological testing brings its own specific requirements. Therefore, a second step was to further define and conduct the experimental work needed to achieve the objective of WP1. This research included assessment of the effect of various test parameters such as water type, inoculum, static/dynamic on the result and elucidation of the practical importance of these results by studying identical products under conditions simulating practice. Also reference materials must be defined.
Research objectives
1. Defining the variables of an ATP-based test method for the microbial growth potential of products in contact with drinking water, with the required detection limit and sufficient reproducibility;
2. Applicability of the method to all types of CPDW;
3. Comparison of results of a number of representative products in the test to be developed with data for these products in experiments simulating practical conditions and in present test methods to obtain information for the interpretation of the data in relation to possible pass/fail criteria.
2 Work programme, test conditions and materials

2.1 Work package structure
The activities included in WP1 had been divided into seven stages. The description of these stages as given in the original project description is presented below. The numbers of stages 4 and 5 have been exchanged in agreement with the numbers used during the investigations. Details of the project stages were discussed in a total of six meetings with the WP1 participants.

2.1.1 Stage 1 Work programme details
Agreement on details of the work to be conducted, including test conditions (viz. test water quality, nature of inoculum, incubation temperature, water replacement scenarios in a batch test, test period, frequency of analysis), selection of representative materials will be obtained in the kick-off meeting of the project. A number of test variables will be selected on the basis of experience with the existing methods.

2.1.2 Stage 2 Harmonisation of the ATP test
The ATP-method will be introduced to the participating institutes. The ATP analysis is the basis for the test method. Kiwa has optimised the ATP analysis and used it for more than 10 years. Detailed instructions will be given to the other participants about the ATP method and about the procedure to remove biomass from solid surfaces of test materials. The quality of the ATP analysis will be tested in three round robin (‘ring’) tests with the participants in the course of the project.
Comment: four ATP ring tests have been performed

2.1.3 Stage 3 Test water quality
Water with a low growth potential (high degree of biological stability) is needed for the test. In various water types the growth of micro-organisms will be assessed with the ATP test over a period of about 2 months. ATP concentrations should be low after 50 to 60 days of incubation. Each participant will test different types of drinking water. The low growth potential of the test water ensures a high sensitivity of the test, as the effects of the feed water are eliminated. This furthermore ensures a wide applicability of the test throughout EU Member States.

2.1.4 Stage 4 Cleaning of product by chlorination
Methods for cleaning products prior to use differ between the various countries. Chlorination is practised in a number of countries and the effect of this treatment will be tested with a number of products in WP4.
Comment: a generally accepted cleaning procedure using tap water without disinfectant was applied.
2.1.5 *Stage 5 Inoculation*

Products in contact with drinking water may contain a large variety of biodegradable compounds. Utilisation of these compounds should not be limited by the physiological properties of the bacterial population of a test water type. Inoculation with a small volume of river water, or with bacteria extracted from sand filters or soil may be needed to quantify the amount of biodegradable compounds releasing from the products. The effects of different types of inoculum on the biomass production will be tested with various types of test water, taking into account the variation in ATP content between prokaryotes and eukaryotes.

*Comment:* The variation in ATP content between prokaryotes and eukaryotes was not studied because the ATP to biomass ratio is fairly constant for both types of organisms (see Chapter 3).

2.1.6 *Stage 6 Static versus dynamic*

Static test conditions make it possible to test the growth of biomass on the product and in the water, but may not reflect the conditions in practice. The effect of various scenarios of water replacement on biomass production, both as attached and suspended growth, will be investigated in semi-batch tests.

2.1.7 *Stage 7 Reproducibility of selected protocol*

Based on the outcome of the investigations on the effects of water replacement scenarios, a series of tests will be conducted by the participants with a number of selected materials to assess the applicability (effects of the selected variables), and the reproducibility of the method. Also experiments will be conducted with these products, simulating practical conditions to enable evaluation of the test results, e.g. by testing pipe segments. In these tests and experiments heterotrophic plate counts, which are used in routine monitoring of drinking water quality, will be used in addition to ATP analysis. Laboratories will apply media that are commonly applied for statutory monitoring in the respective countries. In this way, a translation of these data to existing data will be possible. Furthermore, this will aid the selection of media for the final test procedure. A number of selected materials, representing the range of material types, will also be tested with the existing methods (BS 6920, W270, BPP test, Önorm), for interpretation of the data obtained in the newly designed test in relation to present pass-fail criteria connected to the existing methods.

Materials suggested to be included in the investigations
- Reference/controls: blank (water), glass, PVC-P
- Materials used for comparison: unplasticized PVC (PVC-U), medium density PE, high density PE, cross-linked PE, glass-fibre reinforced polyester, polypropylene, EPDM rubber, natural rubber, Stainless Steel;
- Special materials to show a broad spectrum of applicability: coatings, cementitious materials, copper, lubricants, cement/concrete constituents

*Comment 1:* heterotrophic plate counts (HPC) as biomass parameter were only incidentally applied and the parameter was not further studied because it
does not include all active heterotrophic bacteria (as does ATP), is time consuming and takes a long time before results are available.

Comment 2: Also the Austrian standard method (Önorm 2002) was included for comparison.

Comment 3: The number of materials included in the test development was reduced to obtain sufficient time for determining test variables.

2.2 Test conditions
Details of test conditions and experimental procedures were discussed with the WP1 participants in the project. General aspects are described below; specific conditions and procedures as applied in project stages are described in the next chapters.

2.2.1 Incubation temperature
The temperature for testing differs between the existing methods (Table 1.1). Test water temperature ranges from 6°C (lowest temperature in the W270 method) to 30°C in the MDOD method. The selection of the optimal test temperature is affected by:
- the temperature of drinking water in contact with materials in practice;
- the effect of temperature on the result of the test, viz. the growth of certain types of micro-organisms;
- the practical implications of maintaining a certain temperature in the laboratory/test.

In Europe, the temperature of ‘cold’ tap water may range from values close to 0°C (in winter) to values close to (or even above) 30°C in summer (in southern Europe, inside buildings). Microbiological activity increases at higher temperature. Also at higher temperature (opportunistic) pathogens (P. aeruginosa, Legionella spp., Mycobacterium spp.) can compete more effectively with the ‘normal’ aquatic bacteria than at low temperatures. Applying worst case conditions in the test would lead to the selection of a relatively high incubation temperature. During the meeting in Lisbon (March 13/14, 2001) it was concluded that testing at 30°C may be the best choice. However, at the Schiphol Meeting (May 2, 2001) it appeared that not all participants had the facilities to conduct experiments at 30°C. It was also expected that the difference between the effect of testing at 25 °C and at 30°C on the biomass production (activity) would be small. Therefore it was decided:
- to start the investigations by testing at 25°C;
- to compare testing at 25°C with testing at 30°C (or another temperature) at a later stage of the project.

Comment 1: Materials used in contact with warm tap water should be tested at a temperature higher than 30°C. The effect of warm water temperature on microbial growth on materials is not included in the investigations of WP1.

Comment 2: AT DTU a temperature of 10°C was also tested and at Crecep tests were conducted at 22 ± 1°C. Comparison of the results obtained at these temperatures with results obtained at 25°C provide information about the effect of incubation temperature.
2.2.2 Water composition/quality

The composition/quality of water used in the test must be defined. Tap water which complies with the European quality criteria serves as the basis for the test. However, a number of quality aspects must be defined in more detail. Critical aspects of water composition/quality include:

- inorganic compounds;
- organic growth promoting compounds;
- the types of micro-organisms present.

Inorganic compounds serving as nutrients (N, P) should be present in sufficient concentration. Certain compounds inhibitory to microorganisms should be absent (disinfectants, certain metals). Table 2.1 lists the quality criteria as defined in the UK Standard, BS 6920 and gives guideline values for a number of parameters as based on the discussions with the participants of WP1.

Table 2.1 Criteria proposed for the composition of water to be used in microbial testing

<table>
<thead>
<tr>
<th>Quality parameter</th>
<th>Concentration (range) BS 6920*</th>
<th>Proposal</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>6.5 – 8.5</td>
<td>Preferably between 7.0 and 8.5</td>
</tr>
<tr>
<td>Oxygen (mg/l)</td>
<td>&gt; 6.5</td>
<td>Oxygen depletion is not acceptable; see comment 1</td>
</tr>
<tr>
<td>PO43-P (mg/l)</td>
<td>2.0 – 6.7</td>
<td>≥ 2 mg P/l; See comment 2</td>
</tr>
<tr>
<td>Nitrate-N</td>
<td>5 – 11.3</td>
<td>≥ 5 mg N/l; see comment 3</td>
</tr>
<tr>
<td>Ammonia-N (mg/l)</td>
<td>Not defined</td>
<td>&lt; 0.05 mg/l; see comment 3</td>
</tr>
<tr>
<td>Free residual chlorine (mg/l)</td>
<td>&lt; 0.05</td>
<td>Absent; see comment 4</td>
</tr>
<tr>
<td>Total chlorine (mg/l)</td>
<td>&lt;0.20</td>
<td>Absent; see comment 4</td>
</tr>
<tr>
<td>Copper (mg/l)</td>
<td>&lt; 0.05</td>
<td>&lt; 0.05 mg/l</td>
</tr>
<tr>
<td>Silver (mg/l)</td>
<td>&lt; 0.01</td>
<td>&lt; 0.01 mg/l</td>
</tr>
<tr>
<td>Hardness</td>
<td>Not defined</td>
<td>See comment 5</td>
</tr>
<tr>
<td>Total dissolved organic carbon</td>
<td>Not defined</td>
<td>&lt; 3 mg/l; see comment 6</td>
</tr>
<tr>
<td>Biostability</td>
<td>Not defined</td>
<td>Low ATP maximum/low AOC.</td>
</tr>
<tr>
<td>Micro-organisms</td>
<td>Not defined</td>
<td>A large diversity to enable utilisation of all types of compounds which are biodegradable; comment 8</td>
</tr>
</tbody>
</table>

* as defined in BS 6920

Comment 1.1: Oxygen concentration. Tap water saturated with oxygen should be used. Furthermore, the oxygen concentration in the water should not decrease to a value below 1 mg/l. An oxygen concentration of 10 mg/l is sufficient for the utilisation of about 8 mg of organic-C/l. These rules of thumb define the maximum concentration of biodegradable organic carbon in the test.

Comment 1.2: Water replacement reduces the risk of oxygen depletion.

Comment 2: Phosphate concentration. Phosphorus (P) is an essential nutrient for microorganisms. The P requirement can be derived from the average...
composition of dry biomass. P may constitute up to 5% of the dry matter. With 50% C and assuming a conversion (yield) for biodegradable carbon of 50%, the C to P ratio in the test should not exceed 20. With a maximum of 10 mg C/l, this means that P should be present at a concentration ≥ 0.5 mg PO₄³⁻-P/l (about 1.5 mg PO₄³⁻-P/l). It was decided to add a supplement concentration of 2 mg P/l. For this purpose a stock solution of phosphate (p.a. quality) in demineralised water was prepared. Water replacement will also reduce the possibility of P limitation.

Note: prepare the stock solution (KH₂PO₄) in a borosilicate glass flask (e.g. 500 ml) with screw cap (type of flask identical to those used for testing, see paragraph 2.9) and store in the dark at a temperature between 0 and 6°C for a maximum period of 1 year;

Comment 3: Nitrate concentration. Nitrogen also is an essential nutrient for micro-organisms, constituting about 12% of dry biomass. Consequently, NO₃⁻-N should be present in relation to available C as 10:1. Hence a maximum concentration of 10 mg C/l requires 1 mg of NO₃⁻-N (4.4 mg/l NO₃). It was decided to supplement a concentration of 5 mg of NO₃⁻-N/l from a solution of nitrate (p.a. quality) in demineralised water. Water replacement will also reduce the possibility of N limitation.

Note: prepare a stock solution (KNO₃) in a borosilicate glass flask (e.g. 500 ml) with screw cap (type of flask identical to those used for testing, see paragraph 2.9) and store in the dark at a temperature between 0 and 6°C for a maximum period of 1 year.

Ammonia should not be present, because this compound contributes to oxygen consumption and biomass (ATP) production.

Comment 4: Disinfectant residual. Free residual chlorine; total chlorine, or another other disinfectant should not be present in the water used in the test. If present in tap water, a dechlorination procedure should be applied. GAC filtration can be used to remove the disinfectant. Addition of thiosulphate is not appropriate, because this compound serves as an energy source for certain bacteria.

Comment 5. Hardness. No guideline value is defined for hardness. Carbonate precipitation was observed when the water was aerated during an experiment at OFI, but aeration is not included in the proposed test procedure.

Comment 6: Dissolved organic carbon. The concentration of dissolved organic carbon should be in the normal range for drinking water. Investigations applying BDOC measurements have shown that a certain fraction (10 – 30 %) of DOC may be biodegradable, when in contact with adapted biomass (Joret et al. 1985). A DOC concentration below 3 mg/l is preferred.

Comment 7 Biostability. The water used in the test should have a relatively high degree of biostability, to limit the growth in the blank as much as possible. The biostability of the water to be used will be tested by determining the maximum ATP concentration that may be attained when the water is stored
under conditions as prescribed for the AOC test. The water has a sufficient
degree of biostability when the maximum ATP concentration remains below
10 ng/l. In such water types also the AOC concentration is below 10 µg C/l.
The biostability requirement will be further defined based on experiments
conducted in WP1. With water replacement the growth potential of test water
will be more critical.

Comment 8: Microbiological composition. The water should contain a large
diversity of bacteria naturally occurring in the aquatic environment, ensuring
that growth-promoting compounds come in contact with micro-organisms
with the required physiological properties. The BPP test is conducted in slow
sand filtrate containing the natural bacterial community. In addition, a small
volume (1 ml) of filtered river water is added. In the UK method, 100 ml of
river water is used in a test volume of 1 l of tap water. The following
conclusions were drawn by the WP1 participants:
- the effect of the type of river water most likely is negligible;
- the amount of inoculum should not affect the test result (because of the
  presence of biodegradable compounds);
- the effect of a 1% river water inoculation will be tested (by comparing the
  results with and without inoculum);
- inoculation will be done only at the start of the test.

2.2.3 Test system

2.2.3.1 Surface of test material
Preconditions:
- the surface exposed in the test should represent as much as possible the
  surface exposed in practice;
- the surface of the material in contact with water under the test conditions
  must be clearly defined to enable quantitative measurements;
- the exposure in microbiological testing should follow as much as possible
  the conditions already defined in existing CEN procedures. However, the
  nature of microbial processes requires a number of specific test conditions.

In CEN groups (connected to CEN WG3) it had been concluded that
homogeneous materials can be tested as pieces. This includes pieces of pipe
prepared from homogeneous materials. A list of materials, which are
considered to be homogeneous, is available in CEN WG3 Ad Hoc Groups 1
and 2. Non homogeneous materials should be provided by the manufacturers
in such a way that appropriate testing is possible. Hence, large diameter pipe
materials will not be tested as pipes.

2.2.3.2 Surface (S) to volume (V) ratio
The S/V ratio in pipes equals 4/D (D = diameter). In the MDOD and BPP test
the S/V ratio is 0.15 to 0.16 cm⁻¹ which reflects a pipe diameter of about 25 cm.
In practice, both pipes with smaller and with larger diameters are used. In the
BPP test the S/V ratio is maintained by a proportional decrease (pouring out)
of the volume of water after the removal of a sample piece. The relationship
between S/V ratio and the test result is not clear. Experience at DTU has
shown that growth does not increase proportional with increasing S/V ratio in the test.

Factors affecting the selection of the S/V ratio:
- no oxygen depletion should occur in the test as a result of a too large S/V ratio;
- a minimum surface area is needed to obtain representative results of the biomass (ATP) analysis. Sample surface area should not be less than about 5-10 cm². In combination with a relatively low S/V ratio and the number of test pieces, this has an impact of the volume of the container (and therefore on the volume of the incubator).

The participants decided to maintain the S/V ratio as used in the MDOD test and the BPP test (0.15-0.16 cm⁻¹).

2.2.4 Selection and preparation of the materials

2.2.4.1 Selection of materials

The products selected in the WP1 meetings at Lisbon, Schiphol and Paris are listed in Table 2.2. In the course of the project it appeared that the number of products had to be restricted because the experimental work on analytical methods and test conditions took more time than initially was planned.

Table 2.2 Initially selected materials

<table>
<thead>
<tr>
<th>Category</th>
<th>Material</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference/controls</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- negative control</td>
<td>Borosilicate glass</td>
<td>Use of cylinders instead of plates;</td>
</tr>
<tr>
<td>- positive control</td>
<td>Paraffin wax</td>
<td>Comment 1</td>
</tr>
<tr>
<td>Synthetic materials</td>
<td>PVC-C</td>
<td>Comment 2</td>
</tr>
<tr>
<td></td>
<td>Polyethylene; PE-HD</td>
<td>Comment 3</td>
</tr>
<tr>
<td></td>
<td>Cross-linked PE (PE-X)</td>
<td>Comment 3</td>
</tr>
<tr>
<td></td>
<td>Silicone Rubber</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Glassfibre Reinforced Polyester</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EPDM rubber</td>
<td>Comment 4</td>
</tr>
<tr>
<td></td>
<td>Nitrile Rubber (NBR)</td>
<td>Comment 5</td>
</tr>
<tr>
<td></td>
<td>Epoxy-based coating</td>
<td>Comment 6</td>
</tr>
<tr>
<td></td>
<td>Lubricants</td>
<td>Comment 7</td>
</tr>
<tr>
<td>Metallic materials</td>
<td>Stainless Steel</td>
<td>Comment 8</td>
</tr>
<tr>
<td></td>
<td>Galvanised iron</td>
<td>Comment 8</td>
</tr>
<tr>
<td></td>
<td>Copper</td>
<td>Comment 8</td>
</tr>
<tr>
<td>Cementitious materials</td>
<td>Repair mortar</td>
<td>Comment 9</td>
</tr>
<tr>
<td></td>
<td>Cubes (60x 60 or 50 x 50 mm)</td>
<td>Comment 9</td>
</tr>
</tbody>
</table>

Comment 1 At the Schiphol meeting (May 2, 2001) of WP1 it was concluded that paraffin wax was not a suitable positive control for the BPP test. Plasticized ('soft') PVC (PVC-P) is a potential alternative, provided that differences in the type(s) of plasticizers used (phthalates, adipate) and
concentrations can be controlled. A representative type of PVC-P was selected for inclusion in the experiments (see Chapter 5).

Comment 2. Unplasticized PVC (PVC-U) was not used in the test, because of expected changes in the composition (use of PVC-U without lead stabilisation). Chlorinated PVC (PVC-C) was used instead.

Comment 3. Appropriate types of HDPE and PE-X had been selected, but PE-X was not used in the investigations.

Comment 4. Glass-fibre reinforced plastic (GRP) initially was intended to be included but was not selected.

Comment 5. Two types of rubber were used, a nitrile-based rubber was used by all participants and an EPDM-based rubber was tested by two participants (in stage 7).

Comment 6. Epoxy based coatings/resin was not included in the investigations because preparation of test samples influences the composition of the material.

Comment 7. Lubricants were not included in the investigation.

Comment 8. Only Stainless Steel was used.

Comment 9. Cementitious materials were not included in the investigations, but some experience with such materials was obtained by one of the participants.

2.2.4.2 Cleaning
Cleaning of the material should resemble the conditions as applied in practice, but no disinfectant will be used. A method has been described in the document about testing effect of materials on flavour and odour (EN 1420-1), viz.:
- flush the material with tap water for 15 min;
- stagnation period of 24 h;
- repeat flushing for 15 min prior to use;
- rinsing 3 times with test water.

2.2.4.3 Storage before testing
Procedures described by manufacturers should be followed.

2.2.4.4 Handling and touching
The procedures and experiences as applied/obtained in present testing should be used. A pair of forceps and/or polyethylene gloves should be used; rubber gloves are not suitable. Instruments used for cutting materials (e.g. saw) must be clean (degreased).
2.2.5 Static/dynamic
Water in contact with materials in distribution systems may either be flowing or stagnant. These conditions vary continuously, depending on time and location. Testing under static conditions represents worst case conditions. Testing under continuous flow is not practicable. A number of scenarios will be tested, including no replacement (BPP test; worst case) and twice a week replacement (MDOD test; highest frequency).

2.2.6 Biomass production
Biomass concentrations were determined in water and on the material with ATP analysis, see Chapter 3.

2.2.7 Duration of the test
In the MDOD, Önorm, BPP and W270 methods, the test periods are 7, 12, 16 and 26 weeks respectively. A short test period is preferred, but data will be collected during a period of 26 weeks to enable assessment of the effect of the length of the test period on the result.
3 Adenosine triphosphate (ATP) analysis

3.1 Introduction
An accurate determination of the concentration of biomass produced in the presence of materials in contact with drinking water is an essential component of a harmonised test. Different parameters are used to quantify the growth of microorganisms in the present test methods for determining the microbial growth support potential of products in contact with drinking water (Table 1.1). In an Expert Meeting of specialists and regulators representing the UK, France, Germany and the Netherlands organised by EC/DGIII on February 1, 1999 it was concluded that adenosine triphosphate (ATP) would be the preferred biomass parameter in a specific method for testing the growth-promoting properties of materials. ATP is an energy carrier in all living organisms, linking catabolism and biosynthesis. The amount of metabolically available energy that is momentarily stored in the adenylate system is linearly related to the mole fraction of ATP plus half the mole fraction of adenosine diphosphate (ADP) (Chapman et al., 1971). The turnover time for the intracellular ATP pool in bacteria is very short (< 1 s) enabling a rapid response of the micro-organism to changes inside and outside the cell. A series of investigations have demonstrated that the ATP concentration is 0.1 to 0.2% of the dry weight of micro-organisms and that ATP is a useful estimate of heterotrophic biomass. Generally, a ratio of 250 for biomass carbon and ATP (C/ATP) is used to estimate the active biomass concentration from the ATP concentration (Holm-Hansen and Booth, 1966; Hamilton and Holm-Hansen, 1967; Karl, 1980).

ATP analysis has a number of advantages over the use of other parameters for assessing the presence of microorganisms: (i), it includes all active microorganisms; (ii), is absent in dead (inactive) microorganisms; (iii), enables rapid testing and (iv), has a low detection limit (< 1 ng ATP/l in direct tests of water).

The firefly bioluminescence method is the most rapid, sensitive and reproducible assay for determining the ATP concentration. The principle of this assay is based on using the firefly luciferine-luciferase reaction which results in the emission of one quantum of light for each molecule of ATP that is hydrolysed. ADP does not emit light in this reaction. Equipment and reagents for ATP analysis have been improved considerably in the past two decades. Furthermore, many studies on the presence and behaviour of bacteria in the environment have confirmed the concept of using the ATP concentration as a measure for active biomass. ATP analysis has also been used for assessing the presence of bacteria in drinking water and in filter beds and for the determination of the microbial growth potential of drinking water (Stanfield and Jago, 1987; Van der Kooij et al. 1992). The parameter is also very useful for the assessment of the biofilm concentrations on surfaces exposed to drinking water (Van der Kooij et al. 2003). Apart from the advantages of ATP
analysis itself, the use of a single parameter for assessing biomass concentrations facilitates the interpretation of observations in related environments. For this reason ATP analysis had been introduced for determining the growth potential of materials in contact with drinking water (van der Kooij and Veenendaal, 1994; van der Kooij and Veenendaal, 2001).

ATP has a key position in the harmonised test. Therefore, an instruction day was organised at Kiwa WR in the beginning of the project and round robin (‘ring’) tests with the participating laboratories were included in WP1 to improve and ensure the quality of the ATP measurements in the project. The observations with these ring tests are reported in this chapter.

3.2 Materials and methods

3.2.1 ATP analysis

The equipment and reagents as used by the participants for ATP analysis are shown in Table 3.1.

<table>
<thead>
<tr>
<th>Participant</th>
<th>Instrument</th>
<th>Reagents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Creep</td>
<td>Celsis Biocounter M2500</td>
<td>Celsis Microbial Biomass Reagent Kit (no. 93321); LumiT QM</td>
</tr>
<tr>
<td></td>
<td>Mode of operation: automatic</td>
<td>Celsis ATP Standard Twin Pack (no. 92638)</td>
</tr>
<tr>
<td>DTU</td>
<td>Celsis System Coupe</td>
<td>PCP Kit; Celsis LuminEX/LuminATE (no. 92687)</td>
</tr>
<tr>
<td></td>
<td>Mode of operation: automatic</td>
<td>Celsis Lumin (PM) buffer (no. 92588)</td>
</tr>
<tr>
<td>Kiwa WR</td>
<td>Celsis System TM</td>
<td>Celsis LuminEX/LuminATE kit</td>
</tr>
<tr>
<td></td>
<td>Mode of operation: automatic</td>
<td>Celsis ATP standard (no. 92638)</td>
</tr>
<tr>
<td>OFI</td>
<td>EG &amp; Berthold mini Lumat LB 9506</td>
<td>Perkin Elmer/Life Sciences Cyto-Lux L001-100</td>
</tr>
<tr>
<td></td>
<td>Mode of operation: manually</td>
<td></td>
</tr>
<tr>
<td>TZW</td>
<td>Berthold LB 955, Berthold Autolumat</td>
<td>Celsis LuminEX/LuminATE Kit (no. 92687)</td>
</tr>
<tr>
<td></td>
<td>Mode of operation: automatic</td>
<td>Celsis ATP Standard Twin Pack (no. 92638)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Celsis PM Buffer Twin Pack (no. 92588)</td>
</tr>
<tr>
<td>TWUL</td>
<td>Lumac Biocounter M1500</td>
<td>Celsis LuminEX (no. 92687/92257); Celsis LuminATE (PM) (no. 92380), Celsis PM buffer-92307; Celsis ATP Positive control 129483</td>
</tr>
<tr>
<td></td>
<td>Mode of operation: manually</td>
<td></td>
</tr>
</tbody>
</table>
Assessment of the ATP concentration in water samples with microorganisms includes the following procedures:
- collection of a representative sample;
- extraction of ATP from the microorganisms;
- addition of reagents for the luciferine-luciferase assay;
- recording the light emitted;
- calculation of the ATP concentration from calibration data.

The procedure for determining the ATP concentration in water samples as used by Kiwa WR was introduced at an instruction day at Kiwa WR in April 2001. Detailed descriptions of the techniques as applied at Kiwa WR are given in Appendix 1. A short description is given below.

**Kiwa procedure for ATP analysis:** A sample volume of 100 μl is used in the analytical procedure. ATP contained in this volume is extracted from the cells with a nucleotide-releasing reagent (NRM, Celsis). Subsequently, the sample is mixed with the reagents for enzymatic reaction and the emission of light is measured as relative light units (RLU) without background correction after a 2s delay time and a 10s integration time. Calculation of the ATP concentration from the RLU value is based on calibration curves, which have been made for all (combinations of) instruments and reagents. For calibration a stock solution of ATP is prepared by dissolving a defined amount of ATP in tap water. From this stock solution, dilutions are made in tap water.

**Table 3.2 Water types used for preparing stock solutions and calibration curves for ATP analysis, respectively (B, buffer; D, demineralised or distilled water; T, tap water)**

<table>
<thead>
<tr>
<th>Participant</th>
<th>Test 1</th>
<th>Test 2</th>
<th>Test 3</th>
<th>Test 4</th>
<th>Project stages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crecep</td>
<td>D/T</td>
<td>D/T</td>
<td>T/T</td>
<td>T/T</td>
<td>T/T in all stages</td>
</tr>
<tr>
<td>DTU</td>
<td>B/MQ*</td>
<td>B/B</td>
<td>B/B</td>
<td>B/T</td>
<td>B/B in stages 3,5 and 6, B/T in stage 7**</td>
</tr>
<tr>
<td>Kiwa WR</td>
<td>T/T</td>
<td>T/T</td>
<td>T/T</td>
<td>T/T</td>
<td>T/T in all stages</td>
</tr>
<tr>
<td>OFI</td>
<td>B/D</td>
<td>B/D</td>
<td>B/D</td>
<td>B/D</td>
<td>B/D in all stages, except stage 7 where B/T was used</td>
</tr>
<tr>
<td>TZW</td>
<td>B/B</td>
<td>B/B</td>
<td>B/B</td>
<td>B/B</td>
<td>B/B in all stages except stage 7 where T/T was used and and stage 7 where T/T was used</td>
</tr>
<tr>
<td>TWUL</td>
<td>B/D</td>
<td>B/D</td>
<td>B/D</td>
<td>B/D</td>
<td>B/D in all stages except stage 7 where B/T was applied</td>
</tr>
</tbody>
</table>

*, MQ, Milli-Q-treated tap water; tap water; ** all data corrected to refer to calibration curve in tap water

The participants of WP 1 used different equipment (Table 3.1), different water types for preparing the stock solution and the solutions used for calibration (Table 3.2). Consequently, a comparison of the results of ATP measurements as obtained by the WP1 participants in identical samples is needed to ensure the quality of the analysis.
3.2.2 Ring tests
The ring tests were conducted with a variety of water types containing different concentrations of microorganisms in surface water, tap water and tap water in contact with materials in growth tests. This approach was followed because experience had shown that solutions of free ATP do not remain stable during transportation. Furthermore, the release of ATP from the microorganisms is an essential step in the analysis. Water samples (20 ml) were stored on ice and delivered within 24 hours to the participants. Analysis was conducted within a few hours after arrival in most cases.

3.3 Results of ring tests
Three ring tests had initially been planned but a total of four ring tests with water containing various concentrations of ATP (in microorganisms) were conducted during the project. The results from these ring tests are presented in Table 3.3. These results show both striking similarities and clear differences between the laboratories.

Table 3.3 Results of ATP measurements in samples in 4 ring tests. Values either below or above the average value ± SD are presented in bold.

<table>
<thead>
<tr>
<th>Sample</th>
<th>P1</th>
<th>P2</th>
<th>P3</th>
<th>P4</th>
<th>P5</th>
<th>P6</th>
<th>Average</th>
<th>SD</th>
<th>RSD*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>5.3</td>
<td>17.0</td>
<td>7.1</td>
<td>4.0</td>
<td>11.0</td>
<td>Np**</td>
<td>10.9</td>
<td>5.4</td>
<td>50.1</td>
</tr>
<tr>
<td>1.2</td>
<td>42.6</td>
<td>56.0</td>
<td>40.0</td>
<td>24.0</td>
<td>39.0</td>
<td>Np</td>
<td>40.3</td>
<td>11.4</td>
<td>28.3</td>
</tr>
<tr>
<td>1.3</td>
<td>39.2</td>
<td>56.0</td>
<td>37.0</td>
<td>23.0</td>
<td>40.0</td>
<td>Np</td>
<td>39.0</td>
<td>11.7</td>
<td>30.0</td>
</tr>
<tr>
<td>1.4</td>
<td>87.8</td>
<td>135.0</td>
<td>88.0</td>
<td>66.0</td>
<td>96.0</td>
<td>Np</td>
<td>94.6</td>
<td>25.2</td>
<td>26.7</td>
</tr>
<tr>
<td>2.1</td>
<td>122.0</td>
<td>3.6</td>
<td>4.0</td>
<td>30.0</td>
<td>2.0</td>
<td>1.9</td>
<td>27.3</td>
<td>47.7</td>
<td>175.0</td>
</tr>
<tr>
<td>2.2</td>
<td>78.0</td>
<td>77.0</td>
<td>77.0</td>
<td>59.0</td>
<td>61.0</td>
<td>53.0</td>
<td>67.5</td>
<td>11.1</td>
<td>16.4</td>
</tr>
<tr>
<td>2.3</td>
<td>163.0</td>
<td>16.0</td>
<td>50.0</td>
<td>24.0</td>
<td>14.0</td>
<td>53.4</td>
<td>62.9</td>
<td>117.8</td>
<td></td>
</tr>
<tr>
<td>2.4</td>
<td>87.7</td>
<td>75.0</td>
<td>59.0</td>
<td>84.0</td>
<td>62.0</td>
<td>61.0</td>
<td>71.5</td>
<td>12.6</td>
<td>17.6</td>
</tr>
<tr>
<td>3.1</td>
<td>985.0</td>
<td>995.0</td>
<td>634.0</td>
<td>364.0</td>
<td>1109.0</td>
<td>563.0</td>
<td>775.0</td>
<td>295.9</td>
<td>38.2</td>
</tr>
<tr>
<td>3.2</td>
<td>78.0</td>
<td>58.0</td>
<td>34.0</td>
<td>22.0</td>
<td>77.0</td>
<td>24.0</td>
<td>48.8</td>
<td>25.6</td>
<td>52.5</td>
</tr>
<tr>
<td>3.3</td>
<td>9.4</td>
<td>8.5</td>
<td>6.0</td>
<td>3.9</td>
<td>6.6</td>
<td>9.4</td>
<td>7.3</td>
<td>2.2</td>
<td>30.0</td>
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<td>3.4</td>
<td>7.5</td>
<td>8.8</td>
<td>4.9</td>
<td>5.4</td>
<td>7.0</td>
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<td>21.1</td>
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<tr>
<td>3.5</td>
<td>82.0</td>
<td>86.0</td>
<td>69.0</td>
<td>28.0</td>
<td>100.0</td>
<td>32.0</td>
<td>66.2</td>
<td>29.7</td>
<td>44.9</td>
</tr>
<tr>
<td>3.6</td>
<td>7.0</td>
<td>17.0</td>
<td>12.0</td>
<td>19.0</td>
<td>17.0</td>
<td>12.0</td>
<td>15.7</td>
<td>2.9</td>
<td>18.8</td>
</tr>
<tr>
<td>3.7</td>
<td>654.0</td>
<td>684.0</td>
<td>478.0</td>
<td>374.0</td>
<td>700.0</td>
<td>436.0</td>
<td>554.3</td>
<td>141.6</td>
<td>25.6</td>
</tr>
<tr>
<td>3.8</td>
<td>89.0</td>
<td>97.0</td>
<td>68.0</td>
<td>31.0</td>
<td>103.0</td>
<td>35.0</td>
<td>70.5</td>
<td>31.4</td>
<td>44.5</td>
</tr>
<tr>
<td>4.1</td>
<td>1.5</td>
<td>1.1</td>
<td>0.6</td>
<td>10.8</td>
<td>0.1</td>
<td>3.1</td>
<td>2.9</td>
<td>4.0</td>
<td>140.2</td>
</tr>
<tr>
<td>4.2</td>
<td>647.7</td>
<td>668.0</td>
<td>650.0</td>
<td>1121.0</td>
<td>1259.0</td>
<td>547.0</td>
<td>815.5</td>
<td>296.5</td>
<td>36.4</td>
</tr>
<tr>
<td>4.3</td>
<td>90.4</td>
<td>107.7</td>
<td>115.0</td>
<td>163.0</td>
<td>169.0</td>
<td>81.5</td>
<td>121.1</td>
<td>36.8</td>
<td>30.4</td>
</tr>
<tr>
<td>4.4</td>
<td>45.1</td>
<td>74.0</td>
<td>103.0</td>
<td>97.4</td>
<td>115.0</td>
<td>38.6</td>
<td>78.9</td>
<td>31.7</td>
<td>40.2</td>
</tr>
<tr>
<td>4.5</td>
<td>2.2</td>
<td>10.1</td>
<td>12.0</td>
<td>43.0</td>
<td>16.4</td>
<td>9.5</td>
<td>17.2</td>
<td>12.9</td>
<td>74.8</td>
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<tr>
<td>4.6</td>
<td>17.7</td>
<td>21.4</td>
<td>21.0</td>
<td>47.2</td>
<td>33.0</td>
<td>20.5</td>
<td>26.8</td>
<td>11.3</td>
<td>42.2</td>
</tr>
<tr>
<td>4.7</td>
<td>26.7</td>
<td>56.7</td>
<td>57.0</td>
<td>105.0</td>
<td>77.5</td>
<td>35.0</td>
<td>59.7</td>
<td>28.6</td>
<td>47.9</td>
</tr>
<tr>
<td>4.8</td>
<td>9.6</td>
<td>8.2</td>
<td>8.2</td>
<td>33.1</td>
<td>12.9</td>
<td>16.5</td>
<td>14.8</td>
<td>9.5</td>
<td>64.7</td>
</tr>
</tbody>
</table>

*, RSD, relative standard deviation = 100 x SD/average; ** Np, no participation in this ring test
The relative standard deviations of the average ATP concentrations ranged from 16.4% to 175% with a median value of 41%. The numbers of samples in each laboratory for which the reported value either was ≤ avg. ± SD or ≥ avg. ± SD were 2 (P3), 5 (P2), 6 (P1), 6 (P6), 8 (P5) and 19 (P4), respectively. Further analysis of this data was conducted by plotting the observations of the participants in each sample as a function of the calculated average concentrations (Fig. 3.1). These average concentrations are considerably affected by deviating observations, but in the absence of true values, this approach was used as a compromise.

Fig. 3.1 ATP concentrations as reported by the participants (y-axis), plotted as a function of the average values of the reported ATP concentrations (x-axis). A, 1st ring test; B, 2nd ring test; C, 3rd ring test and D, 4th ring test. Broken lines represent 100% similarity with the calculated average values.

For each participant linear regression analysis was undertaken for each ring test (Table 3.4). The values of the slopes of the calculated relationships between the observations of the participants were clearly different. However, the regression coefficients were relatively high, indicating that most of the differences are due to systematic differences in the analysis. A main factor is the (preparation of the) calibration curve. A few laboratories prepared the
standard solution by dissolving ATP in buffer, others used tap water or demineralised water (Table 3.2).

### Table 3.4 Correlations between results (linear regression) in 4 ATP ring tests*

<table>
<thead>
<tr>
<th>Participant**</th>
<th>Ring test</th>
<th>A (slope)</th>
<th>B (intercept)</th>
<th>r²</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1 (D/T)</td>
<td>1</td>
<td>0.864 ± 0.02</td>
<td>6.3 ± 1.1</td>
<td>0.998</td>
</tr>
<tr>
<td>P2 (B/MQ)</td>
<td>1</td>
<td>1.41 ± 0.02</td>
<td>0.61 ± 1.3</td>
<td>0.999</td>
</tr>
<tr>
<td>P3 WR (T/T)</td>
<td>1</td>
<td>0.954</td>
<td>-1.07 ± 2.4</td>
<td>0.996</td>
</tr>
<tr>
<td>P4 (B/D)</td>
<td>1</td>
<td>0.747 ± 0.02</td>
<td>5.2 ± 1.2</td>
<td>0.998</td>
</tr>
<tr>
<td>P5 (B/B)</td>
<td>1</td>
<td>1.01 ± 0.02</td>
<td>-0.53 ± 1.2</td>
<td>0.999</td>
</tr>
<tr>
<td>P1 (D/T)</td>
<td>2</td>
<td>-1.00 ± 1.2</td>
<td>167 ± 68</td>
<td>0.26</td>
</tr>
<tr>
<td>P2 (B/B)</td>
<td>2</td>
<td>1.7 ± 0.18</td>
<td>-42 ± 10.5</td>
<td>0.989</td>
</tr>
<tr>
<td>P3 (T/T)</td>
<td>2</td>
<td>1.51 ± 0.59</td>
<td>-43.9 ± 34</td>
<td>0.76</td>
</tr>
<tr>
<td>P4 (B/D)</td>
<td>2</td>
<td>1.02 ± 0.33</td>
<td>-0.51 ± 18.4</td>
<td>0.83</td>
</tr>
<tr>
<td>P5 (B/B)</td>
<td>2</td>
<td>1.42 ± 0.27</td>
<td>-40.1 ± 15.5</td>
<td>0.93</td>
</tr>
<tr>
<td>P5 (B/D)</td>
<td>2</td>
<td>1.33 ± 0.39</td>
<td>-40.9 ± 22.5</td>
<td>0.85</td>
</tr>
<tr>
<td>P1 (T/T)</td>
<td>3</td>
<td>1.31 ± 0.11</td>
<td>-0.16 ± 5.3</td>
<td>0.968</td>
</tr>
<tr>
<td>P2 (B/B)</td>
<td>3</td>
<td>1.34 ± 0.05</td>
<td>-2.47 ± 2.57</td>
<td>0.992</td>
</tr>
<tr>
<td>P3 (T/T)</td>
<td>3</td>
<td>0.99 ± 0.1</td>
<td>-3.3 ± 4.5</td>
<td>0.96</td>
</tr>
<tr>
<td>P4 (B/D)</td>
<td>3</td>
<td>0.18 ± 0.17</td>
<td>7.5 ± 7.8</td>
<td>0.21</td>
</tr>
<tr>
<td>P5 (B/B)</td>
<td>3</td>
<td>1.38 ± 0.05</td>
<td>-0.95 ± 2.26</td>
<td>0.996</td>
</tr>
<tr>
<td>P6 (B/D)</td>
<td>3</td>
<td>0.408 ± 0.01</td>
<td>-5.2 ± 0.77</td>
<td>0.993</td>
</tr>
<tr>
<td>P1 (T/T)</td>
<td>4</td>
<td>0.80 ± 0.01</td>
<td>-7.5 ± 0.4</td>
<td>0.998</td>
</tr>
<tr>
<td>P2 (B/T)</td>
<td>4</td>
<td>0.82 ± 0.01</td>
<td>2.05 ± 2.45</td>
<td>0.999</td>
</tr>
<tr>
<td>P3 (T/T)</td>
<td>4</td>
<td>0.79 ± 0.02</td>
<td>8.3 ± 6.6</td>
<td>0.995</td>
</tr>
<tr>
<td>P4 (B/D)</td>
<td>4</td>
<td>1.36 ± 0.016</td>
<td>9.9 ± 4.76</td>
<td>0.999</td>
</tr>
<tr>
<td>P5 (B/B)</td>
<td>4</td>
<td>1.55 ± 0.006</td>
<td>-10.6 ± 2.0</td>
<td>0.999</td>
</tr>
<tr>
<td>P6 (B/D)</td>
<td>4</td>
<td>0.67 ± 0.009</td>
<td>-1.42 ± 2.7</td>
<td>0.998</td>
</tr>
</tbody>
</table>

*, (ATP participant) = A x (ATP avg.) + B; **, between brackets: water types used for preparing calibration solution.

3.4 Calibration curves

Dissolved inorganic compounds (salts) have a large impact on light emission, with a decreasing yield at increasing salt concentrations (Karl and LaRock, 1975). Consequently, preparing ATP dilutions for calibration in buffer leads to an overestimation of the ATP concentration in tap water (with low concentrations of salts, cf. Table 4.3). Preparation of ATP dilutions in demineralised or distilled water may lead to relatively high RLU/ATP values, and result in an underestimation of the ATP concentrations in the samples (low slope), depending on the concentration of inorganic compounds in the test water. A number of additional factors can also affect the ATP analysis (maintenance of equipment; automatic of manually operating equipment; accuracy of procedures etc.).

As a result of the observations in the ring tests, further attention was paid to the preparation of the solutions for calibration. The effects of preparing the stock solutions and further dilutions in buffer and/or in tap water are shown in Fig. 3.5 and in Table 3.5.
Fig. 3.5 ATP calibration curves with stock solutions/dilutions prepared in tap water (TW), in buffer or in demineralised (Demi) water, respectively (results Kiwa WR).

Table 3.5 ATP calibration characteristics in 4 water types (Kiwa WR)

<table>
<thead>
<tr>
<th>Concentration range (ng ATP/l)</th>
<th>Slope (RLU/ATP)</th>
<th>R²</th>
<th>Slope (RLU/ATP)</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tap water/tap water</td>
<td>0.9973</td>
<td>demineralised water/demineralised water</td>
<td>0.975</td>
</tr>
<tr>
<td>1.6 - 40</td>
<td>5.22± 0.085</td>
<td></td>
<td>5.58 ± 0.28</td>
<td></td>
</tr>
<tr>
<td>1.6 - 100</td>
<td>5.11 ± 0.041</td>
<td>0.9992</td>
<td>5.50 ± 0.100</td>
<td>0.996</td>
</tr>
<tr>
<td>1.6 - 200</td>
<td>5.27 ± 0.029</td>
<td>0.9995</td>
<td>5.649 ± 0.053</td>
<td>0.9987</td>
</tr>
<tr>
<td>1.6 - 1000</td>
<td>5.29± 0.016</td>
<td>0.9998</td>
<td>5.62 ± 0.012</td>
<td>0.999927</td>
</tr>
<tr>
<td></td>
<td>Buffer/buffer</td>
<td></td>
<td>Buffer/tap water</td>
<td></td>
</tr>
<tr>
<td>1.6 - 40</td>
<td>3.52± 0.057</td>
<td>0.9973</td>
<td>5.20 ± 0.1007</td>
<td>0.996</td>
</tr>
<tr>
<td>1.6 - 100</td>
<td>3.52 ± 0.041</td>
<td>0.9983</td>
<td>5.17 ± 0.043</td>
<td>0.9992</td>
</tr>
<tr>
<td>1.6 - 200</td>
<td>3.37 ± 0.0027</td>
<td>0.9990</td>
<td>5.11 ± 0.0296</td>
<td>0.9995</td>
</tr>
<tr>
<td>1.6 - 1000</td>
<td>3.44 ± 0.0066</td>
<td>0.9994</td>
<td>5.25 ± 0.033</td>
<td>0.9993</td>
</tr>
</tbody>
</table>

*, tap water, ATP dissolved in tap water and diluted in tap water; demineralised water, ATP dissolved in demi water and diluted in demi water; Buffer/buffer, ATP dissolved in buffer and diluted in buffer; Buffer/tap water, ATP dissolved in buffer and diluted in tap water. For tap water composition, see Table 4.3.

The average values of the RLU/ATP yields presented in Table 3.5 were 5.22 (tap water/tap water), 5.18 (Buffer/tap water), 5.58 (demineralised water/demineralised water) and 3.46 (Buffer/Buffer), respectively. The relative values (compared to the combination of tap water/tap water) are: 0.99 (B/T), 1.07 (D/D), 0.66 (B/B). Hence, the ATP concentration in tap (test) water calculated from the calibration curve in demineralised water would be 93% of the concentration calculated from the calibration curve in tap water (at Kiwa WR) and 1.5 times this value when using the calibration data obtained in buffer (LuminATE(Ph)).

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Fig. 3.6 Calibration curves for ATP analysis in different water types (data TZW).

Similar experiments have been conducted by all participants to assess the relationship between the RLU/ATP yields in demineralised water, buffer and tap (test) water, respectively (Fig. 3.6). The derived conversion factors are shown in Table 3.6.

Table 3.6 Relative RLU/ATP yields in tap (test) water and in demineralised water in comparison to the yield in buffer.

<table>
<thead>
<tr>
<th>Participant</th>
<th>Buffer</th>
<th>Tap water</th>
<th>Demineralised water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crecep</td>
<td>1</td>
<td>1.19</td>
<td>1.29</td>
</tr>
<tr>
<td>DTU</td>
<td>1</td>
<td>1.21</td>
<td>1.32</td>
</tr>
<tr>
<td>Kiwa WR</td>
<td>1</td>
<td>1.42</td>
<td>1.52</td>
</tr>
<tr>
<td>OFI</td>
<td>1</td>
<td>1.49</td>
<td>1.43</td>
</tr>
<tr>
<td>TZW</td>
<td>1</td>
<td>1.16</td>
<td>1.65*</td>
</tr>
<tr>
<td>TWUL</td>
<td>N.a.</td>
<td>0.92**</td>
<td>1**</td>
</tr>
</tbody>
</table>

*, tap water provided by Kiwa; **demineralised water was taken as reference; N.a., not analysed.

Additional investigations have been conducted by DTU and OFI where the effect of different equipment using identical reagents (LumiATE/LuminEX) was tested. The results demonstrated 10 to 20 % differences between the ATP concentrations observed with different equipment. These differences may have been affected by a high intercept and a low regression coefficient of the calibration curve at OFI. A high intercept indicates that the water used for calibration was not ATP free. No investigation was performed with reagents and samples from the same vials/bottles.

3.5 Miscellaneous
At TZW cuboids (10 x 15 x 4 cm) of cementitious products had been incubated in tap water for about 12 months. Samples were placed in water (600 to 850 ml) contained in sterile plastic bags to limit the volume of water for biomass detachment. Subsequently, these bags were placed in a water bath and a 2 min
low energy sonication treatment (see Chapter 5) was applied. The obtained suspension had a high turbidity, which hampered ATP analysis. Therefore a standard addition procedure with ATP was applied for measuring ATP. These observations revealed that products to be tested may release compounds which inhibit ATP analysis.

3.6 Discussion
The results of the ring tests show that relatively large differences were observed between ATP concentrations in similar samples as distributed in the ring test (median RSD value: 41%). Deleting extreme values may show a better picture, but it is clear that further standardisation is needed. Water samples had been transported on ice. Observations on water temperature in the samples upon arrival revealed that temperature in the containers used for distributing the samples had not increased. Therefore it is assumed that changes in ATP concentrations were negligible, although some incidental differences cannot be excluded. Hence, the observed differences were due to differences in the ATP analysis, e.g. related to procedure, equipment and chemicals. The water type used for calibration seems to be a major source of differences. Also differences between calibration curves over time or between persons within one laboratory is a source of errors. At Kiwa it had been observed that the slopes of calibration curves on average differed about 4.5% between experienced technicians using automatic equipment. In the past, with less experienced technicians, differences over 10% were observed. Since ATP calibration is both time consuming and costly, the frequency of calibration can be restricted provided that the equipment and reagents remain the same together with factors such as room temperature. Inclusion of controls in the sample series enables verification of the analytical procedures and chemicals (see Appendix 1).

![Graph](image1)

**Fig. 3.7** Effects of conductivity and Ca concentration in tap water on the relative light yield in the ATP assay. Relative light yield is RLU/ATP in water compared to RLU/ATP in buffer. Data on conductivity and Ca concentrations in tap water are given in Table 4.3.

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The observations in the ring tests, which had been conducted over a period of about 1.5 years, showed that improvement was achieved when tap water was used for the preparation of the calibration curve. The participants P1, P2 and P3 obtained similar results in ring test 4 (Table 3.3). Also, recalculation of the P5 data of ring tests 3 and 4 using Kiwa tap water for calibration gave results which were much closer to those reported by the participants P1, P2 and P3. However, the calibration curves determined at TZW (Fig. 3.6) and the yield factors in tap water in comparison to the yield in buffer reveal that different types of tap water also give different yield factors. The concentration of inorganic constituents, and Ca in particular, has a strong inhibitory effect on light emission in the ATP assay (Karl and LaRock, 1975). Fig. 3.7 shows the inhibitory effect of the concentration of Ca as present in the types of tap water used in the tests (Table 4.3) on the relative light yield. These observation implicate that identical results cannot be obtained in ring tests when the locally available tap water is used for ATP calibration purposes. Demineralised water or the buffer can be used for this purpose. However, it should be realised that the use of buffer, with a low RLU/ATP yield, gives an overestimation of the ATP concentration in test water and also that demineralised water, with a high RLU/ATP yield, gives an underestimation of the ATP concentration in test water. Hence, calibration of the ATP analysis for application in regular tests should be done in the test water to obtain the correct ATP concentration. Buffer of demineralised water may be used but the conversion factor should be established on a regular basis. Another option is to use standard addition to account for the matrix effects on the ATP analysis.

The differences in ATP analysis as observed in the ring test have an impact on the results of the investigations as conducted with the materials in the various project stages and hamper comparisons between BPP values as observed with identical materials in different laboratories. However, the differences in ATP analysis are less relevant for tests aiming at determining effects of certain test conditions within each laboratory in the course of the investigation. In stage 7 all laboratories used test (tap) water for preparing the calibration curve.

3.7 Conclusions.
- Reported ATP concentrations in samples which had been prepared and distributed under conditions ensuring similarity and stability showed relatively large differences between the participants;
- The use of different water types for preparing calibration curves seems a main source of error, but other factors (e.g. differences over time in preparing ATP solutions, manually operated versus automated equipment) may also play a role;
- Improvement was observed in the course of the project, with laboratories using the same equipment and reagents and preparing the calibration solutions in tap water;
- For ATP ring tests, calibration should be done in water with a defined composition, viz. demineralised water of buffer;
- For determining the ATP concentration in test water and on materials, calibration should be done in test water. Buffer of demineralised water may be used provided that the conversion factor is determined on a regular basis;
- A more detailed standardisation of the ATP analysis is needed to improve the quality of the analysis.
4 Selection of test water, effect of inoculum and jar type

4.1 Introduction
Tap water that complies with the European Directive 80/778/EEC is the basis for the test water. Essential additional water quality aspects of test water as compared to drinking water include the presence of inorganic compounds which may serve as nutrients (P,N), as a source of energy (ammonia), or inhibit growth (metals, disinfectants). Furthermore, the concentration of (organic) growth-promoting compounds should be strictly limited. Table 2.2 lists the quality criteria as defined in the UK Standard, BS 6920 and gives guideline values for a number of parameters as based on the discussions in WP1. The need to increase the diversity of the microbial community by adding an inoculum obtained from surface water is an issue that needs to be tested. Selection of appropriate water by each participant was needed prior to conducting experiments with materials. Also, an appropriate container type for testing had to be selected. The investigations, which cover stages 3 and 5 of the project are described in this Chapter.

4.2 Selection of test water (stages 3)
Each participant selected a few water types to determine the biomass production potential (BP) with the ATP test. This test included incubation of the water sample (600 ml) contained in thoroughly-cleaned 1 litre borosilicate glass bottles (Schott Duran) with polyethylene (blue) cap. These flasks had been cleaned by soaking in a phosphate free detergent, rinsing ten times with hot tap water, rinsing three times with demineralised water, drying at 100 °C in an oven and rinsing twice with test water before use. Different laboratories used different cap types and different cleaning procedures (see stage 3). At Kiwa WR, also thoroughly cleaned glass-stoppered Erlenmeyer flasks (heated at 550 °C) were used for comparison with the Schott flasks. Nitrates (5 mg N/l) and phosphates (2 mg P/l) were added from autoclaved stock solutions prepared in demineralised water) to the selected water types to obtain the necessary conditions for testing of materials.

The selection of the water type was combined with testing of the effect of a 1 % inoculum. For this purpose samples of river water were collected. After membrane filtration (1.2 μm pores) of this water a 1% v/v inoculum was added to the test water. At TZW glass fibre filtration was applied.

The flasks were incubated at 25 ± 2 °C and ATP analysis was conducted at a regular basis for a period up to two weeks. At Crecep samples were incubated at 22 ± 2 °C. Table 4.1 shows the Biomass Production (BP) values (maximum ATP concentrations) as observed by the participants in the selected water types.
### Table 4.1 Growth potential of test water types without and with inoculum (Stage 3/5)

<table>
<thead>
<tr>
<th>Participant</th>
<th>Water type</th>
<th>Maximum BP (ng ATP/l)</th>
<th>Without inoc</th>
<th>With inoc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Creep</td>
<td>Tap water Choisy water treatment plant</td>
<td>23.7 ± 3.5</td>
<td>46.4 ± 2.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tap water Vanne Aqueduct</td>
<td>9.2 ± 0.3</td>
<td>9.4 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>DTU</td>
<td>Tap water</td>
<td>3.1 ± 0.3</td>
<td>4.6 ± 0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tap water + P + N</td>
<td>3.5 ± 0.1</td>
<td>5.5 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>Kiwa WR</td>
<td>Slow sand filtrate + P + N</td>
<td>7.3 ± 1.3</td>
<td>9.9 ± 2.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Slow sand filtrate + PN, red cap#</td>
<td>Nt</td>
<td>7.6 ± 0.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Slow sand filtrate (E)*</td>
<td>5.3 ± 0.5</td>
<td>Nt</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Slow sand filtrate (E) + P+N</td>
<td>6.7 ± 1.3</td>
<td>Nt</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tap water + P + N</td>
<td>5.9 ± 2.7</td>
<td>8.8 ± 1.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tap water (E) + P + N</td>
<td>3.8 ± 0.6</td>
<td>Nt</td>
<td></td>
</tr>
<tr>
<td>OFI</td>
<td>Tap water (without P/N)</td>
<td>8.6 ± 0.9</td>
<td>10.8 ± 1.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Milli Q + P + N</td>
<td>35.4 ± 0.3</td>
<td>55.4 ± 2.9</td>
<td></td>
</tr>
<tr>
<td>TZW</td>
<td>Tap water Karlsruhe + P + N</td>
<td>1.5</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tap water B ### + P + N</td>
<td>15</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tap water C &amp; ^ + P + N</td>
<td>17</td>
<td>Nt</td>
<td></td>
</tr>
<tr>
<td>TWUL</td>
<td>Tap water</td>
<td>71 ± 24</td>
<td>76 ± 31</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Laboratory treated water</td>
<td>9.8 ± 0.3</td>
<td>5.9 ± 0.3</td>
<td></td>
</tr>
</tbody>
</table>

*, (E) = tested in glass-stoppered Erlenmeyer flasks; # Schott bottles with red cap; ## tap water prepared from lake water; &, tap water prepared from ground water; Nt, not tested

### Table 4.2 Water types selected for further use in the investigations

<table>
<thead>
<tr>
<th>Participant</th>
<th>Test water</th>
<th>Source of inoculum *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Creep</td>
<td>Tap water (Vanne Aqueduct), derived from underground spring water. Chlorine is removed from the water before being stored in reservoirs</td>
<td>River Seine (above Ivy Water treatment plant)</td>
</tr>
<tr>
<td>DTU</td>
<td>Tap water prepared from ground water, Prepared from anaerobic ground water by aeration and rapid sand filtration (no chemical disinfection)</td>
<td>Lake Arresoe</td>
</tr>
<tr>
<td>Kiwa WR</td>
<td>Tap water as available at the laboratory. Prepared from anaerobic ground water by aeration and rapid sand filtration (no chemical disinfection)</td>
<td>River Rhine (Lek) at Nieuwegein</td>
</tr>
<tr>
<td>OFI</td>
<td>Tap water (untreated mountain water)</td>
<td>River Danube</td>
</tr>
<tr>
<td>TZW</td>
<td>Tap water in Karlsruhe is a mixture of 4 different ground water works**</td>
<td>River Rhine at Karlsruhe-Maxau</td>
</tr>
<tr>
<td>TWUL</td>
<td>Laboratory treated water - tap water treated on-site using the following sequence - de-ionisation, activated carbon, 5 micron filtration, reverse osmosis, electronic ion exchange, 0.45 micron filtration, ultra violet disinfection and finally 0.2 micron filtration.</td>
<td>Lowland river water (River Kennet taken from the abstraction point of Fobney water treatment works)</td>
</tr>
</tbody>
</table>

* 1% of test water volume, membrane (1.2 µm) filtered; ** one works applies aeration for iron removal; one water type is aerobic and is not treated.

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Table 4.1 shows that all participants had at least 1 water type available with a BP value below 10 ng ATP/l. In a few types of tap water this BP value was exceeded. At TWUL it was concluded that laboratory treated tap water was a suitable alternative. The data presented in Table 4.1 demonstrate that the effect of the addition of a 1% inoculum was negligible. Addition of the inorganic nutrients (P, N) also had a negligible effect on the BP values.

Table 4.3 Chemical composition of water types used in the tests

<table>
<thead>
<tr>
<th>Water quality parameter</th>
<th>Crecep</th>
<th>DTU</th>
<th>Kiwa</th>
<th>OFI</th>
<th>TW</th>
<th>Lab treated TW</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vanne Aqueduct</td>
<td>TW*</td>
<td>TW</td>
<td>TW</td>
<td>TW</td>
<td>TW</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>12-20</td>
<td>10 (8-12)</td>
<td>12.8</td>
<td>10.9</td>
<td>23</td>
<td>9-21</td>
</tr>
<tr>
<td>pH</td>
<td>7.6</td>
<td>7.5</td>
<td>7.86</td>
<td>7.4</td>
<td>7.35</td>
<td>6.5</td>
</tr>
<tr>
<td>Conduct. (μS/cm)</td>
<td>554</td>
<td>792</td>
<td>388</td>
<td>349</td>
<td>634</td>
<td>483-645</td>
</tr>
<tr>
<td>CO₂ (mg/l)</td>
<td>8.5</td>
<td>&lt;2</td>
<td>6.5</td>
<td>27</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HCO₃ (mg/l)</td>
<td>24°F</td>
<td>305</td>
<td>257</td>
<td>311</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cl⁻ (mg/l)</td>
<td>23</td>
<td>70</td>
<td>9.4</td>
<td>0.5-2.5</td>
<td>15.3</td>
<td></td>
</tr>
<tr>
<td>SO₄²⁻ (mg/l)</td>
<td>17</td>
<td>54</td>
<td>&lt;0.1</td>
<td>2.8</td>
<td>57</td>
<td></td>
</tr>
<tr>
<td>Ammonia (mg N/l)</td>
<td>&lt;0.1</td>
<td>&lt;0.05 &lt;0.05</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>&lt;0.06</td>
<td></td>
</tr>
<tr>
<td>Na (mg/l)</td>
<td>6.5</td>
<td>28</td>
<td>0.9</td>
<td>&lt;1</td>
<td>9.3</td>
<td></td>
</tr>
<tr>
<td>K (mg/l)</td>
<td>1.8</td>
<td>3.5</td>
<td>0.9</td>
<td>&lt;1</td>
<td>1.5</td>
<td>1.9</td>
</tr>
<tr>
<td>Ca (mg/l)</td>
<td>119</td>
<td>109</td>
<td>71.5</td>
<td>37</td>
<td>112</td>
<td>95</td>
</tr>
<tr>
<td>Mg (mg/l)</td>
<td>3.5</td>
<td>16</td>
<td>5.8</td>
<td>7.2</td>
<td>8.9</td>
<td>2.5</td>
</tr>
<tr>
<td>Hardness (mmol Ca/l)</td>
<td>28°F</td>
<td>3.4</td>
<td>2.0</td>
<td>1.6</td>
<td>3.2</td>
<td></td>
</tr>
<tr>
<td>NO₃ (mg N/l)</td>
<td>40-42</td>
<td>2</td>
<td>0.6</td>
<td>2.0-6.0</td>
<td>3.8</td>
<td>19.5-22.1</td>
</tr>
<tr>
<td>Total phosphate (mg/l)</td>
<td>&lt;0.1</td>
<td>&lt;0.02</td>
<td>0.1</td>
<td>0.0</td>
<td>0.01</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Copper (μg/l)</td>
<td>&lt;3</td>
<td>&lt;1</td>
<td>1.2</td>
<td>&lt;10</td>
<td>4.5</td>
<td></td>
</tr>
<tr>
<td>Total organic carbon (mg C/l)</td>
<td>1.6-1.8</td>
<td>1.85</td>
<td>2.0</td>
<td>0.84</td>
<td>1.0</td>
<td>1.2</td>
</tr>
<tr>
<td>Total chlorine</td>
<td>0.1-0.3</td>
<td>0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.08-0.42</td>
</tr>
</tbody>
</table>

* TW, tap water

Tables 4.2 and 4.3 give information about the origin and composition of the water types that were selected for further testing. Table 4.3 includes the composition of the tap water tested at TWUL because this water was used in stage 7 of the project (see Chapter 6). Tap water as tested at TWUL is supplied by Thames Water from their Fobney advanced water treatment plant - water abstracted the River Kennet (a navigable waterway) and subsequently treated by primary filtration, slow sand filtration with a sandwich of granular activated carbon, optional ozonisation, and final disinfection using chlorine - dechlorinated with sodium thiosulfate before use in the test laboratory.

4.2.1 Effect of container type

The possible effect of using a less thoroughly cleaned flask with a plastic cap was tested at Kiwa WR by conducting additional tests with water in the thoroughly cleaned glass-stoppered Erlenmeyer flasks. As shown in Table 4.1 Microbial growth support potential of CPDW - 49 - July 2003
no significant differences were observed between BP values of water incubated in the Erlenmeyer flasks and those incubated in the Schott flasks. At DTU the potential of various plastic caps to promote growth was determined with the AOC test. It was observed that the growth-promoting properties of the red caps (thermosetting plastics material) were less pronounced than such properties of the blue caps (thermoplastics polyolefin).

4.3 Effect of inoculum (stage 5)

The effect of inoculum was tested in all laboratories using the selected water type and a selection of materials including: glass (control), Silicone Rubber and Nitrile Rubber. These materials had been prepared and distributed by Kiwa WR, whereas TWUL had provided the Nitrile Rubber material. The test was conducted in Schott bottles under static conditions (no replacement of the water). Test conditions were similar to those in the experiment for test water selection (incubation at 25°C).

<table>
<thead>
<tr>
<th>Participant</th>
<th>Material tested</th>
<th>Day</th>
<th>Biomass Production (pg ATP/cm²)</th>
<th>Without inoculum</th>
<th>With inoculum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Creep</td>
<td>Tap water + glass</td>
<td>54</td>
<td>50 (10)*</td>
<td>70 (20)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+ Silicone Rubber</td>
<td>54</td>
<td>500 (200)</td>
<td>600 (250)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+ Nitrile Rubber</td>
<td>54</td>
<td>4500 (3500)</td>
<td>5500 (4000)</td>
<td></td>
</tr>
<tr>
<td>DTU</td>
<td>Tap water + glass</td>
<td>78</td>
<td>20 (2)</td>
<td>24 (4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+ Silicone Rubber</td>
<td>78</td>
<td>188 (96)</td>
<td>152 (58)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+ Nitrile Rubber</td>
<td>78</td>
<td>292 (92)</td>
<td>22 (3)</td>
<td></td>
</tr>
<tr>
<td>Kiwa WR</td>
<td>Tap water + glass</td>
<td>54</td>
<td>70 (15)</td>
<td>50 (5)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+ Silicone Rubber</td>
<td>54</td>
<td>520 (180)</td>
<td>480 (250)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+ Nitrile Rubber</td>
<td>54</td>
<td>4800 (4600)</td>
<td>5500 (5200)</td>
<td></td>
</tr>
<tr>
<td>OFI</td>
<td>Tap water + glass</td>
<td>28</td>
<td>263 ± 30</td>
<td>251 ± 51</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+ Silicone Rubber</td>
<td>28</td>
<td>3523 ± 79</td>
<td>4008 ± 880</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+ Nitrile Rubber</td>
<td>28</td>
<td>4023 ± 930</td>
<td>3486 ± 2831</td>
<td></td>
</tr>
<tr>
<td>TZW</td>
<td>Tap water + glass</td>
<td>54</td>
<td>11 (7)</td>
<td>21 (4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+ Silicone Rubber</td>
<td>54</td>
<td>491 (253)</td>
<td>391 (184)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+ Nitrile Rubber</td>
<td>54</td>
<td>4463 (3708)</td>
<td>5851 (4256)</td>
<td></td>
</tr>
<tr>
<td>TWUL</td>
<td>Tap water + glass</td>
<td>14</td>
<td>1434 (1420)</td>
<td>NT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+ Silicone Rubber</td>
<td>14</td>
<td>39605 (39405)</td>
<td>NT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+ Nitrile Rubber</td>
<td>14</td>
<td>483072(482900)</td>
<td>NT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lab treated water (LTW) + glass</td>
<td>14</td>
<td>402 (401)</td>
<td>375 (372)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LTW + Silicone Rubber</td>
<td>14</td>
<td>50024 (50005)</td>
<td>89496 (89384)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LTW + Nitrile Rubber</td>
<td>14</td>
<td>143 (141)</td>
<td>4916 (4773)</td>
<td></td>
</tr>
</tbody>
</table>

* data between brackets represent attached biomass; ** NT, not tested

The data presented in Table 4.4 show that addition of an inoculum had no significant effect on most BP values. A few exceptions were observed with Nitrile Rubber, which gave higher BP values in water with inoculum. Continued testing at DTU, where low BP values were found with Nitrile
Rubber in a test period of 78 days, also indicated that growth in the presence of this material was affected by the type of inoculum. The effect of the inoculum on the growth in the glass control was very limited, thus confirming the observations with the blank water. The introduction of biodegradable compounds with the inoculum obviously is negligible. Based on these observations and the theoretical consideration that a materials test should include a wide variety of micro-organisms covering a wide range of metabolic properties it was concluded to add an inoculum to the test water in further investigations.

4.4 Conclusions
- All participants had selected a water type with a low BP value for further testing;
- Thoroughly cleaned Schott Duran bottles were considered suitable for use in further investigations;
- Addition of an inoculum did not increase the BP value of the test water but may have an effect with certain materials. A 1% river water inoculum will be used in further tests.
5 Effect of water replacement

5.1 Introduction
The materials are in contact with continuous flowing tap water in the W270 method and no water replacement is applied in the BPP test. The MDOD test is in between these extremes with a water replacement twice a week (Table 1.1). Discussion by the WP1 participants had led to the conclusion that a discontinuous flow in a test gives is a more realistic simulation of conditions in practice than a static test or a continuous-flow test. Therefore, it was decided to compare the effects of various water replacement frequencies on the biomass production of a number of selected materials. The frequencies ranged from no replacement (original BPP test) to two replacements each week as applied in the MDOD test (Table 5.1). The objectives of the experiments in stage 6 were: (i), to establish whether or not water replacement has an effect on the biomass production of a material and if so, (ii) to select the most appropriate water replacement scheme.

Table 5.1 Water replacement schemes as tested by the participants

<table>
<thead>
<tr>
<th>Participant</th>
<th>Scheme 1 (BPP)</th>
<th>Scheme 2 (MDOD)</th>
<th>Scheme 3</th>
<th>Scheme 4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No replacement</td>
<td>Replacement 2x/week</td>
<td>Replacement 1x/week</td>
<td>Replacement 1x/2 week</td>
</tr>
<tr>
<td>CrecceP</td>
<td>X*</td>
<td>-**</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>DTU</td>
<td>X</td>
<td>-</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Kiwa WR</td>
<td>X</td>
<td>X</td>
<td>-</td>
<td>X</td>
</tr>
<tr>
<td>OFI</td>
<td>X</td>
<td>-</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>TZW</td>
<td>X</td>
<td>-</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>TWUL</td>
<td>X</td>
<td>X</td>
<td>-</td>
<td>X</td>
</tr>
</tbody>
</table>

* X, test conducted; ** - , not conducted

5.2 Materials and methods

5.2.1 Materials

The materials applied in project stage 6 are listed in Table 5.2. The selection of materials in this project stage was based on the following considerations:
- include plastic materials with a high production of biomass (PVC-P) and with a low production of biomass (PVC-C), respectively;
- include a rubber type.

Glass cylinders (length, 15 mm; outer diameter 18 mm; wall thickness 2 mm; total external surface area 15 cm²), which had been heated in an oven at 550° for 4 hours, served a negative control. PVC-P hose (outer diameter 16 mm; wall thickness 2 mm) with 36% of phthalates was selected for use as a positive control. The positive control and test materials were rinsed with flowing tap water for 15 minutes, stored for 24 h in tap water and again 15 minutes rinsed in flowing tap water prior to use.
Table 5.2 Materials used in stage 6

<table>
<thead>
<tr>
<th>Material</th>
<th>Preparation</th>
<th>Nominal exposed surface (cm²)</th>
<th>Surface (S) to volume (V) ratio (cm⁻¹)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glass cylinders</td>
<td>Provided (precut)</td>
<td>8.6</td>
<td>0.172</td>
</tr>
<tr>
<td>PVC-C pipe</td>
<td>Provided (precut)</td>
<td>13.6</td>
<td>0.254</td>
</tr>
<tr>
<td>Silicone Rubber hose</td>
<td>Cut with knife</td>
<td>8 - 9</td>
<td>0.16 - 0.18</td>
</tr>
<tr>
<td>Nitrile Rubber ‘O’ seals</td>
<td>As produced</td>
<td>10.3</td>
<td>0.21</td>
</tr>
<tr>
<td>PVC-P hose</td>
<td>Cut with knife</td>
<td>7.5 - 12.6</td>
<td>0.15 - 0.25</td>
</tr>
</tbody>
</table>

* at TZW a value of 0.16 cm⁻¹ was obtained by adaptation of the volume of test water (e.g. 11 pieces of PVC-C each of 14 cm² in 962.5 ml of test water)

5.2.2 Test water, inoculum and test system

Table 5.3. Test water and inoculum as used in stage 6

<table>
<thead>
<tr>
<th>Participant</th>
<th>Test water</th>
<th>Source of inoculum *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crecop</td>
<td>Tap water (Vaince Aqueduct), dechlorinated</td>
<td>River Seine</td>
</tr>
<tr>
<td>DTU</td>
<td>Tap water prepared from ground water</td>
<td>Lake Arresoe**</td>
</tr>
<tr>
<td>Kiwa WR</td>
<td>Tap water prepared from ground water</td>
<td>River Rhine (Lek) at Nieuwegein</td>
</tr>
<tr>
<td>OFI</td>
<td>Tap water (untreated mountain water)</td>
<td>River Danube</td>
</tr>
<tr>
<td>TZW</td>
<td>Tap water prepared from ground water</td>
<td>River Rhine at Karlsruhe-Maxau</td>
</tr>
<tr>
<td>TWUL</td>
<td>Laboratory treated water - tap water treated on-site using the following sequence - demineralization, activated carbon, 5 micron filtration, reverse osmosis, electronic ion exchange, 0.45 micron filtration, ultra violet disinfection and finally 0.2 micron filtration</td>
<td>Lowland river water (River Kennet taken from the abstraction point of Fobney water treatment works)</td>
</tr>
</tbody>
</table>

* 1% of test water volume, membrane (1.2 µm) filtered; **DTU also inoculated a set of Nitrile Rubber samples with Kiwa inoculum (see report on stage 6)

Volumes of 600 ml of the selected test water were introduced into cleaned 1-litre containers (Schott borosilicate flask) and supplements of phosphate (2 mg P/l) and nitrate (5 mg N/l) were added from autoclaved stock solutions in demineralized water. Twelve test pieces, each having a nominal surface area of about 8 cm² were added; 6 ml of inoculum was then added to the containers. At DTU 6 ml were added to make up a final volume of 600 ml. After closing with blue thermoplastic caps the test containers were incubated, in the absence of light at 25°C (22°C at Crecop). At DTU red caps with Teflon inlays were used. All test systems were set up in duplicate.

The following water replacement schemes were applied:
- Scheme 1 (no replacement): no water changes were undertaken;
- Scheme 2 (replacement twice a week): the water in each test container was poured to waste and replaced with an identical volume of test water plus supplements (but without inoculum). When ATP measurements were
undertaken these were carried out immediately before the change of water;
-  Scheme 3 (replacement every week); see scheme 2;
-  Scheme 4 (replacement every 2 weeks); see scheme 2.

5.2.3 Sampling and biomass analysis

Suspended biomass. At each test period 50 ml of test water was removed for ATP analysis (assessment of planktonic growth). Flasks were gently swirled directly prior to sampling of the water. (At TZW 87.5 ml of test water was removed with PVC-C). At OFI a volume of 1 ml was collected into a sterile Eppendorf pipette and 49 ml was decanted. At TWUL a 50 ml sample was removed from the test jar for suspended biomass measurement, after first gently swirling the test jars. The disposable micropipette tip used for the suspended biomass sample was sold as ‘ATP free’ and sterile.

Attached biomass. One test piece was removed from each test container using sterilised tongs and placed into a glass test tube or beaker (Nitrile Rubber ‘O’ seals) with 10 ml of sterile water. The tube with the test piece was placed into an ultrasonic bath and exposed to ultrasonic energy for 2 minutes. At the end of this period the sonicate was collected in a separate container standing in ice, and the sonication of the test piece was repeated with a further 10 ml of sterile water. The treatment was repeated to give the following total number of sonication periods: glass – three; all other materials – six.

The total volume of sonicate collected for each sample was 30 ml and 60 ml, respectively. At TZW the materials were sonicated in 15 ml and the total volume of the sonicates was collected. At OFI the NBR O rings were sonicated in 20 ml of water in a 100 ml beaker. The combined sonicates were assessed for ATP concentration. The sonication equipment used for sonication is presented in Table 5.4.

<table>
<thead>
<tr>
<th>Participant</th>
<th>Sonication equipment</th>
<th>Operational conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crecep</td>
<td>Bransonic Ultrasonic DTH 3210</td>
<td>Max power 335 W; HF 130 W; Heating Power 205 W; 47 kHz</td>
</tr>
<tr>
<td>DTU</td>
<td>Bransonic Ultrasonic Cleaner 2210</td>
<td>Max Power: 234 W; RF Power 125 W; Heating Power 109 W; 47 kHz</td>
</tr>
<tr>
<td>Kiwa WR</td>
<td>Ultrasonic Cleaner Branson 5501</td>
<td>Output 135 Watts, 42 kHz</td>
</tr>
<tr>
<td>OFI</td>
<td>Sonorex RK 514</td>
<td>Output 135 Watts, 42 kHz</td>
</tr>
<tr>
<td>TZW</td>
<td>Ultrasonic Cleaner Branson M 5510</td>
<td>Operating frequency 38.15 kHz; Power Output: 21 Watts/litre, volume to fill: 2000 ml</td>
</tr>
<tr>
<td>TWUL</td>
<td>Ultrawave Limited, Model U300</td>
<td></td>
</tr>
</tbody>
</table>

ATP analysis. The procedures for ATP analysis have been described in Chapter 3. The ATP concentrations had been calculated from yield factors as obtained in the calibration of the equipment and reagents. Different water types were used in stage 6 for preparation of the calibration solutions.

Microbial growth support potential of CPDW

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- Crecep: ATP analysis was calibrated with tap water;
- DTU: buffer was used for ATP calibration. Data were recalculated using a normalisation factor to correct for the difference between ATP yield in buffer and in tap water respectively;
- Kiwa WR: calibration in tap (= test) water;
- OFI: calibration in demineralised tap water;
- TZW: calibration in buffer;
- TWUL: calibrated ATP in laboratory treated water (= test water).

5.2.4 Calculation of BP and BPP values
The BP values of the materials were obtained from the biomass concentration on the material sample and the concentration of suspended biomass divided by the S/V ratio of the involved material. In this way all biomass present is related to the surface area of the exposed material. The BPP values are the averages of the BP values obtained after 56, 84 and 112 days of incubation.

5.2.5 Statistics
Analysis of variance (F-test) was applied on the results obtained for each material in the different water replacement schemes. The water replacement schemes were considered the treatments and the days were considered the blocks. The null hypothesis tested was that there is no effect of the replacement scheme on the BPP value.

5.3 Results
The selected materials behaved differently in the tests. Silicone Rubber initially had high BP values (about 2500 pg ATP/cm²), which declined during prolonged incubation, but the BP values of Nitrile Rubber increased over time (Fig. 5.1). The obtained BPP values of all tests are listed in Table 5.5 together with the relative standard deviations (RSD) of the average BP value, which is calculated from the BP values on days 56, 84 and 112 days. The average RSD values in the laboratories ranged from 24% (TZW) to 49% (OFI). A declining or increasing BP value within the test period will increase the RSD value. The test was not completed at TWUL because very little growth was observed with Nitrile Rubber and with PVC-P in scheme 2. The three months readings were completed only for Scheme 1.

Fig. 5.1 Effect of water replacement on the biomass production of Silicone Rubber (A) and Nitrile Rubber (B) at 25°C (duplicate samples; results Kiwa WR).
In Fig. 5.2 the BPP values are arranged per material type to enable comparisons on a linear scale between the applied water replacement schemes. The results demonstrate that the BPP values of the materials ranged from less than 50 pg ATP/cm² for glass (not corrected for the effect of the test water) to values above 6 x 10⁴ pg ATP/cm² for PVC-P. Hence a range of more than 3 orders of magnitude is covered with these tests. BPP values for identical materials differed between laboratories and between water replacement schemes, respectively, but values for one type of material were of the same order of magnitude in most cases. The differences between materials tested in different laboratories may be due to a number of factors, i.e. ATP calibration, nature of bacteria, sonication for removal of bacteria and water type. These aspects will not be discussed in this chapter, which aims at comparing the effects of water replacement schemes.

Table 5.5  BPP values with relative standard deviations (rsd) as observed in the various water replacement schemes.

<table>
<thead>
<tr>
<th>Particulate</th>
<th>Scheme/ (Replacement)*</th>
<th>Water**</th>
<th>Glass</th>
<th>PVC-C</th>
<th>Silicone Rubber</th>
<th>Nitrile Rubber</th>
<th>PVC-P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Creep</td>
<td>1 (0)</td>
<td>20.4</td>
<td>40 (60)</td>
<td>161 (43)</td>
<td>592 (37)</td>
<td>6238 (37)</td>
<td>54475 (44)</td>
</tr>
<tr>
<td></td>
<td>3 (1)</td>
<td>35 (46)</td>
<td>27.3 (44)</td>
<td>115 (49)</td>
<td>374 (16)</td>
<td>4859 (19)</td>
<td>63995 (16)</td>
</tr>
<tr>
<td></td>
<td>4 (24)</td>
<td>34 (17)</td>
<td>44.7 (50)</td>
<td>107 (36)</td>
<td>524 (36)</td>
<td>5346 (30)</td>
<td>57490 (39)</td>
</tr>
<tr>
<td>DTU</td>
<td>1 (0)</td>
<td>14 (62)</td>
<td>19.7 (18)</td>
<td>64.2 (52)</td>
<td>346 (3)</td>
<td>2868 (10)</td>
<td>5374 (36)</td>
</tr>
<tr>
<td></td>
<td>3 (1)</td>
<td>23.8 (4)</td>
<td>36.8 (12)</td>
<td>74.7 (13)</td>
<td>225 (2)</td>
<td>1823 (43)</td>
<td>8138 (78)</td>
</tr>
<tr>
<td></td>
<td>4 (24)</td>
<td>27.8 (15)</td>
<td>36.7 (15)</td>
<td>87.3 (20)</td>
<td>219 (25)</td>
<td>1495 (30)</td>
<td>21993 (75)</td>
</tr>
<tr>
<td>Kiwa WR</td>
<td>1 (0)</td>
<td>104 (57)</td>
<td>165 (89)</td>
<td>132 (19)</td>
<td>659 (16)</td>
<td>7782 (42)</td>
<td>71225 (36)</td>
</tr>
<tr>
<td></td>
<td>2 (2)</td>
<td>35.3 (36)</td>
<td>105 (64)</td>
<td>99.5 (8)</td>
<td>284 (10)</td>
<td>8422 (28)</td>
<td>46088 (74)</td>
</tr>
<tr>
<td></td>
<td>4 (24)</td>
<td>36.8 (24)</td>
<td>72.2 (42)</td>
<td>85.2 (47)</td>
<td>213 (5)</td>
<td>4514 (28)</td>
<td>29586 (46)</td>
</tr>
<tr>
<td>OFI</td>
<td>1 (0)</td>
<td>103 (72)</td>
<td>191 (81)</td>
<td>244 (65)</td>
<td>1977 (31)</td>
<td>1994 (8)</td>
<td>16331 (57)</td>
</tr>
<tr>
<td></td>
<td>3 (1)</td>
<td>44.8 (45)</td>
<td>103 (42)</td>
<td>148 (51)</td>
<td>1159 (32)</td>
<td>2252 (93)</td>
<td>17213 (6)</td>
</tr>
<tr>
<td></td>
<td>4 (24)</td>
<td>66.2 (88)</td>
<td>66 (34)</td>
<td>147 (41)</td>
<td>730 (62)</td>
<td>1985 (20)</td>
<td>27332 (60)</td>
</tr>
<tr>
<td>TZW</td>
<td>1 (0)</td>
<td>12.3 (18)</td>
<td>17.9 (40)</td>
<td>93.8 (28)</td>
<td>361 (17)</td>
<td>5371 (6)</td>
<td>17227 (21)</td>
</tr>
<tr>
<td></td>
<td>3 (1)</td>
<td>11.6 (9)</td>
<td>19.0 (37)</td>
<td>41.8 (8)</td>
<td>336 (41)</td>
<td>9860 (31)</td>
<td>72886 (21)</td>
</tr>
<tr>
<td></td>
<td>4 (24)</td>
<td>12.4 (28)</td>
<td>17 (28)</td>
<td>47.7 (25)</td>
<td>252 (33)</td>
<td>6314 (14)</td>
<td>50556 (28)</td>
</tr>
<tr>
<td>TWUL##</td>
<td>1 (0)</td>
<td>14</td>
<td>51</td>
<td>1979</td>
<td>70</td>
<td>34665</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 (2)</td>
<td>14.8</td>
<td>88.1</td>
<td>832</td>
<td>141</td>
<td>121</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4 (24)</td>
<td>7.4</td>
<td>11</td>
<td>810</td>
<td>23.7</td>
<td>10510</td>
<td></td>
</tr>
</tbody>
</table>

* between brackets: replacements/week; **, BPP values in water is only planktonic growth; BPP value calculated with the S/V ratio as applied with glass (0.16 cm²); #, values between brackets are relative standard deviations (= 100 x std/avg); ##, results of day 56; experiment terminated

For each laboratory the results were analysed with analysis of variance (using the F-test). The data available for this analysis was however not the same for all these laboratories. The power of the analysis was less for the laboratories that provided only average BP values than for the laboratories that provided the individual BP values. The results of the tests are presented in Table 5.6.
Fig. 5.2 Biomass production potential (BPP) of materials in the replacement schemes in the BPP test at 25°C. TWUL data are values observed on day 56 only.

Table 5.6 Effect of water replacement scheme on the BPP values of the materials as determined with analysis of variance (F-test). Numbers indicate replacement schemes (see Table 5.1).

<table>
<thead>
<tr>
<th>Participant</th>
<th>Data</th>
<th>Water</th>
<th>Glass</th>
<th>PVC-C</th>
<th>Silicone Rubber</th>
<th>Nitrile Rubber</th>
<th>PVC-P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crecep</td>
<td>Avg.</td>
<td>n.a.**</td>
<td>n.s.***</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>DTU</td>
<td>Ind.</td>
<td>1&lt;3, 1&lt;4</td>
<td>1&lt;3, 1&lt;4</td>
<td>n.s.</td>
<td>1&gt;3, 1&gt;4</td>
<td>1&gt;3, 1&gt;4</td>
<td>n.s.</td>
</tr>
<tr>
<td>Kiwa</td>
<td>Ind.</td>
<td>1&gt;2; 1&gt;4</td>
<td>1&gt;4</td>
<td>1&gt;2; 1&gt;4</td>
<td>1&gt;2; 1&gt;4</td>
<td>1&lt;4; 2&gt;4</td>
<td>1&gt;2; 1&gt;4</td>
</tr>
<tr>
<td>WR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1&gt;4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OFI</td>
<td>Ind.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>1&gt;3; 1&gt;4</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>TZW</td>
<td>Avg.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>1&gt;3; 1&gt;4</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>TWUL</td>
<td>Avg.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

* Avg, average BP values of used; Ind, individual BP values (at days 56, 84 and 112) used; **, n.a., not available; *** , n.s., not significant (p > 0.05); > , scheme x gave significant higher value than scheme y; < opposite of “>”.

Table 5.6 shows that water replacement schemes had significant effects on the BPP values of a number of materials in four of the six laboratories, viz. DTU.
Kiwa WR, OPI and TZW, respectively. Scheme 1 differed from scheme 3 in seven cases and from scheme 4 in 12 cases, respectively. This differences were not consistently higher or lower. Scheme 1 gave significantly higher values than scheme 2 with a few materials (only compared at Kiwa WR and TWUL). In one case (TZW), PVC-P a significant difference was observed between schemes 3 and 4. Also in one case (Kiwa WR, Nitrile Rubber) a significant difference was observed between schemes 2 and 4. In all other cases no significant differences were recorded between schemes 2, 3 and 4.

5.4 Discussion
The results of the experiments show that the tested materials covered a wide range of BPP values. Furthermore, biomass production as a function of time clearly differed in the presence of the various materials. BP values increased even after more than 100 days in the presence of Nitrile Rubber but a strong decrease was observed within a few weeks with Silicone Rubber. With the latter material stable values were reached after about 50 days of incubation at 25°C. A continuing increase or decrease of the growth (BP) in the period after 50 days may result in a relative standard deviation of the BPP value. A changing BP value in the test period depends on the material type (e.g. Nitrile Rubber) and may be affected by the test system (water replacement), the type of test water, e.g. composition of the microbial community and/or inorganic nutrients.

Water replacement had an effect on the BPP values (i.e. the sum of the biomass concentration as present on the material and the biomass concentration in the water) in a number of cases, but not all participants observed significant differences. This may be due to the relatively large RSD values of the mean BP value (BPP) in the tests. The impact of water replacement on the BPP values depends on a number of effects:

- Depletion of nutrients (and/or oxygen) when water is not replaced. In such a situation the BPP value as obtained with water replacement would be higher than without replacement. This may explain that the BPP values for PVC-P and NBR at TZW in scheme 3 (water replaced 1 x/week) were higher than those with scheme 1 (no replacement). Also the BPP level observed with Nitrile Rubber in scheme 2 (2 replacements/week) at Kiwa WR was higher than the value obtained with scheme 4. However, these observations were not confirmed by the other participants and oxygen concentrations were not monitored;

- Accumulation of growth-inhibiting compounds. The increase of the BPP values with water replacement as observed at Kiwa for Nitrile Rubber may be due to growth inhibition with this material, although differences between batches of material may have been involved. Nitrile Rubber also showed highly variable results in the investigations in stage 4 (effect of inoculum; no water replacement). The results of the follow up investigations on stage 4 at DTU further suggest that the type of inoculum could have an effect (see Chapter 4.3);

- Removal of released biodegradable compounds when the water is replaced. Such a removal would result in a lower BPP value, because the released compounds are not available for (further) utilisation. This process
may play a role in situations where a relatively large proportion of the biodegradable material is rapidly released from the material. This aspect may be relevant for Silicone Rubber (cf. Fig. 5.1) and also for PVC-P, provided that there is no nutrient/oxygen limitation in the test. Also here, the observations are not conclusive;

- No replacement of the water may give an increased biomass production when the air in the incubator or the test laboratory contains biodegradable compounds, e.g. solvents or volatile organic compounds arising from other sources. The use of incubators reserved only for testing of materials is advisable;
- Water replacement makes the test more laborious.

The effects observed with the few types of materials and water as included in this investigation may be stronger with other combinations of water types and materials. Hence, water replacement in the BPP test is needed to prevent these problems. A replacement frequency of once a week is a realistic simulation of conditions in water systems in practice and is also practical in the laboratory.

5.5 Conclusions

- Replacement of the water as compared to no water replacement had a limited but significant effect on the BPP values of the tested materials. With some materials lower BPP values were observed with replacement and with some materials a higher BPP value was observed with replacement. These effects depend on the BPP level.
- In a few cases with materials with a high BPP value (> 5000 pg ATP/cm²), significantly different BPP values were observed between water replacement frequencies of twice a week, once a week and once in two weeks, respectively. A higher replacement scheme gave a higher BPP value. This effect may have been caused by oxygen limitation, but oxygen concentrations have not been monitored;
- Water replacement should be applied in the BPP test for the following reasons: (i) prevention of growth limitation by inorganic nutrients and/or oxygen; (ii) prevention of the accumulation of growth-limiting compounds; (iii) limiting the effect of compounds which are released from the material to a period which reflects situations in practice.
- A water replacement frequency of once a week has been selected because a one week contact time reflects situations in practice and is easily manageable in the laboratory. However, it remains uncertain whether or not oxygen limitation may occur with certain materials at this replacement frequency;
- Water replacement requires the use of water with a high degree of biostability to maintain a low detection level for the effect of the material on biomass production.
6 Reproducibility of the BPP test with the revised protocol

6.1 Description and objectives of stage 7

A revised protocol for the BPP test is evaluated in stage 7 of the project. The participants of WP1 decided that a revised protocol was needed for a number of reasons:

- water replacement of the water during the test (based on the results of stage 6; see Chapter 5);
- size and number of sample pieces. Material samples with an external nominal surface area of about 8 cm² are relatively small and with the disadvantage of a relatively large proportion of cut surfaces and are not applicable to all types of products, e.g. cementitious materials, coatings. Therefore the ability to test larger samples was preferable. A consequence of larger materials samples was the need to use containers with a wider neck than the Schott bottles (or Erlenmeyer flasks).
- incubation temperature. The incubation temperature should be 30°C because this temperature would reflect worst case conditions in water systems in Southern Europe and in buildings in Northern Europe.

These aspects were included in the tests as conducted in stage 7 of the project in which the BPP test with a revised protocol was tested in all participating laboratories with a number of selected materials. The main objective of this stage was to assess the reproducibility of the method. To improve the similarity of the test conditions, all participants used river Rhine water as the inoculum.

The BPP test with the revised protocol and selected materials was conducted at 25°C (five laboratories), at 30°C (three laboratories), at 22°C (one laboratory) and at 10°C (one laboratory), respectively. Furthermore, the tests were compared with the MDOD test, the W270 test, the original BPP test and Önorm B5018 (see table 1.1). Stage 7 also included experiments with the materials in different conditions of (semi)continuous flow to obtain information about the relationship between BPP values as observed in the test and biofilm concentrations as may occur under conditions prevailing in practice. This information may serve as background information for defining pass-fail criteria for the BPP values of materials in contact with drinking water.

The objectives for stage 7 are summarised below:

- testing of the reproducibility of the revised BPP test;
- comparison of effect of the incubation temperatures 25°C, 30°C and 10°C;
- comparison of the results of the revised BPP test with the MDOD test, the W270 test and the original BPP;
testing of materials under conditions simulating practical conditions to obtain background information about the BPP values. The results of the BPP test with the revised protocol as obtained by the participants are described below.

6.2 Materials and methods

6.2.1 Materials

It was concluded to use larger samples than those applied in the original BPP test (about 8 cm²) and in the previous project stages. Large samples would reduce the impact of cutting edges of material samples and also facilitate the application of the test to other products. Furthermore, the surface (S) to volume (V) ratio as applied in the MDOD and BPP test should be maintained. This S/V ratio is about 0.15 cm⁻¹ (MDOD) to 0.166 cm⁻¹ (BPP test). Also it was decided to maintain the test period as used in the BPP test (16 weeks) in stage 7 and to determine BP values after 8, 12 and 16 weeks. Consequently, 3 material pieces (each with a surface area of 50 to 55 cm²) were placed in a total of 900 ml of test water in each test container.

A number of different materials were selected for testing. The following descriptions apply:
- glass cylinders (control): cut to length and then the cut edges ground; provided by Kiwa;
- Nitrile Rubber “O” seals: tested as received;
- Silicone Rubber hose: cut to length with cleaned sharp scissors (or with a knife in some laboratories);
- PVC-C: provided cut to length by Kiwa, cut edges smooth;
- PVC-P: cut to length with cleaned sharp scissors (or cut with a knife in some laboratories), cut edges smooth;
- HDPE: cut to length with a cleaned plastics pipe cutter, cut edges smooth
- Stainless Steel (SS), provided sawn to length and pre-cleaned by Kiwa.

Test pieces were cut as accurate as possible. However, exact determination of the size of the pieces is needed after taking it from the flasks (after ATP analysis). SS was cleaned to remove attached grease with the following procedure: pieces were dipped in methanol (99% v/v) for 30 seconds, dipping was repeated two times with fresh methanol. Subsequently, the pieces were rinsed with hot tap water, and with cold tap water. All materials had been cleaned with flowing tap water as described in Chapter 5. At TZW all materials had been in contact with flowing tap water for 60 min, 24 h storage in tap water, and again 60 min of flushing with tap water.

6.2.2 Test jars

A commercially available container was selected (Fig. 6.1), with a volume of one litre. This container has an external diameter of 10 cm and a length of 18 cm. The internal diameter of the opening is 5.7 cm. Lids with a PTFE inlay (prepared and provided by Kiwa WR) were used to improve the sealing of the jar. At TWUL the (UK) “Le Parfait” test jars were used for comparison.
6.2.3 Water type and inoculum
All participants used tap water supplemented with inorganic nutrients (N and P) and a once a week water change for the duration of this testing. All participants used river Rhine water provided by Kiwa WR as inoculum (1% v/v), after membrane filtration (pores of 1.2 μm).

Fig. 6.1 The test jar as used for conducting the revised protocol. Volume: 1 liter; height: 18 cm; external diameter bottom: 10 cm; diameter neck opening: 5.7 cm; Lid: bakelite, with PTFE inlay.

6.2.4 Effect of temperature
The effect of the incubation temperature was tested by incubating a number of the selected materials at 30°C (see Table 6.1). At DTU a temperature of 10°C was used and compared with testing at 25°C.

6.2.5 ATP analysis
ATP analysis was done as described in Chapter 3. Tap water was used to prepare dilutions of the ATP standard for calibration purposes. The larger samples required sonication in larger volumes. Samples with an external surface of about 50 cm² were sonicated in a volume of 40 ml (50 ml at DTU) in glass beakers. In the BPP method, samples of about 8 cm² external surface were sonicated in 10 ml of water.

Suspended biomass - at each test period a volume of test water (300 ml at Kiwa WR and CRECEP, 1 ml at DTU) was removed for ATP analysis (assessment of planktonic growth) immediately before the test water in the test containers was changed.

Attached biomass – at the appropriate time period (8, 12 and 16 weeks) one test piece was removed from each test container using sterilised tongs, and placed into either a glass test tube (Silicone Rubber hose, PVC-C pipe, PVC-P hose and Stainless Steel) or tall form 100 ml glass beakers (glass, Nitrile Rubber “O”
seals, polyethylene pipe) with 40 ml of tap water. At DTU the same type was used for all samples, with 50 ml of tap water). A volume of 300 ml of the test water in the container was removed (and used for suspended biomass analysis) and discarded.

The beaker or test tube was placed into an ultrasonic bath and exposed to ultrasonic energy for 2 minutes. At the end of this period the sonicate was transferred to another test tube, standing in ice, and the sonication repeated with a further 40 ml of tap water. The sequence was repeated to give the following total number of sonication periods:
- glass – three;
- all other materials – six.

6.2.6 Scheme for stage 7

An overview of the materials used and the activities conducted by each participant in stage 7 is given in Table 6.1.

Table 6.1 Activity scheme of stage 7

<table>
<thead>
<tr>
<th>Participant</th>
<th>Test with revised protocol at 25°C.</th>
<th>Materials tested at 30°C (10°C at DTU)</th>
<th>Comparison of methods</th>
<th>Simulation of practical conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRECEP</td>
<td>PVC-P, NBR, PVC-C, HDPE, SILR, SS</td>
<td>PVC-P, PVC-C, HDPE</td>
<td>** 3 EPDM</td>
<td>types tested at 22°C (see TZW)</td>
</tr>
<tr>
<td>DTU</td>
<td>PVC-P, NBR, PVC-C, HDPE, SILR, SS</td>
<td>PVC-P, PVC-C, HDPE</td>
<td>Test rig with PVC-P, PVC-C and SS</td>
<td></td>
</tr>
<tr>
<td>KIWA WR</td>
<td>PVC-P, NBR, PVC-C, HDPE, SILR, SS</td>
<td>PVC-P, PVC-C, HDPE</td>
<td>BPP test with all selected materials</td>
<td>Biofilm monitor with glass, PVC-C, PVC-P and Silicone Rubber</td>
</tr>
<tr>
<td>OFI</td>
<td>PVC-P, NBR, PVC-C, HDPE, SILR, SS</td>
<td>PVC-P, NBR, PVC-C, HDPE, SILR, SS</td>
<td>Austrian method with PVC-C, PVC-P, HDPE or Silicone Rubber</td>
<td></td>
</tr>
<tr>
<td>TZW</td>
<td>PVC-P, NBR, PVC-C, HDPE, SILR, SS</td>
<td>** 3 EPDM types were tested at 25°C (see Crecep)</td>
<td>Comparison with W270 using 3 types of EPDM **</td>
<td></td>
</tr>
<tr>
<td>TWUL</td>
<td>PVC-P, NBR, PVC-C, HDPE, SILR, SS</td>
<td>PVC-P, PVC-C, HDPE</td>
<td>MDOD, with all materials selected</td>
<td></td>
</tr>
</tbody>
</table>

* revised protocol is BPP test with weekly water replacement and incubation at 25°C; at Crecep the incubation temperature was 22 °C; ** 3 types of EPDM rubber which were selected for testing in the W270 method (at TZW) were tested in the BPP test with the revised protocol;
6.3 Results

6.3.1 Reproducibility of the BPP test with the revised protocol
The main objective of stage 7 was to test the reproducibility of the revised protocol using a number of selected materials. The reported BPP values are presented in Fig. 6.2 and in Table 6.2 respectively. The BPP values of the materials differed clearly from each other, confirming the observations of stage 6. The relative standard deviations (RSD) of the mean of the BP values obtained after 56, 84 and 112 days of incubation (BPP) obtained with the tested materials ranged from 3.0 % to 77 %. The median RSD value for all BPP tests with the revised protocol conducted at 25°C by the participants was 33%. These RSD values differed between the laboratories, with average RSD values of 12% (TWUL), 29% (DTU), 31% (TZW and Kiwa WR), 55% (Crecep) and 54% (OFI), respectively. The RSD value is also affected by the increase or decrease of growth (BP value) in the test period (56 – 112 days). Materials that gave the highest RSD values were: PVC-P, Nitrile Rubber and HDPE, respectively.

Table 6.2 Biomass Production Potential (BPP) values (= average of BP values on day 56, 84 and 112) of the materials tested at 25°C with the revised protocol. The standard deviations (std) of the mean value are shown between brackets.

<table>
<thead>
<tr>
<th>Material</th>
<th>Crecep*</th>
<th>DTU</th>
<th>Kiwa WR</th>
<th>OFI</th>
<th>TZW</th>
<th>TWUL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>19.1</td>
<td>15.7</td>
<td>53.6</td>
<td>224</td>
<td>21.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(4.5)</td>
<td>(4.5)</td>
<td>(28.6)</td>
<td>(115)</td>
<td>(9.0)</td>
<td></td>
</tr>
<tr>
<td>Glass</td>
<td>102</td>
<td>25.3</td>
<td>116</td>
<td>220</td>
<td>26.1</td>
<td>336</td>
</tr>
<tr>
<td></td>
<td>(16.2)</td>
<td>(7.8)</td>
<td>(41)</td>
<td>(67.4)</td>
<td>(5.0)</td>
<td>(37.9)</td>
</tr>
<tr>
<td>Stainless</td>
<td>333</td>
<td>39</td>
<td>142</td>
<td>321</td>
<td>54</td>
<td>316</td>
</tr>
<tr>
<td>Stocl</td>
<td>(245)</td>
<td>(7)</td>
<td>(36)</td>
<td>(120)</td>
<td>(11.6)</td>
<td>(18)</td>
</tr>
<tr>
<td>PVC-C</td>
<td>188</td>
<td>46.0</td>
<td>149</td>
<td>278</td>
<td>50</td>
<td>393</td>
</tr>
<tr>
<td></td>
<td>(40)</td>
<td>(8)</td>
<td>(22)</td>
<td>(123)</td>
<td>(6.5)</td>
<td>(22)</td>
</tr>
<tr>
<td>Silicone</td>
<td>691</td>
<td>262</td>
<td>556</td>
<td>744</td>
<td>327</td>
<td>948</td>
</tr>
<tr>
<td>Rubber</td>
<td>(190)</td>
<td>(67)</td>
<td>(38)</td>
<td>(322)</td>
<td>(89)</td>
<td>(60)</td>
</tr>
<tr>
<td>HDPE</td>
<td>332</td>
<td>137</td>
<td>608</td>
<td>676</td>
<td>230</td>
<td>691</td>
</tr>
<tr>
<td></td>
<td>(245)</td>
<td>(1.0)</td>
<td>(135)</td>
<td>(195)</td>
<td>(119)</td>
<td>(19)</td>
</tr>
<tr>
<td>Nitrile</td>
<td>4,151</td>
<td>3018</td>
<td>22,860</td>
<td>4,065</td>
<td>6,206</td>
<td>3,526</td>
</tr>
<tr>
<td>Rubber</td>
<td>(1,119)</td>
<td>(203)</td>
<td>(9,344)</td>
<td>(1,479)</td>
<td>(1463)</td>
<td>(435)</td>
</tr>
<tr>
<td>PVC-P</td>
<td>40,582</td>
<td>8,344</td>
<td>34,578</td>
<td>11,659</td>
<td>10,728</td>
<td>24,681</td>
</tr>
<tr>
<td></td>
<td>(5,560)</td>
<td>(260)</td>
<td>(16,094)</td>
<td>(2,550)</td>
<td>(5,527)</td>
<td>(1,230)</td>
</tr>
</tbody>
</table>

*, test at 22°C; ** n.a., not analysed.

The BPP values presented in Table 6.2 include the effect of the water on the biofilm formation. Fig. 6.2 shows that the quality of the test water had a clear impact at low BPP values as observed with glass, steel and PVC-C. The BPP values of glass exceeded 100 pg ATP/cm² at Kiwa WR, OFI and TWUL, respectively. Glass had been heated at 550 °C and this material is expected to have a negligible potential for biomass production. Consequently, the biofilm developing on the glass surface is caused by the utilisation of biodegradable compounds present in the water, which was replaced weekly and by other external effects (e.g. air quality). Correction of the BPP values of the tested materials for these effects was done by subtracting the obtained BPP values.

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with those observed with glass. These net BPP values (Table 6.3) showed better similarities between the participants. From these BPP values, the reproducibility of the test, defined as the coefficient of variation of reproducibility (VC\textsubscript{R}), was calculated. These VC\textsubscript{R} values ranged from 34 - 107\%, with the highest values for Stainless Steel and for Nitrile Rubber.

![Graph A](image)

Fig. 6.2 A BPP values of water, glass, Stainless Steel and PVC-C as observed in the BPP test at 25\degree C with the revised protocol. The BPP values of water were calculated on the basis of a hypothetical S/V of 0.16. Numbers indicate the participants: 1, Crecep; 2, DTU; 3, Kiwa WR; 4, OFI; 5, TZW; 6, TWUL. Bars indicate average values with standard deviations.

![Graph B](image)

![Graph C](image)

Fig. 6.2 B/C BPP values of Silicone Rubber (SiIR), high density polyethylene (HDPE), Nitrile Rubber (NiR) and plasticized PVC (PVC-P) in the BPP test at 25\degree C with the revised protocol. Numbers indicate the participants: 1, Crecep; 2, DTU; 3, Kiwa WR; 4, OFI; 5, TZW; 6, TWUL. Bars indicate average values with standard deviations.
Table 6.3 BPP values of selected materials tested at 25°C (analysis at Crecep at 22°C) after subtraction of the BPP values obtained with glass.

<table>
<thead>
<tr>
<th>Material</th>
<th>Crecep</th>
<th>DTU</th>
<th>Kiwa WR OFI</th>
<th>TZW</th>
<th>TWUL</th>
<th>Average</th>
<th>( \text{VC}_{\text{A}} ) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glass</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Stainless Steel</td>
<td>7.2</td>
<td>13.3</td>
<td>26.5</td>
<td>101</td>
<td>28.1</td>
<td>-18.7</td>
<td>64</td>
</tr>
<tr>
<td>PVC-C</td>
<td>86.1</td>
<td>20.7</td>
<td>33</td>
<td>57.8</td>
<td>23.7</td>
<td>57.7</td>
<td>47</td>
</tr>
<tr>
<td>Silicone</td>
<td>590</td>
<td>237</td>
<td>440</td>
<td>524</td>
<td>301</td>
<td>613</td>
<td>450</td>
</tr>
<tr>
<td>Rubber</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HDPE</td>
<td>278</td>
<td>112</td>
<td>492</td>
<td>457</td>
<td>204</td>
<td>356</td>
<td>316</td>
</tr>
<tr>
<td>Nitrile Rubber</td>
<td>4050</td>
<td>2993</td>
<td>22,744</td>
<td>3845</td>
<td>6180</td>
<td>3191</td>
<td>7167</td>
</tr>
<tr>
<td>PVC-P</td>
<td>40,480</td>
<td>8319</td>
<td>34,463</td>
<td>11,439</td>
<td>10,703</td>
<td>24,346</td>
<td>21,069</td>
</tr>
</tbody>
</table>

* \( \text{VC}_{\text{A}} \) is the coefficient of variation of reproducibility, with a high reproducibility at low \( \text{VC}_{\text{A}} \) values.

At TWUL the BPP test had also been conducted using an inoculum from river Kennet. With the seven test materials no statistical differences were observed between BPP values with this inoculum and the BPP values with the river Rhine inoculum. Also with the tests conducted in the Le Parfait jars no consistent and significant differences were seen between the results obtained with the results in the other test containers.

6.3.2 Effect of incubation temperature
A selection of materials was also been tested at 30°C in three different laboratories (Fig. 6.3). Table 6.4 shows that in the majority of cases BPP values were lower at 30°C than at 25°C. The BPP values observed at 30°C at Kiwa WR were about 60-65% of the BPP values obtained at 25°C for most materials except for HDPE. The BPP value of this material at 30°C was similar to the BPP value observed at 25°C. Furthermore, BPP values at 10°C at DTU were higher than those observed at 25°C. These observations suggest an increase in biomass production at decreasing temperature and vice versa.

![Graph](image)

Fig. 6.3 Average BPP values (with standard deviations) of selected materials at 30°C. 1, Crecep, 3, Kiwa WR, 6, TWUL.
Table 6.4: Comparison of BPP values of materials tested at 25°C (22°C), 30°C and 10°C, respectively.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>19.1</td>
<td>25</td>
<td>26.3</td>
<td>15.7</td>
<td>53.6</td>
<td>32.8</td>
<td>na*</td>
<td>na</td>
</tr>
<tr>
<td>Glass</td>
<td>102</td>
<td>66</td>
<td>51.7</td>
<td>25.3</td>
<td>116</td>
<td>61.5</td>
<td>335</td>
<td>270</td>
</tr>
<tr>
<td>PVC-C</td>
<td>188</td>
<td>149</td>
<td>109</td>
<td>46</td>
<td>149</td>
<td>103</td>
<td>392</td>
<td>351</td>
</tr>
<tr>
<td>HDPE</td>
<td>201</td>
<td>188</td>
<td>69</td>
<td>137</td>
<td>608</td>
<td>649</td>
<td>691</td>
<td>673</td>
</tr>
<tr>
<td>PVC-P</td>
<td>49,582</td>
<td>44,213</td>
<td>16,911</td>
<td>5,011</td>
<td>34,578</td>
<td>22,335</td>
<td>24,681</td>
<td>31,785</td>
</tr>
</tbody>
</table>

*, n.a., not reported

The results obtained at 30 °C also gave information about the reproducibility of the revised BPP protocol. For this purpose, the BPP values reported for PVC-C, HDPE and PVC-P respectively were corrected for the BPP values reported for with glass, which represents the effect of water on the BPP value. The VCR value of the corrected BPP values, which covered a wide range, ranged from 20 to 34% (Table 6.5).

Table 6.5: BPP values of selected materials tested at 30°C, corrected for BPP values of glass.

<table>
<thead>
<tr>
<th>Material</th>
<th>Crecep</th>
<th>Kiwa WR</th>
<th>TWUL</th>
<th>Average</th>
<th>SD</th>
<th>VCR (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0</td>
<td>34</td>
</tr>
<tr>
<td>Glass</td>
<td>83</td>
<td>42</td>
<td>81</td>
<td>69</td>
<td>23</td>
<td>34</td>
</tr>
<tr>
<td>PVC-C</td>
<td>442</td>
<td>587</td>
<td>403</td>
<td>478</td>
<td>97</td>
<td>20</td>
</tr>
<tr>
<td>HDPE</td>
<td>444,147</td>
<td>22,273</td>
<td>31,514</td>
<td>32,644</td>
<td>10,980</td>
<td>34</td>
</tr>
<tr>
<td>PVC-P</td>
<td>44,147</td>
<td>22,273</td>
<td>31,514</td>
<td>32,644</td>
<td>10,980</td>
<td>34</td>
</tr>
</tbody>
</table>

6.4 Discussion

The BPP values with the revised protocol (25 °C) reported by the participants ranged from less than 50 pg ATP/cm² (for water, glass, stainless steel and PVC-C) to about 40,000 pg ATP/cm² for PVC-P. BPP values decreased with increasing incubation temperature with most of the materials tested. This decrease does not indicate less growth, but most likely is the result of the effect of an increasing endogenous respiration at increasing temperature. The effect of temperature on endogenous respiration can be estimated with the quotient of the reaction rates at a temperature difference of 10°C (Q₁₀). A Q₁₀ value of 2.5 is generally observed with biological processes at normal temperatures. With this value it can be calculated that the BPP value at 30°C would be 1.5 times lower as compared to the value at 25°C. This estimation fits well with most of the observations. An increasing temperature may also accelerate biodegradation and the release of biodegradable compounds from some materials. Incubation at a temperature of 30°C therefore is preferred over incubation at 25°C. An estimation for a BPP value at a lower temperature can be derived using the Q₁₀ value, but more data are needed to determine this value.
The RSD values of the mean BP value in the individual tests are affected by the type of material, the test conditions in the laboratories and probably also by variations in the ATP analysis in the test period. The highest RSD values were observed with a material with a low BPP value (Stainless Steel) and materials with the highest BPP values (Nitrile Rubber, PVC-P), respectively. A decreasing or increasing growth (BP) in the incubation period gives a high RSD of the BPP value. The differences in RSD values between laboratories indicate that improvement of procedures, including ATP analysis, is possible.

The BPP values of glass exceeded 100 pg ATP/cm² in some laboratories. These values were higher than the BPP values observed for Stainless Steel and PVC-C in the laboratories with low BPP values for glass. The high BPP value of glass is caused by the water quality and the effect of other external conditions (e.g. presence of volatile biodegradable compounds in the air). Consequently, determination of BPP values at the low range requires the use of water with a high degree of biostability. Incubation of the jars with the materials in incubators that are also used for cultivation of microorganisms on solid or liquid media (as was done at Kiwa WR) or opening and handling the test containers/jars in a laboratory where volatile substances (e.g. solvents) may be present, may be other sources of errors. The use of the glass-stoppered Erlenmeyer flasks (cleaned by heating at 550 °C) may be more efficient in preventing both the introduction of contaminants from the air or from the container material. Subtraction of the BPP values obtained for glass from those of the materials enables to deal with the effect of the test water, container and the air. Still, an accurate assessment of low BPP values requires that growth in the glass control should be limited, e.g. less than 100 pg ATP/cm².

The BPP values of the materials, after subtraction of the BPP values for the glass controls, clearly show differences between the materials. The coefficient of variation of the reproducibility (VC_R) of the test conducted at 25 °C ranged from 34 % to 107% and from 20 to 34% when conducted at 30°C (in three laboratories). These VC_R values may be regarded as satisfying given the fact that the test with the revised protocol was done for the first time. The VC_R values are affected by the properties of the materials and by factors which also have an impact on the RSD values of the mean BP value but differences in analytical procedures between participants will have a larger effect on VC_R than on RSD values. Effects of materials include:

- A changing BP value during the test. The BP values may not have reached a stable level at the days selected for analysis. This aspect may be affected by differences in the composition of the water or the microbial community;
- Differences between batches of materials. This aspect may explain some of the variations of BPP values obtained with Nitrile Rubber. The value of 107% for Nitrile Rubber is mainly caused by the high BPP value for this material observed at Kiwa WR. This material had a much lower BPP value in the original BPP test without water replacement. Furthermore, observations in stages 4 and 6 suggested that either batches of this material show large differences or growth-limiting compounds are released from the material. It is unknown to what extent water composition, including
the present microbial community may have been responsible for this deviating BPP value;

- For Stainless Steel also a high RSD value was found. In a number of laboratories the BPP values of Stainless Steel are the result of the difference between two values which are relatively large in comparison to the net BPP value. Nevertheless, the standard deviation as absolute value is low.

Factors related to differences in analytical procedures and test conditions:
- ATP analysis. Further standardisation of the analysis is needed (see Chapter 3);
- The sonication procedure was not calibrated (e.g. different characteristics of sonication equipment; position in water bath) and may have given different yields at different laboratories. Observations at Kiwa WR and DTU have shown that application of High Energy Sonication (HES) can release a significant additional amount of biomass from some materials, particularly PVC-P and HDPE. Also this aspect requires further investigation and standardisation;
- Some uncertainty about the effect of water composition remains. In stage 6 limited growth was observed with a few materials at TWUL when using the laboratory treated water despite the addition of N and P. Some other inorganic nutrients may be limiting the growth. The relatively low BPP values of PVC-P, which is the strongest growth promoting compounds applied, as observed in some water types may also be related to nutrient limitation. Consequently, the effect of adding a mixture of inorganic nutrients to the growth yield should be tested.

6.5 Conclusions
- The RSD values of the mean of the BP values determined in the test period ranged from 3 % to 77 % with a median value of 33%. Differences between average RSD values for laboratories indicate that improvement of the BPP test is achievable. With some materials relatively high RSD values will remain because biomass production is not stabilised within the test period of 56 to 112 days;
- A relatively high BPP value as observed with glass (control) by some participants is the result of the growth-promoting properties of the test waier. A high BPP value in the control hampers the accurate assessment of low BPP values of materials. Further improvement in water selection is needed;
- The BPP values as observed by the participants, after correction with the BPP values for glass (effect of the test water), were of the same order of magnitude for identical materials;
- Temperature has a limited but distinct effect on the BPP value, with lower values at increasing temperatures when temperature does not enhance biodegradation and/or the release of growth promoting compounds;
- An incubation temperature of 30 °C is preferred to account for the effects of temperature on biodegradation and release of growth promoting compounds;
- The coefficient of variation of the reproducibility (VCR) of the BPP test (values after subtraction of BPP of glass control), ranged from 34 to 107% at 25°C and from 20 to 34 % at 30 °C. These observations indicate that a
reproducibility of about 30 to 35% is achievable with the described test protocol;
- The highest $VCR$ value were observed with a material with a low BPP value (Stainless Steel; due to the effect of the blank) and Nitrile Rubber (which also gave large differences in growth promoting properties in earlier project stages);
- Further standardisation of the revised BPP test is needed. Such standardisation includes the procedures for ATP analysis (calibration), test water composition, quality of the air in the test laboratory and the incubator, addition of a larger variety of inorganic nutrients to the test water and improvement of the sonication procedure.
7 Comparison of test methods

7.1 Introduction
A number of materials which had been selected and tested to determine the reproducibility of the revised BPP test were also tested with the methods described in Chapter 1 and in two continuous flow situations. The objective of these investigations was to obtain background information needed for the interpretation of the BPP values as obtained with the revised BPP test.

7.2 Methods and selected materials

7.2.1 Test methods
The revised BPP test was compared to the MDOD test, the Slime Production test, the original BPP test and the Austrian test, respectively (see Table 1.1) with a number of selected materials.

The MDOD measurements were undertaken over the seven week test period using the procedures described in BS 6920-2.4:2000. Oxygen concentration measurements were made using a YSI model 58 dissolved oxygen meter with a temperature compensating stirred probe (type 5905), calibrated at the time of use by Winkler titrations of selected reference samples.

The Slime production analysis was conducted as described in W270. The Slime production test required the application of additional materials from which sheets had been prepared. Hence, the materials selected for the testing of the reproducibility could not be used. Three different EPDM rubber compounds had been selected for this purpose. These materials had also been tested in the BPP test (revised protocol) at TZW and Crecep.

The BPP test was conducted following the original description (with Erlenmeyer flasks, test pieces of about 8 cm² and without water replacement).

The Austrian test (Önorm B5018), was applied on a selection of pipe materials. No air supply was provided because the diameter of the provided pipes was less than the diameter prescribed in the test procedure.

7.2.2 Biofilm monitor Kiwa
The materials glass (control), PVC-C, Silicone Rubber and PVC-P have been tested in the biofilm monitor (Van der Kooij et al. 1995). In this system, pieces of material had been placed on top of each other in a glass column (length: 60 cm; internal diameter: 2.5 cm). Tap water was flowing downward through this column (empty column flow velocity 0.2 m/s). Material samples were collected weekly. Attached biomass was removed with low energy sonication and the biomass concentration subsequently was determined with ATP measurements as described in Chapter 3. The test was conducted for a period of about 150 days. The Biofilm Formation Rate was calculated from the
increase of the BP values over time. The composition of the tap water is given in Table 4.3. No compounds were added to the water. The temperature of tap water ranged from 17.5°C to 12.6°C (at the end of the test).

7.2.3 **Test rig DTU**

Drinking water distribution systems were simulated in three parallel model systems set up with either PVC-C, PVC-P or with Stainless Steel (reference material). Each model system consisted of an inlet line, a recirculation ring, an outlet line and two types of sampling points (Fig. 7.1).

![Diagram of test rig DTU](image)

**Fig. 7.1 Test rig at DTU**

The models were built in Stainless Steel with segments of the polymeric materials to be tested. In the recirculation ring the test material was inserted in the pipeline giving a S/V ratio of approximately 1 cm⁻¹. The three systems had a continuous water feed (controlled by a needle valve) at a flow of approximately 1 ml/min. These conditions resulted in residence time of approximately one day in the recirculation ring. The quality of the feed water is described in Table 4.3. No compounds were added to the water. Water temperature was 10°C in the test period.

The inlet and outlet line had a relatively low flow. The flow in the recirculation ring was approximately 0.08 m/s. Thus the inlet line simulated a short travelling distance at low flow, the recirculation ring a long travelling distance at high flow and the outlet line a long travelling distance at low flow.

Polymeric pipe segments (test pieces) were inserted in the inlet line, the recirculation ring and in the outlet line, and these segments can be sampled over time. Every test piece removed was replaced by a new one, which made it possible to run investigations for extensive time periods. The sampling
points consisted of pipe segments either fully submerged in the water or with only inner surfaces in contact with water.

**Type 1 sampling point**
In the inlet line, 3 times 2 pieces of the test material, each with a total surface area of 50 cm², was enclosed in Stainless Steel pipe segments, allowing both inner and outer surface to be in contact with the water. This sampling point allowed for a direct comparison between a constant low rate water exchange and the batch experiments performed in stage 6 and 7. The three steel segments were coupled with a (mappress) fitting, which had an O-seal at both openings, giving a water-tight seal, but still allowed for the removal of pipe segments for sampling purposes.

**Type 2 sampling point**
In the inlet line, the recirculation ring and in the outlet line, three pieces of test material were inserted as a part of the pipeline, allowing only the inner surfaces of 50 cm² to be in contact with the water phase. With these sampling points, the effects of a short travelling distance at low flow rate, a 24 h travelling at a flow rate of 0.08 m/s and a long travelling distance at low flow rate could be compared.

The pieces were connected with unions where the ferrules were exchanged with O-seals allowing for sampling each material piece and its replacement with a new one.

All steel materials were acid rinsed prior to installation and all polymeric materials were rinsed according to the cleaning procedure described in 2.2.4.2. The O-seals in the systems represented a potential source of contamination, but the contact between the water and the O-seals was very limited and it was estimated that the leaching of compounds into the water would have had little overall impact. If, despite all precautions the O-seals affected the water, such an effect was taken into account when each test material was compared to the model system with the reference material – Stainless Steel.

The biomass production on the test pieces was measured by ATP. In the case of the fully submerged test pieces (sampling point type 1) the biomass was removed as in stage 7. The test piece was transferred to a test tube with 50 ml of water and treated 6 x 2 minutes with low energy sonification (LES). The LES treatment was followed by three minutes high energy sonification (HES). For the test pieces only exposed to the water at their inner surfaces (sampling point type 2), the biomass was removed by swabbing. Each test piece was swabbed with five sterile cotton sticks, which were transferred to a test tube with 50 mL of water. The cotton sticks were treated 6 x 2 minutes with LES followed by 1 x 3 min HES. Five sterile cotton sticks were sonificated as controls. When disconnection type 2 sampling points in the recirculation ring, a water sample was collected, representing water from the recirculation ring and outlet.
7.2.4 Selected materials
The materials used in the tests are listed in Table 6.1.

7.3 Results

7.3.1 MDOD test
MDOD tests were done in parallel with the BPP tests at TWUL. The results of these MDOD tests with the materials, which had also been tested in previous stages, are given in Table 7.1. The MDOD values ranged from 0.3 mg O₂/l (glass control) to 5.9 mg O₂/l for PVC-P.

<table>
<thead>
<tr>
<th>Project Stage</th>
<th>Glass Stainless Steel</th>
<th>PVC-C Silicone Rubber</th>
<th>HDPE NBR O seals</th>
<th>PVC-P</th>
<th>Wax</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.1</td>
<td>-</td>
<td>0.7</td>
<td>-</td>
<td>2.3</td>
</tr>
<tr>
<td>6</td>
<td>0.2</td>
<td>-</td>
<td>0.6</td>
<td>1.2</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>0.3</td>
<td>0.7</td>
<td>1.2</td>
<td>0.6</td>
<td>1.4</td>
</tr>
<tr>
<td>7a</td>
<td>0.3</td>
<td>0.9</td>
<td>-</td>
<td>1.1</td>
<td>1.4</td>
</tr>
</tbody>
</table>

The MDOD values are plotted as a function of the BPP results as obtained in stage 7 at TWUL (Fig 7.2). This figure suggests that materials with a BPP value > 1500 pg ATP/cm² exceed the pass-fail criterion of 2.3 mg O₂/l as defined for the MDOD test. However, the relationship between the obtained values for BPP and MDOD is not proportional and further data are needed to establish the true relationship.

![ATP vs. MDOD](image-url)

Fig. 7.2 Comparison of the MDOD results with the BPP values in stage 7 (results at TWUL).
7.3.2 Slime Production method (W270)

The German standard method W270 was applied on a few selected materials at TZW and another laboratory in Germany. For this purpose, three types of EPDM rubber were used. Testing was conducted in flowing tap water at ambient temperature. The composition of the tap water as used at TZW is given in Table 4.3.

The selected EPDM materials were also tested with the revised BPP protocol at TZW and at Crecep. For this purpose, samples pieces of 50 cm² were prepared which were fixed on glass holders. The results of the investigation are presented in Table 7.2.

Table 7.2 Results of W270 test and revised BPP protocol on three different EPDM types (numbers between brackets are RSD values of the test result).

<table>
<thead>
<tr>
<th>Material</th>
<th>Participant</th>
<th>BPP (pg ATP/cm²)</th>
<th>SP (W270) (ml/800 cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPDM-1</td>
<td>TZW</td>
<td>6582 ± 1242 (19%)</td>
<td>3.4 ± 0.2 (7%)</td>
</tr>
<tr>
<td>EPDM 1</td>
<td>Crecep</td>
<td>12,908 ± 3259 (25%)</td>
<td>9.3 ± 2.6 (28%)</td>
</tr>
<tr>
<td>EPDM 2</td>
<td>TZW</td>
<td>3323 ± 210 (6%)</td>
<td>0.2 ± 0.1 (47%)</td>
</tr>
<tr>
<td>EPDM 2</td>
<td>Crecep</td>
<td>1410 ± 432 (29%)</td>
<td>0.3 ± &lt; 0.1 (&lt;)</td>
</tr>
<tr>
<td>EPDM 3</td>
<td>TZW</td>
<td>17,282 ± 11,980 (69%)</td>
<td>1.3 ± 0.9 (74%)</td>
</tr>
<tr>
<td>EPDM 3</td>
<td>Crecep</td>
<td>53,478 ± 14,907 (28%)</td>
<td>2.7 ± 0.5 (19%)</td>
</tr>
</tbody>
</table>

The results presented in Table 7.2 show that the three EPDM rubbers had relatively high and clearly different BPP values. BPP values observed with EPDM 1 and EPDM 2 at Crecep were clearly higher than the BPP values reported by TZW. However, ranking of the materials was the same at TZW and Crecep, (viz. EPDM 2 < EPDM 1 < EPDM 3).

The material with the lowest BPP value gave the lowest Slime Production (SP) in the W270 test, but the material with the highest BPP value did not give the highest SP value. Furthermore, the SP values also differed between the laboratories. The acceptance criterion for SP production is 0.1 ml, but for certain materials with a low surface/volume ratio values <0.3 and < 0.5 ml are accepted. Only EPDM 2 complies with the latter criterion.

7.3.3 BPP test

At Kiwa WR the revised protocol BPP test was compared with the original BPP test (with no replacement of the water, test samples of about 8 cm² and Erlenmeyer flasks) (Table 7.3). Statistical analysis revealed that the BPP values as observed in both tests were significantly different for a number of the materials thus confirming the observations in stage 6 (Chapter 5). BPP values of water and glass in the original BPP test were lower than those in the revised test, indicating that external introduction of biodegradable compounds in the original BPP test is more limited than in the revised BPP test (water replacement; wide neck jars). The higher BPP values for Stainless Steel, PVC-C, Silicone Rubber and HDPE may have been caused by the effect of biodegradable compounds remaining present in the test water, but these
effects were only significant with HDPE. The BPP value for Nitrile Rubber in the revised BPP test was more than 20 times higher than in the original BPP test (no water replacement). The higher BPP value of PVC-P as obtained with water replacement may indicate an effect of nutrient (oxygen) limitation. No pass-fail criteria have been defined for the BPP value of materials in contact with drinking water.

<table>
<thead>
<tr>
<th>Material</th>
<th>Revised Protocol</th>
<th>Original method</th>
<th>p-value *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>53.6 ± 29</td>
<td>26 ± 7</td>
<td>0.005</td>
</tr>
<tr>
<td>Glass</td>
<td>116 ± 41</td>
<td>41 ± 16</td>
<td>0.002</td>
</tr>
<tr>
<td>Stainless Steel</td>
<td>142 ± 36</td>
<td>228 ± 97</td>
<td>0.063 (n.s.)</td>
</tr>
<tr>
<td>PVC-C</td>
<td>149 ± 22</td>
<td>197 ± 55</td>
<td>0.073 (n.s.)</td>
</tr>
<tr>
<td>Silicone Rubber</td>
<td>555 ± 38</td>
<td>672 ± 209</td>
<td>0.214 (n.s.)</td>
</tr>
<tr>
<td>HDPE</td>
<td>608 ± 135</td>
<td>905 ± 40.2</td>
<td>0.011</td>
</tr>
<tr>
<td>Nitrile Rubber</td>
<td>22,860 ± 9344</td>
<td>1074 ± 23</td>
<td>0.000</td>
</tr>
<tr>
<td>PVC-P</td>
<td>34,578 ± 16,094</td>
<td>19,034 ± 4089</td>
<td>0.026</td>
</tr>
</tbody>
</table>

*, significant at 95% level when p value < 0.05

7.3.4 ÖNORM B 5018

The Austrian method was applied on four different materials (including the glass control). The test was conducted without air supply for the materials PVC-C, Silicone Rubber, and HDPE because of the small diameter of the test samples. In this respect the methods differed from standard method. At the end of the test period (three months of incubation) the ATP concentrations of the test water and the pipe material were determined (Table 7.4). For comparison, the BPP values as reported by OFI in the reproducibility test are included in this table.

<table>
<thead>
<tr>
<th>Material</th>
<th>Sample area (cm²)</th>
<th>Biofilm (pg ATP/cm²)</th>
<th>Suspended biomass (ng ATP/l)</th>
<th>BP A*</th>
<th>BP B**</th>
<th>BPP# (pg ATP/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glass</td>
<td>1257</td>
<td>56</td>
<td>56</td>
<td>180</td>
<td>124</td>
<td>220</td>
</tr>
<tr>
<td>PVC-P 1</td>
<td>377</td>
<td>453</td>
<td>1511</td>
<td>1461</td>
<td>1009</td>
<td>11659</td>
</tr>
<tr>
<td>PVC-P 2</td>
<td>377</td>
<td>516</td>
<td>1721</td>
<td>2032</td>
<td>1516</td>
<td></td>
</tr>
<tr>
<td>PVC-C 1</td>
<td>471</td>
<td>67</td>
<td>178</td>
<td>351</td>
<td>284</td>
<td>278</td>
</tr>
<tr>
<td>PVC-C 2</td>
<td>471</td>
<td>50</td>
<td>133</td>
<td>340</td>
<td>290</td>
<td></td>
</tr>
<tr>
<td>Silicone 1</td>
<td>314</td>
<td>489</td>
<td>1944</td>
<td>1636</td>
<td>1147</td>
<td>744</td>
</tr>
<tr>
<td>Silicone 2</td>
<td>314</td>
<td>412</td>
<td>1639</td>
<td>1856</td>
<td>1444</td>
<td></td>
</tr>
<tr>
<td>HDPE 1</td>
<td>408</td>
<td>35</td>
<td>54</td>
<td>310</td>
<td>275</td>
<td>676</td>
</tr>
<tr>
<td>HDPE 2</td>
<td>408</td>
<td>32</td>
<td>53</td>
<td>272</td>
<td>240</td>
<td></td>
</tr>
</tbody>
</table>

*, analysis after 3 months of exposure and calculation according to BPP method; **B, analysis after 3 months of exposure; calculation according to Önorm B 5018; #, BPP values reported by OFI in the reproducibility test (Chapter 6, Table 6.2)

Microbial growth support potential of CPDW

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Heterotrophic plate count (HPC) values of the water were also determined (Table 7.5). These HPC values were determined in accordance with EN ISO 6222 (incubation at 22°C). The BP values and the HPC values reveal distinct differences between materials. The highest biofilm concentrations were observed with PVC-P and Silicone Rubber; the lowest values with HD-PE and PVC-C.

The biomass production values as observed with PVC-C and glass with the Austrian method are close to the BPP values as observed by OFP with the BPP method (Table 6.2). Furthermore, the BP values for HDPE and PVC-P were lower than those observed with the BPP test and the BP value of Silicone Rubber was higher. It is not clear which factors are responsible for these differences.

Table 7.5. Heterotrophic plate counts (CFU/ml) as observed in the Austrian test with a series of materials

<table>
<thead>
<tr>
<th>Material</th>
<th>Day 7</th>
<th>1 month</th>
<th>2 months</th>
<th>3 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glass</td>
<td>6.7 x 10⁴</td>
<td>1.7 x 10⁵</td>
<td>2.7 x 10⁴</td>
<td>1.4 x 10⁵</td>
</tr>
<tr>
<td>PVC-P 1</td>
<td>1.9 x 10⁶</td>
<td>1.7 x 10⁶</td>
<td>2.6 x 10⁶</td>
<td>9.1 x 10⁶</td>
</tr>
<tr>
<td>PVC-P 2</td>
<td>4.8 x 10⁶</td>
<td>7.4 x 10⁶</td>
<td>7.0 x 10⁶</td>
<td>4.9 x 10⁶</td>
</tr>
<tr>
<td>PVC-C 1</td>
<td>6.7 x 10⁴</td>
<td>8.3 x 10⁴</td>
<td>7.0 x 10⁴</td>
<td>7.1 x 10⁶</td>
</tr>
<tr>
<td>PVC-C 2</td>
<td>6.3 x 10⁴</td>
<td>2.9 x 10⁵</td>
<td>8.9 x 10⁵</td>
<td>2.0 x 10⁵</td>
</tr>
<tr>
<td>Silicone Rubber 1</td>
<td>1.9 x 10⁶</td>
<td>1.1 x 10⁶</td>
<td>2.1 x 10⁶</td>
<td>8.1 x 10⁶</td>
</tr>
<tr>
<td>Silicone Rubber 2</td>
<td>1.2 x 10⁶</td>
<td>7.4 x 10⁵</td>
<td>3.2 x 10⁶</td>
<td>6.1 x 10⁶</td>
</tr>
<tr>
<td>HD-PE 1</td>
<td>1.3 x 10⁵</td>
<td>6.1 x 10⁴</td>
<td>4.2 x 10⁴</td>
<td>2.0 x 10⁴</td>
</tr>
<tr>
<td>HD-PE 2</td>
<td>1.0 x 10⁶</td>
<td>1.4 x 10⁵</td>
<td>1.2 x 10⁵</td>
<td>7.0 x 10⁴</td>
</tr>
</tbody>
</table>

The ÖNORM B 5018 requires glass values 10 times lower than the positive control (PVC-P). Materials pass the test when BP values do not exceed 5 times the value of the negative control. HDPE and PVC-C pass the test, but Silicone Rubber failed according to ÖNORM B 5018 part 2. However, the S/V ratio of the silicone hose was about 4 fold higher than the S/V ratio normally used in the test.

7.3.5 Biofilm monitor (Kiwa)

Biofilm formation was determined at Kiwa in the Biofilm Monitor at a continuous tap water flow of 0.2 m/s. Biofilm formation was slow on glass, PVC-C and rapid on Silicone Rubber and PVC-P. Fig. 7.3 shows that the level of biofilm formation on PVC-C is similar to the biofilm formation on glass caused by the biodegradable compounds present in the continuously supplied tap water. The increased biofilm concentration observed after 2 weeks on PVC-C may reflect the initial presence of some biodegradable compounds on this material. With Silicone Rubber and PVC-P a fast increase of the biofilm concentration was observed, followed by a strong decline on Silicone Rubber reaching minimum values after about 70 days. Thereafter the biofilm concentration on this material increased at a higher rate than on glass. The biofilm concentration on PVC-P remained at a level between 25,000 – 100,000 pg ATP/cm². These values are similar to the BFP and BPP values observed in
the (revised) BPP test. The Biofilm Formation Rates and Biomass concentrations observed with the tested materials are given in Table 7.6.

![Graphs showing biofilm formation on selected materials exposed to a continuous flow of tap water (v = 0.2 m/s) in the biofilm monitor. Results Kiwa WR.](image)

Fig. 7.3 Biofilm formation on selected materials exposed to a continuous flow of tap water (v = 0.2 m/s) in the biofilm monitor (results Kiwa WR). For PVC-P a log scale vertical axis is used to facilitate comparison with glass. Stars indicate the biofilm concentrations obtained after application of high-energy sonication on the material samples following the normal (low energy) sonication procedure (see Chapter 8).

<table>
<thead>
<tr>
<th>Material</th>
<th>Biofilm Formation Rate</th>
<th>Biomass concentration*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(pg ATP/cm²/day)</td>
<td>(pg ATP/cm²)</td>
</tr>
<tr>
<td>Glass</td>
<td>5.4 ± 0.6 (r² = 0.90)</td>
<td>804 (116)**</td>
</tr>
<tr>
<td>PVC-C</td>
<td>5.0 ± 0.4 (r² = 0.96)</td>
<td>633 (149)</td>
</tr>
<tr>
<td>Silicone Rubber (hose)</td>
<td>11.1 ± 1.5 (r² = 0.84)</td>
<td>1,490 (556)</td>
</tr>
<tr>
<td>From day 0 to day 13:221</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PVC-P</td>
<td>From day 0 to day 27:4050</td>
<td>46,340 (34,578)</td>
</tr>
</tbody>
</table>

*on day 153, single samples for glass, PVC-C and PVC-P; duplicate for Silicone Rubber; **, data between brackets are BPP values as observed with the revised protocol at 25°C.

The BFR values of Silicone Rubber and PVC-P at the beginning of the test period are based on single observations and therefore only indicative. Table 7.6 shows that biomass concentrations (biofilm only) on glass, PVC-C and Silicone Rubber in the biofilm monitor were higher than the BPP values in the revised protocol. The differences are caused by the contribution of water to the biofilm formation in the biofilm monitor and the effect of temperature on the BP value, with lower values at lower temperature (see Table 6.4). Furthermore, it was observed that treatment of the materials with high energy sonication (HES) after to the low energy sonications (LES) in the water bath yielded a significant amount (50%) of additional biomass with PVC-P.
7.3.6 Test rig (DTU)

One series of samples was collected from the test rig after 40 days of incubation. Results of the type 2 sampling points must be considered inconclusive, since they were given for single test pieces. Control samples: extraction water treated with LES and HES and sonificated sterile cotton sticks did not have a detectable background. Hence the observed ATP values were a direct measure for biomass on material surfaces.

The ATP content of the inlet water was 6.6 pg ATP/ml. The ATP content in the water sampled from the model system with Stainless Steel was lower than in the inlet (2.2 pg ATP/ml), suggesting that bacteria present in the water attached to the surfaces. In the model systems containing PVC-C and PVC-P the ATP concentrations were 13.5 and 69 ng/l, respectively, indicating growth on (substrates from) the materials. The fully submerged steel test pieces in the model inlet line had slightly (but not significantly) higher biomass concentrations than the steel samples in the batch test. All other test pieces had lower biomass concentrations in the model systems compared with the batch test, which may have been caused by the continuous flow in the model systems. Table 7.7 gives biomass concentrations as observed on the materials after an operation period of 40 days.

Table 7.7 Biomass concentrations on surfaces in the test rig after 40 days of exposure to tap water at 10 °C

<table>
<thead>
<tr>
<th>Sample</th>
<th>Biomass concentration (pg ATP/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Steel</td>
</tr>
<tr>
<td>Inlet line *</td>
<td>48.6</td>
</tr>
<tr>
<td>Recirculation *</td>
<td>7.3</td>
</tr>
<tr>
<td>Outlet line *</td>
<td>28.8</td>
</tr>
<tr>
<td>Submerged **</td>
<td>40 ± 22</td>
</tr>
<tr>
<td>Revised BPP test (day 56) **</td>
<td>34 ± 1</td>
</tr>
</tbody>
</table>

* Biomass was removed with cotton sticks;
** Biomass was removed from test pieces with LES.

In all three model systems the highest biomass concentrations (BP) were observed in the inlet line, which had a low flow rate. For steel and PVC, the lowest BP values were in the recirculation ring, where the higher flow rate may have limited the attachment of microorganisms to the surfaces. In the outlet, where the flow rate was low, the BP values were higher for steel and PVC-C than in the recirculation ring, but lower than in the inlet. PVC-P also had lower BP values in the ring than in the inlet, but had even lower BP values in the outlet. Since no nutrients (e.g. P, N) were added to the water phase, the lower BP value in the outlet could be a result of nutrients limitation due to the high growth in inlet and recirculation ring.

The biomass concentration was significantly higher on PVC-P with only the inner surfaces exposed to the water phase than on fully submerged test pieces. This difference may be explained by the difference in the procedure for removing biomass rather than a result of sampling point type. Experiences
from the stage 6 and 7 showed that biomass from PVC-P is not easily removed by LES treatment. Swabbing with cotton sticks removed a larger amount of the biomass from the test pieces than low energy sonication (LES) treatment. Also, the biomass was easier to remove from the cotton sticks than from the material surfaces.

7.4 Discussion

7.4.1 Comparison of test methods
The results obtained in the different test conditions with a limited number of materials clearly demonstrate the complexity of assessing and evaluating biomass production on the surface of products in contact with drinking water. Test conditions have a large impact on biomass production of a material. The use of a single parameter, which enables assessment of the biomass concentration in different situations, facilitates the elucidation of the impact of different conditions in the test and in practice. However, the limited set of data as obtained in these investigations only gives indications of these effects.

7.4.1.1 UK MDOD test
The relationship between MDOD and BPP does not seem to be proportional, but a straight line relationship can be obtained by plotting the BPP values on a log scale with the MDOD values on a linear scale (Fig. 7.2). Growth-mediated reductions in dissolved oxygen concentrations in the test water will eventually result in the suppression of further growth as the system approached anaerobic conditions. This oxygen limitation in the MDOD test had an impact on the assessment of the MDOD values. This effect was less for the BPP value, despite a lower water replacement frequency (once a week) in the revised BPP test. A range of about a factor 1000 as covered with the BPP test cannot be observed with the MDOD test. The results presented in Fig. 7.2 suggest that the MDOD pass/fail criterion of 2.3 mg/l would approximate a BPP value of about 1600 pg cm⁻³ ATP. It is difficult to derive the true relationship between ATP concentrations and MDOD values until a much larger range of materials have been assessed. The relationship between MDOD and BPP also deserves more investigation.

7.4.1.2 German W270 Test
The interpretation of the comparison between the Slime Production (SP) test and the BPP test is complicated by a number of factors. Only three materials were tested and the BPP values of identical materials as determined in two laboratories differed from each other. These differences were larger than the VCₚ values reported in the reproducibility tests with other materials (Chapter 6). The nature of the materials and the procedure applied for sample preparation may have had an impact on the reproducibility. Also the SP values of the materials as reported by two laboratories showed relatively large differences. From the data obtained no indications for a relationship between SP values and BPP values can be derived. The pass-fail criterion for W270 was exceeded at the BPP values obtained for the EPDM materials.

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7.4.3 Austrian method

The comparison between the Austrian method and the revised BPP test was complicated by the fact the diameters of the tested pipe material were not in agreement with the prescribed diameter (4 cm). Consequently the S/V ratio in the experiment was higher than prescribed and also no air supply was possible under these conditions. The BP values for PVC-P, and HDPE were lower than the BPP values reported by O'H in the revised BPP test. However, for Silicone Rubber the BP value was higher than in the BPP test. The low BP values of PVC-P may have been due to nutrient and/or oxygen limitation, as a result of the high S/V ratio. The HPC values in the water showed distinct differences between the materials. The HPC values in water in contact with HDPE were about 100 to 1000 times below those observed in the presence of Silicone Rubber and PVC-P, but the BP values of these materials showed less difference. Differences in release of biodegradable compounds into the water may explain this phenomenon. A complicating factor with the HPC parameter is that differences in the composition of the microbial community have an impact on the HPC value, even when total numbers of viable bacteria are similar. This aspect does not play a role with ATP analysis.

7.4.4 Original Dutch method

The comparison between the revised BPP test and the original BPP method (no water replacement; glass stoppered Erlenmeyer flasks) showed a number of similarities and differences. Materials with low BPP values gave higher BPP values in the revised BPP than in the original method. This effect is probably caused by the introduction of biodegradable compounds with the replaced water and the less effective closing function of the lid of the jars (as compared to the glass-stoppered Erlenmeyer flasks). Intermediate BPP values were similar in both tests, but at high BPP values, the revised protocol gave much higher BPP values than the original BPP test. The difference observed with PVC-P probably is caused by growth limitation due to a lack of oxygen. With Nitrile Rubber additional factors may have played a role because the BPP value of this material in the test without water replacement was much less than the BPP value observed with PVC-P under these conditions. This difference may have been due to differences between the used O seals (e.g. different batches) and/or the presence of growth-inhibiting compounds in this material. The effect of the latter factor would also explain the poor growth observed with this material in tests without water replacement (see Chapters 4 and 5).

7.4.2 Continuous flow conditions

Continuous flow conditions were applied in the biofilm monitor (Kiwa) and in the test rig (DTU). The observations with the biofilm monitor show that the materials tested had clearly different properties regarding biomass production. The biomass ('biofilm') production on the materials Silicone Rubber and PVC-P clearly reflected the biomass production as observed in the (semi) static BPP test; a continuous flow rate of 0.2 obviously did not reduce biomass formation on these materials. The biofilm concentrations on the material in the biofilm monitor were the result of the combined effect of the water (biofilm formation rate) and the material. With the tap water used (BFR value of 5 pg ATP/cm².day) it was not possible to determine the effect of
PVC-C (with a BPP value < 100 pg ATP/cm²) but clear impacts of Silicone Rubber and PVC-P were observed. After about 70 days the biofilm concentration on Silicone Rubber had reached a minimum value, indicating that it took about this period before the release of biodegradable compounds had stabilised. After this period, the BFR value on Silicone Rubber was about twice the value on glass. These observations indicate that the biofilm monitor could serve as an alternative method for determining the BPP value (only biofilm) of materials. One disadvantage of this approach is the relative large impact of the water quality on the biofilm formation. Also, temperature control is not possible and a different temperatures will give different BP values.

With the test rig only preliminary data were obtained. In this system the biomass concentration on PVC-P remained relatively low, suggesting that lack of nutrients and/or oxygen limited growth. The differences between the BP values on the materials in the various parts of the test rig further demonstrate that (hydraulic) conditions in practice may have a large impact on the actual biomass production. The short test period (40 days) in combination with the low water temperature do not allow further conclusions to be drawn from the reported observations.

7.5 Conclusions
- The comparison of the results of different test methods was hampered by the limited number of materials, and deviations from prescribed procedures;
- MDOD values increased with increasing BPP values, but the relationship between MDOD values and BPP values was not proportional. More tests are needed to establish the true relationship between the results of these tests;
- Comparison between the revised BPP test and the W270 method, as conducted with three EPDM rubber samples, did not give the same ranking of materials. Both the BPP values and the SP values of identical EPDM types showed large differences between laboratories;
- The Austrian method was not conducted in accordance with the prescribed procedure. The low BP value of PVC-P is probably due to limitation of growth by nutrients/oxygen under the conditions used (high S/V ratio, no aeration);
- No clear relationships could be derived from the results obtained with the different test methods. More tests with more materials are needed to establish the degree of such relationships;
- The comparison with the original BPP test (no water replacement confirmed the earlier observations (stage 6) on the effect of water replacement. The revised protocol BPP test gave higher BPP values than the original method at low levels and at high levels, respectively;
- Biofilm formation on materials in the biofilm monitor using continuous flow (0.2 m/s) showed a clear distinction between materials. Materials with low BPP values showed a low or no increase of the biofilm formation rate (BFR). Furthermore, the similarities between biofilm formation on PVC-P and Silicone Rubber and the BPP value of these materials
demonstrate that the BPP test (revised protocol) reflects biofilm formation/biomass production when these materials are exposed to flowing water.

- Biofilm formation on materials in the test rig system with a flow of 0.08 m/s and a retention time of one day was similar to the BPP values of the Stainless Steel and PVC-C (low BPP values) but was much lower with PVC-P. Growth limitation by a lack of nutrients may have caused this difference;

- A period of more than 70 days was needed before the release of biodegradable compounds from Silicone Rubber stabilised, suggesting that a shorter test period may not be suitable for some materials.
8 Removal of attached biomass from materials with ultrasound

8.1 Introduction
A precondition for a robust ATP-based assessment of microbial growth on CPDW is the complete removal of the accumulated biomass for the ATP measurements. In this respect, the sonication procedure (6 x 2 min in a water bath sonicator), as conducted in the proposed test method at stage 7, may be improved. The application of ultrasonic energy (ultrasound) is a convenient physical detachment method, but its power range is device-dependent. A low-energy water bath sonicator (LES) applies waves with relatively low (and fixed) amplitude, whereas a (sonotrode-)tip high energy system (HES) delivers waves with higher (and adjustable) amplitude into the sample. This implies that less sonication treatments per material are required when using HES. Moreover, incidental observations in stage 7 experiments indicated that HES treatment may result in additional biomass release. Additional research, which was not included in the project plan, was conducted to elucidate the importance of this phenomenon. The experiments conducted on this topic are described below.

8.2 Materials and methods
HES testing was performed on pieces (small and large) of selected materials that had been incubated up to 30 weeks at 10°C (DTU) or at 25°C (DTU, Kiwa, TZW) following the protocol of stage 7. LES and HES treatments were performed in triplicate on pieces originating from the same bottle (Kiwa, TZW), or in duplicate on pieces originating from two parallel bottles (DTU).

8.2.1 HES equipment
The sonotrode devices used were a Branson Sonifier W-250 (DTU and Kiwa) and a B. Braun Labsonic U (TZW), all with a working frequency of 20 kHz. Tip diameters (mm) were 3 (DTU), 6.5 (Kiwa) and 16 (TZW), respectively. The smaller the tip diameter, the higher the wave amplitude range and thus the ultrasonic energy (cavitation) potential.

At the start, in between different material samples and when finished, the tip was cleaned with a disinfectant solution and subsequently sterile tap water (if it was being used for the same material (Kiwa) or fresh MilliQ (DTU). At Kiwa, small material pieces (14-17 cm²) were treated (on ice) in polypropylene tubes (50 ml Greiner, conical ending) containing 20 ml of autoclaved tap water, while large pieces (46-51 cm²) were treated in glass beakers of 100 ml (for PE) or 150 ml (for PVC-P) containing 80 ml of autoclaved tap water. At DTU and TZW, sonication of large pieces was performed in glass beakers containing respectively 50 ml and 40 ml of autoclaved tap water. Tip immersion was at least 1 cm below the water surface. Pieces were exposed to high-energy ultrasound for one to three period of 1-4 min (see tables, water

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replaced each run) at an amplitude setting of 10-50% (power reading of 25-60 W). During a treatment session, when necessary, the microtip was cooled down (on ice) in between individual runs.

8.3 Results

8.3.1 Observations at Kiwa WR

<table>
<thead>
<tr>
<th>Treatment sequence</th>
<th>PE small</th>
<th>PVC-P small</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25%</td>
<td>40%</td>
</tr>
<tr>
<td>1st 2 min</td>
<td>983</td>
<td>608</td>
</tr>
<tr>
<td>2nd 2 min</td>
<td>0.69</td>
<td>57.2</td>
</tr>
<tr>
<td>Total 2 x 2 min</td>
<td>984</td>
<td>665</td>
</tr>
<tr>
<td>1 x 4 min</td>
<td>1,210</td>
<td>1,446</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Treatment sequence</th>
<th>PE large</th>
<th>PVC-P large</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25%</td>
<td>40%</td>
</tr>
<tr>
<td>1st 3 min</td>
<td>Nt</td>
<td>557</td>
</tr>
<tr>
<td>2nd 3 min</td>
<td>Nt</td>
<td>24.1</td>
</tr>
<tr>
<td>3rd 3 min</td>
<td>Nt</td>
<td>Nt</td>
</tr>
<tr>
<td>Total 2/3 x 3 min</td>
<td>nt</td>
<td>581</td>
</tr>
</tbody>
</table>

*, Nt, not tested

HES (1x) following LES (6x) treatment of week-4 test pieces (PVC-C, Silicone Rubber, HDPE and PVC-P) revealed that only PVC-P released significant amounts of additional biomass (> 25%) for both small and large pieces. The additional release was negligible (< 5%) for PVC-C, small (in between 5 and 10%) for Silicone Rubber and PE-small and for PE-large it was zero (Fig 8.1). Notably, when LES was performed on small pieces in plastic (Greiner) tubes (like with HES), the subsequent release by HES was more than 50% for all materials tested (week-2 pieces, data not shown). For large pieces of PVC-P and PE at week 2 (LES in glass beakers) the extra release by HES was, like at week 4, respectively significant and negligible (i.e. > 25% - for PVC-P and < 5% - for PE), confirming the previous observations.

To determine an optimal HES setting initial testing at Kiwa on relatively hard (PE) and soft (PVC-P) materials focussed on sonication energy, time and treatment repetition (Table 8.1). Increasing the energy resulted in a higher biomass release for PVC-P, but this was not obvious for PE. For PVC-P, treatment repetition (in fresh replaced water) gave a higher yield improvement than extending a single run. For PE, one run appeared to be sufficient (≥ 95% release), but a second treatment enables verification of the efficiency of the first. Therefore, a two- or threefold HES treatment with a setting of 40% (27 W) for 2 or 3 min was applied in subsequent tests. Test pieces of two soft materials (Silicone Rubber and PVC-P) and two hard materials (PVC-C and PE) were analysed after two and four weeks of incubation, with glass rings as the negative control. At week 2, in all cases most biomass (69% < BP < 96%) was released after the first 3-min treatment.
(Table 8.2). The additional release with the second HES was more than 10% for PVC-P and significantly around 10% for SILR. For PVC-C, it was insignificant—around 10%, and for PE, it was negligible (≤ 5%). At week 4, small and large pieces were treated for respectively 2 and 3 min (Table 2B). A two-fold treatment appeared to be sufficient for PVC-C, but this was not the case for PE-small. Three HES treatments were adequate (> 90%) for Silicone Rubber but still insufficient for PVC-P.

**Table 8.2. Biomass release (pg ATP/cm²) with two- or threefold HES treatment of 40% (27W), sm=small pieces and la=large pieces (data Kiwa)**

<table>
<thead>
<tr>
<th></th>
<th>SILR sm</th>
<th>PVC-P sm</th>
<th>PVC-P la</th>
<th>PVC-C</th>
<th>PE sm</th>
<th>PE la</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Week 2 pieces (n=3), 3 min HES</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total 2x</td>
<td>518±50.1</td>
<td>24,825±3,370</td>
<td>20,654±779</td>
<td>132±14.3</td>
<td>905±202</td>
<td>478±134</td>
</tr>
<tr>
<td>% 2nd</td>
<td>13.3±2.9</td>
<td>18.5±15.7</td>
<td>31.3±4.5</td>
<td>11.9±11.4</td>
<td>3.8±1.6</td>
<td>7.0±1.3</td>
</tr>
<tr>
<td>HES</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. Week-4 pieces (n=3), 2-min HES-sm and 3 min HES-la</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total 3x</td>
<td>415±107</td>
<td>25,826±5,47</td>
<td>27,515±2,187</td>
<td>882±290</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% 2nd</td>
<td>22.2±4.2</td>
<td>25.5±5.7</td>
<td>15.0±1.7</td>
<td>18.5±4.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HES</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% 3rd</td>
<td>10.2±3.7</td>
<td>19.1±8.8</td>
<td>7.5±2.4</td>
<td>9.0±6.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HES</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

![Fig. 8.1. Sequential biomass release (ATP) by 6x LES (L1-L6) and one HES (H1) for soft (SILR and PVC-P) and hard (PVC-C and PE) materials (data Kiwa).](image)

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8.3.2 **Observations at DTU**

At DTU, preliminary tests with HES treatment of PVC-P (after 6x 2-min LES treatment) showed a significant effect of increasing the output control setting from 10 to 30% as well as of increasing the treatment time from 1 to 3 minutes (see Table 8.3). Of the other materials tested, only Nitrile Rubber significantly released additional biomass with HES after LES (up to 25%, not shown). Subsequently, one set of test pieces was repeatedly treated with HES at 30% for 3 minutes each time (see Table 8.4). Another set of test pieces was repeatedly treated for 1 minute at 30%. With 3-min treatment time, the third treatment resulted in less than 10% extra biomass release for most of the samples (12% for PE #1, 13% for PVC-P #2). Likewise with the 1-minute treatment time, the third treatment resulted in less than 10% further release for all materials except PVC-P, and a fourth treatment only resulted in a limited further release. For both treatment settings, the first treatment gave 74-93% of the total release (except for PVC-P). However, with the 3x 3-min treatment (9 min in total) the total amount of released biomass was larger than with the 4x 1-min treatment (4 min in total).

Earlier investigations (stage 4 and 6) had shown that biofilm formation was limited after week 16. Therefore the effect of different treatments were compared between test pieces from week 16 (Table 8.3) and week 30, indicating that highest total amounts were obtained with three 3-min 30% HES treatments (step 3). Comparing the effect of the 6x LES treatment with the 1- and 3-min HES treatments (Tables 8.4 and 8.5) revealed that one HES treatment (both 1 min and 3 min) could replace the 6x LES treatment.

**Table 8.3. PVC-P biomass release (µg/cm² ATP) with LES followed by HES (data DTU)**

<table>
<thead>
<tr>
<th></th>
<th>PVC-P week 12, 25°C</th>
<th></th>
<th>PVC-P week 12, 10°C</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LES 6x 2-min</td>
<td>5,715</td>
<td>1,978</td>
<td>LES 6x 2-min</td>
<td>20,394</td>
</tr>
<tr>
<td>1. HES 10%, 1-min</td>
<td>344</td>
<td>806</td>
<td>1. HES 30%, 1-min</td>
<td>9,798</td>
</tr>
<tr>
<td>2. HES 20%, 1-min</td>
<td>530</td>
<td>1,727</td>
<td>2. HES 30%, 1-min</td>
<td>3,883</td>
</tr>
<tr>
<td>3. HES 30%, 1-min</td>
<td>1,338</td>
<td>1,650</td>
<td>3. HES 30%, 1-min</td>
<td>2,676</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4. HES 30%, 1-min</td>
<td>1,611</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5. HES 30%, 2-min</td>
<td>1,647</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>6. HES 30%, 2-min</td>
<td>1,399</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>7. HES 30%, 3-min</td>
<td>1,752</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>8. HES 30%, 3-min</td>
<td>1,975</td>
</tr>
<tr>
<td>Total HES</td>
<td>2,211</td>
<td>4,183</td>
<td>Total HES</td>
<td>24,243</td>
</tr>
<tr>
<td>Total LES+HES</td>
<td>7,926</td>
<td>6,161</td>
<td>Total LES+HES</td>
<td>44,637</td>
</tr>
<tr>
<td>% HES of LES</td>
<td>39</td>
<td>211</td>
<td>% HES of LES</td>
<td>119</td>
</tr>
</tbody>
</table>

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Table 8.4 Biomass release (pg/cm² ATP) from different materials (week 30, 10°C) when treated with HES. (A) at 30% for 3 min each time, (B) at 30% for 1 min each time (data DTU). Experiments in duplicate.

<table>
<thead>
<tr>
<th></th>
<th>Glass</th>
<th>Steel</th>
<th>PE</th>
<th>PVC-C</th>
<th>PVC-P</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HES 30%, 1st 3-min</td>
<td>24</td>
<td>14</td>
<td>51</td>
<td>35</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>2nd 3-min</td>
<td>2.0</td>
<td>1.0</td>
<td>3.5</td>
<td>6.1</td>
</tr>
<tr>
<td></td>
<td>3rd 3-min</td>
<td>0.8</td>
<td>0.7</td>
<td>2.4</td>
<td>2.0</td>
</tr>
<tr>
<td>Total</td>
<td>27</td>
<td>16</td>
<td>57</td>
<td>42</td>
<td>79</td>
</tr>
<tr>
<td>% 3rd HES</td>
<td>3</td>
<td>4</td>
<td>4</td>
<td>5</td>
<td>12</td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HES 30%, 1x 1-min</td>
<td>16</td>
<td>14</td>
<td>45</td>
<td>32</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>2x 1-min</td>
<td>1.9</td>
<td>2.3</td>
<td>3.3</td>
<td>3.8</td>
</tr>
<tr>
<td></td>
<td>3x 1-min</td>
<td>1.4</td>
<td>1.9</td>
<td>2.1</td>
<td>3.3</td>
</tr>
<tr>
<td></td>
<td>4x 1-min</td>
<td>0.5</td>
<td>0.7</td>
<td>0.6</td>
<td>1.3</td>
</tr>
<tr>
<td>Total</td>
<td>19</td>
<td>19</td>
<td>51</td>
<td>41</td>
<td>61</td>
</tr>
<tr>
<td>% 4th HES</td>
<td>3</td>
<td>4</td>
<td>1</td>
<td>3</td>
<td>6</td>
</tr>
</tbody>
</table>

Table 8.5 Biomass release (pg/cm² ATP) from different materials (week 16, 10°C) with 3-min HES (30%) after 6x LES (data DTU).

<table>
<thead>
<tr>
<th></th>
<th>Glass</th>
<th>Steel</th>
<th>PE</th>
<th>PVC-C</th>
<th>PVC-P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LES, 6x 2-min*</td>
<td>28</td>
<td>12</td>
<td>26</td>
<td>44</td>
<td>22</td>
</tr>
<tr>
<td>HES 30%, 1x 3-min</td>
<td>6.5</td>
<td>6.2</td>
<td>8.7</td>
<td>2.2</td>
<td>1.8</td>
</tr>
<tr>
<td>Total</td>
<td>35</td>
<td>18</td>
<td>34</td>
<td>46</td>
<td>23</td>
</tr>
<tr>
<td>% HES of LES</td>
<td>23</td>
<td>51</td>
<td>34</td>
<td>5</td>
<td>8</td>
</tr>
</tbody>
</table>

*3 x 2-min for glass

8.3.3 Observations at TZW

At TZW, a different HES system with a sonotrode tip of large diameter (16 mm) was used. Nevertheless, results were comparable with those at Kiwa and DTU. HES (1x min at “39W”) following LES (6x) treatment of week-12 test pieces revealed that only PVC-P released considerably additional biomass (88.8% of 6x LES value). Pieces of Silicone Rubber with young biofilms also released significantly extra biomass when HES was applied after LES (Table 8.6). The HES effect was again large for PVC-P, but unclear for Silicone Rubber and insignificant for PVC-C, when comparing the outcome of the triple HES treatment as well as the additional HES treatment with the standard LES treatment result (week 1 and 3; Table 8.6).
Table 8.6. Evaluation of LES/HES-comparison (data TZW).

<table>
<thead>
<tr>
<th>Material</th>
<th>Time</th>
<th>LES pg ATP/cm²</th>
<th>% of LES</th>
<th>LES + HES pg ATP/cm²</th>
<th>% of LES</th>
<th>3x HES pg ATP/cm²</th>
<th>% of LES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glass</td>
<td>1 week</td>
<td>&lt; LOD*</td>
<td></td>
<td>&lt; LOD</td>
<td></td>
<td>4.1</td>
<td></td>
</tr>
<tr>
<td>Glass</td>
<td>3 weeks</td>
<td>&lt; LOD</td>
<td></td>
<td>&lt; LOD</td>
<td></td>
<td>3.5</td>
<td></td>
</tr>
<tr>
<td>Silicone R</td>
<td>1 week</td>
<td>1592</td>
<td>100</td>
<td>1921</td>
<td>121</td>
<td>1412</td>
<td>89</td>
</tr>
<tr>
<td>Silicone R</td>
<td>3 weeks</td>
<td>527</td>
<td>100</td>
<td>716</td>
<td>136</td>
<td>1277</td>
<td>242</td>
</tr>
<tr>
<td>PVC-P</td>
<td>1 week</td>
<td>7253</td>
<td>100</td>
<td>7775</td>
<td>107</td>
<td>10500</td>
<td>144</td>
</tr>
<tr>
<td>PVC-P</td>
<td>3 weeks</td>
<td>23790</td>
<td>100</td>
<td>29280</td>
<td>123</td>
<td>30710</td>
<td>129</td>
</tr>
<tr>
<td>PVC-C</td>
<td>1 week</td>
<td>76</td>
<td>100</td>
<td>83</td>
<td>109</td>
<td>65</td>
<td>86</td>
</tr>
<tr>
<td>PVC-C</td>
<td>3 weeks</td>
<td>20</td>
<td>100</td>
<td>22</td>
<td>110</td>
<td>29</td>
<td>145</td>
</tr>
</tbody>
</table>

* LOD, limit of detection

8.4 Discussion

In the investigations in stages 5, 6 and 7 a six-step LES treatment was used to achieve complete biomass removal with various materials (Fig. 8.1). For the materials tested at this extra stage, it appeared that only for PVC-P the 6x-LES treatment is largely insufficient; HES revealed that a significant fraction (about 50%) remains on the material after the 6th LES. For Silicone Rubber this is not observed. One HES treatment can completely (± 10%) replace 6x LES with the hard materials PVC-C and PF, but because of deviation and to verify completeness, a second treatment is favoured. The HES setting can be further optimised, but longer runs and/or high(er) power coincide with extra heat development and thus possibly (ATP) deterioration. Nevertheless, soft materials need at least a second HES treatment. Performing respectively two and three ‘individual’ HES treatments per hard and soft material piece is rather laborious, whereas a waterbath sonicator can handle multiple samples. Therefore, a sequential use of the two sonication devices may become the standard, e.g. 3x 3-min LES (>90% sufficient for hard materials, Fig. 8.1) followed by either 1x 3-min or 2x 3-min HES (respectively hard and soft materials).

Experience at Kiwa has shown that it takes 1.5-2.5 hours to subject 36 small-material samples to the standard 6x LES treatment, with 12 sample tubes during each waterbath run. About 1.5 hours are needed to subject these samples to an additional single HES treatment of 1 min each, i.e. ~3 min per sample including the preceding water replacement and tube exchange. At TZW, the time needed for 6x LES (60 min for 11 samples) and a single 1-min HES (40 min for 11 samples) was more or less the same. When extending the HES treatment to 3 min, the time needed for the HES session with one run per material is already slightly longer than the duration of the whole LES session. Although the total time is doubled with a double HES treatment, instead of LES, the disadvantage of extra time may be considered compensated by the extra biomass yield and the fewer operations (water replacements and sampling).
All in all, LES treatment seems satisfying for most materials and the required equipment is present in most laboratories. Applying HES treatment requires specific equipment, and also a special room to avoid noise problems. Intensive use of the equipment may lead to probe damage. The conducted experiments demonstrate the impact of (additional) HES treatment for biomass detachment, but not all aspects involved have been elucidated. At TZW relatively large sample pieces (10 x 15 x 4 cm) of cementitious materials were sonicated in sterile plastic bags. This approach may be useful, but the efficacy of LES and/or HES under these conditions needs further attention. Also alternative treatments may be considered for the removal of attached biomass from products, e.g. swabbing with sterile cotton sticks, or the use of chemicals.

8.5 Conclusions
- HES treatment after six LES treatments removed additional biomass from PVC-P but for other materials tested (PVC-C, PE, Silicone Rubber) this effect was insignificant. It cannot be excluded that also with other (soft) materials, LES treatment is insufficient for complete biomass removal;
- For the removal of attached biomass with high-energy ultrasound, different HES devices (regarding tip diameters) are appropriate, but for large pieces larger tip diameters (having a larger sample volume range) are preferred;
- Regarding absolute biomass yield, one HES treatment can replace the 6x LES treatment sequence;
- Relatively hard materials would need a second HES treatment for verification of total (> 95%) biomass removal, while for relatively soft materials (at least) three treatments are required;
- Application of HES instead of LES does not reduce the time needed for conducting the analysis. Furthermore, it requires specific equipment;
- The suitability of alternative methods for biomass removal needs further investigation.
9 General discussion

9.1 Design of the test
The objective of WP1 of the CPDW project was to develop a test method for determining the microbial growth promoting properties of these products based on existing methods. Such a harmonised method would serve as the basis for further standardisation in CEN. A number of aspects, which are critical for a suitable test, have been addressed in discussions and investigations in WP1. Much progress has been made and a first draft for a standard based on the collected information has been prepared (Appendix 2). The first application of this protocol by the participants using a number of selected products covering a wide range of BPP values (Tables 6.3 and 6.5) suggests that a coefficient of variation of reproducibility of about 30% is achievable. This level is similar to the reproducibility indicated for the MDOD test. A number of aspects related to the test require further attention to improve reproducibility. These critical aspects are discussed below.

An essential aspect of the test is the use of ATP as the biomass parameter. As described in Chapter 3, ATP represents active biomass. This parameter may correlate with the oxygen consumption of biomass, but relationships with HPC values and other parameters for determining the microbiological quality of drinking water are not clear (see below). The value of using ATP as biomass parameter is clearly demonstrated by this research. The analysis is rapid and has a low detection limit. The main factors causing differences in the ATP measurements have been identified in the course of the project. The concentration of inorganic compounds in the calibration solution should be identical to the concentrations of these compounds in test water. Problems may be expected when products release compounds inhibitory to the ATP analysis, e.g. Ca by cementitious materials or metals by metallic products. In such cases either dilution of the test water in contact with the product and the water used for sonication or standard addition may be needed to obtain a reliable measurement. Hence, further standardisation of the analytical procedures for ATP measurements is needed to ensure the accuracy of the observations with CPDW. Aspects that need further consideration include quality assurance within the laboratory (calibration, use of controls, maintenance and operation of equipment, selection of reagents) and between laboratories (organisation of ring tests).

In relation to the ATP analysis, the method for collecting the biomass present on the surface of the material needs further improvement. Ultrasound procedures are effective but time consuming. These methods can be used and further improved/standardised, but the potential utility of alternative procedures (swabbing, chemicals) also needs attention.

A further critical test condition is the contact between the product and the water in the test. This contact is affected by the degree of test dynamics and
the surface to volume contact ratio. The aim of the test is to assess the potential of a product to promote microbial growth. Consequently, test conditions may differ from practice to ensure that the potential is properly determined. Interpretation of the data obtained in such a test is a different issue (see below). A number of test conditions need to be realistic in comparison to situations in practice (e.g. water composition, water temperature). The investigations showed that a batch test approach in combination with water replacement is a suitable basis for the test. Water replacement is relevant to prevent limitation of growth by a lack of nutrients, oxygen, and/or accumulation of growth inhibiting compounds. Furthermore, the removal of slowly biodegradable compounds from the test system, which do not play a (significant) role in practice, is achieved with this proposed method. A water replacement frequency of once a week was found both suitable and easy to control in the laboratory. Some uncertainty remains however, about the possibility of oxygen depletion in the presence of products with a strong growth potential (> 20,000 pg ATP/cm²). It is possible that such materials do not comply with pass-fail criteria, but an accurate assessment of the growth potential may be useful to draw conclusions about conditions in which the material can be used without causing water quality problems. Investigations carried out in the UK have clearly shown that the MDOD values obtained in their microbial growth potential test are not linearly related to the surface area to volume of the test system. These observations may be explained by the impact of the S/V ratio on growth limitation by factors mentioned above. The use of a relatively high S/V ratio in combination with a long retention time in the test rig and in the Austrian method demonstrated limited growth on products with a large growth potential. These conditions may resemble situations in practice, but hamper the assessment of the growth potential of the material involved. Based on these considerations it may be concluded that the surface area used in the test system should be sufficiently large to enable a quantitative assessment of low concentrations of biomass. With ATP analysis, surface areas of a few cm² would be sufficiently large. However, samples with small surface areas have the disadvantage that a relatively large proportion of exposed surface is the cutting edge. Consequently, sample sizes as used in this investigation (10-50 cm²) in combination with an S/V ratio of about 0.16 cm⁻¹ are a good compromise.

The quality of the surface exposed in the test is another concern. Homogeneous materials can be tested with representative samples. Non homogeneous materials (multiple layers; outside surface different from inside surface) can pose a problem. This problem has also been recognised in other tests with materials and needs to be addressed along existing lines, e.g. the manufacturers provide representative samples. An alternative approach for certain materials would be to collect the biomass only from those parts of the surface, which would also be exposed to drinking water in practice. Swabbing is a possibility for removing biomass from these surfaces, but is only applicable to materials with a smooth surface. The surface to be investigated is swabbed several times with sterile cotton swabs, which subsequently are placed in ATP free sterile water and sonicated to remove the biomass from the swabs.

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A further aspect related to the sample surface is cleaning of the materials prior to testing. Also here the procedure generally applied will be used without the contact with disinfectants.

Water quality is another critical aspect of the test. The water may be growth limiting despite the addition of inorganic N and P, or growth promoting as the result of the presence of biodegradable compounds. The biostability of the water has an impact on the detection of low levels of growth promotion. At an S/V ratio of 0.16, an ATP yield of the water of 10 ng/l may cause a BP value of about 60 pg ATP/cm², when all available growth promoting compounds are utilised by the microorganisms in the biofilm. Replacement of the water would further enlarge this effect. The results presented show that certain materials (Stainless Steel, PVC-C) have a BPP value below 50 pg ATP/cm². Consequently, the maximum ATP level of the test water, as determined in the blank without materials, should not exceed 10 ng/l. In addition, introduction of biodegradable compounds from the air, the jar (lid) or the pipettes should be strictly limited to ensure proper analysis of materials with a low growth potential. The procedures followed to achieve this goal need attention in further standardisation of the proposed method.

Temperature has an effect on the growth potential, with decreasing BPP values at increasing temperature. However, certain materials may have higher BPP values at elevated temperatures (and vice versa) due to enhanced biodegradation and/or accelerated release of biodegradable compounds. These phenomena need further scientific elucidation, but for the time being it is proposed to apply an incubation temperature of 30°C because this level can be attained in ('cold') water systems.

The test period as used in the present tests range from seven weeks (MDOD) to six months (W270), with intermediate periods for the Austrian method (three months) and the BPP test (16 weeks), respectively. The investigations have shown that the BP value of Nitrile Rubber did not reach a stable level in the test period of 16 weeks, and that BP values of Silicone Rubber were still declining in a period of six to 10 weeks (Figs. 5.1 and 7.3). Given the limited number of materials included in the investigations it seems justified to conclude that a test period of 16 weeks is a good compromise. When more information becomes available, the use of a different test period for specific materials can be considered.

9.2 **Interpretation of test results**
A correct interpretation of the results of the test requires information about the relationship between the amount of biomass produced by a material under the test conditions and the quality of drinking water in contact with this material in practice. As already mentioned, the ATP concentration associated with a material cannot be related directly to microbial water quality criteria and problems; this is also the case with the MDOD value and the Slime Production in the W270 test. One approach is ranking of the materials and in combination with practical experience derive a pass-fail criterion. In the MDOD test, the
The ATP parameter has the advantage however, that it can also be used in assessing the concentration of active biomass in tap water and in biofilms on surfaces exposed to tap water. This approach has been followed in the Netherlands and databases of these concentrations are available (van der Kooij, 1992; van der Kooij et al. 1999). Furthermore, with the use of a biofilm monitor information has been collected about the Biofilm Formation Rate (BFR, pg ATP/cm².d) of treated water and the potential of treated water to produce biofilms (pg ATP/cm²) on exposed surfaces. This approach, the Unifying Biofilm Analysis, results in a framework that is helpful for the interpretation of individual data (van der Kooij et al., 2003). As an example it can be mentioned that the BPP value of unplasticized PVC (PVC-U) is typically (less than) about 50 pg ATP/cm². Similar BPP values have been obtained in this study with Stainless Steel and PVC-C (Tables 6.3 and 6.5). The observations with the biofilm monitor confirmed that PVC-C did not enhance biofilm formation when exposed to continuously flowing tap water without disinfectant (Fig. 7.3). However, increased biofilm formation was observed with Silicone Rubber for which an average BPP value of 450 pg ATP/cm² was found (Table 6.3). The MDOD test did not differentiate between these materials for which MDOD values were reported ranging from 0.6 to 1.1 mg/l (Table 7.1). These levels are below the pass-fail criterion of this test. In unchlorinated supplies in the Netherlands, with more than 50% PVC-U pipes, no regrowth problems occur when the water is biologically stable (van der Kooij, 1992). Consequently, products with BPP values below 100 pg ATP/cm² do not seem to cause problems when applied in long lengths. However, it is uncertain at what BPP level CPDW will start to cause problems. Relatively high BPP values may be acceptable at short contact times, but problems with growth of *Legionella* on natural rubber demonstrate that caution is needed (Colbourne et al. 1984; Hengesbach et al. 1993). A further aspect is the distribution of drinking water with a disinfectant residual, which will limit biofilm formation. Given these differences in water quality and the lack of quantitative information about the relationships between regrowth problems and Biomass Production by CPDW, it is concluded that further studies are needed to enable the establishment of scientifically sound and practicable pass-fail criteria.
10 Conclusions and recommendations

10.1 Conclusions
- A harmonised method for determining the growth promoting properties of CPDW has been developed on the basis of existing methods;
- The test determines the Biomass Production Potential (BPP) of CPDW using ATP as the biomass parameter. Product samples are incubated in tap water which is replaced at a frequency of once a week.
- The coefficient of variation of reproducibility (CVr) of the test ranged from 34 to 107 % (at 25 °C) and from 20 to 34% at 30 °C in the first application of the test protocol with selected products. The BPP values of these products covered a wide range (from less than 50 to more than 40,000 pg ATP/cm²).
- The main factors affecting the reproducibility include: deviations in ATP analysis, the quality of the test water and procedures for biomass detachment;
- BPP values decrease with increasing temperature, which may be explained by the effect of endogenous respiration. A temperature of 30°C is proposed for incubation;
- The number of samples tested in the MDOD test, the W270 test and the Austrian method was too limited to establish quantitative relationships between the results of these tests and the revised BPP test;
- Some indications about the practical significance of the BPP values can be derived from results observed with similar materials in the MDOD tests in this investigation, MDOD values and W270 values reported for other materials and biofilm concentrations as observed in distribution systems.

10.2 Recommendations for further research
As explained above, further investigations are needed to improve the quality of the proposed test procedure and to establish pass-fail criteria. Main aspects of such investigations include:
- optimisation and standardisation of ATP analysis;
- optimisation of procedures for the removal of biomass (ATP) from CPDW;
- selection of test water (biostability; growth-limiting compounds);
- establishment of the effect of temperature (Q10 value);
- growth promoting properties of materials which have been in use for a long period (e.g. > 10 years);
- relationship between biofilm concentrations and water quality problems (includes a survey on biofilm concentrations).
These investigations should include practical situations, test rigs and laboratory conditions.
11 References


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1 Determination of the ATP content of water and suspensions

*Kiwa internal procedure*

1 **Subject**
These procedures describe a method for the determination of the adenosine triphosphate (ATP) content of water and suspensions with the aid of a luminometer.

2 **Scope**
The ATP content is a measure of the quantity of active biomass. The procedures described in this document apply to water, such as groundwater, surface water, water types at various stages of treatment, drinking water, mineral water, etc., and also to extracts in water obtained from products in contact with water using a pretreatment method as described in Kiwa internal procedure LMB-010. The detection limit in water is 1 ng ATP/l. The detection limit of pipe and construction materials is always calculated on the basis of the surface area processed. Excessive (8.0) or inadequate pH (7.5), excessive chloride, cadmium, copper or zinc concentrations and the colour of the sample may act as interfering factors. Other interfering factors may be a too low (< 18°C) or a too high (> 23°C) temperature of the sample and the reagents.

3 **Definitions**
Adenosine triphosphate is an energy-rich compound that is present in all living cells and consequently provides a measure of active biomass.

4 **Principle**
The ATP measurement is based on the reaction of luciferin with luciferase (originating from fireflies) which occurs in the presence of free ATP. During this process, light is produced which is measured and expressed in Relative Light Units (RLUs). The sample to be analysed is transferred to a cuvette and placed in the luminometer. Reagents are then added in an automated reactions occurring:

\[
\begin{align*}
\text{Mg}^{2+} & \\
\text{luciferin} + \text{luciferase} \rightarrow & \text{(luciferin-luciferase-AMP)} \\
+ \text{ATP} & + \text{pyrophosphate} \\
\text{(luciferin-luciferase-AMP)} \rightarrow & \text{Oxyluciferin} + \text{luciferase} \\
+ \text{ATP} & + \text{CO}_2 + \text{AMP} + \text{light}
\end{align*}
\]

Under optimal conditions, 1 photon of light is produced per molecule of ATP.

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The light generated is measured using a sensitive photometer and expressed in Relative Light Units (RLUs). The ATP content of the sample is then calculated with the aid of a conversion factor.

5 Safety and environment
Not applicable

6 Checks

6.1 Equipment and reagent checks
Before measuring, check whether the luminometer and the reagents meet the following criteria:
- the mean value of the blank cuvettes must be < 10 RLUs;
- the mean value for Nucleotide Release Reagent (LuminEX) and LuminATE-PM must be ≤ 25 RLUs.

6.2 Perform multiple analyses
Perform all analyses in duplicate. Calculate the variation coefficient for the two RLU values. The variation coefficient (vc) must not exceed:

<table>
<thead>
<tr>
<th>Results (RLUs)</th>
<th>vc (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 - 100</td>
<td>30</td>
</tr>
<tr>
<td>&gt; 100</td>
<td>20</td>
</tr>
</tbody>
</table>

If this requirement is not met, this should be specified in the lab report and the final analysis report.

6.3 Blank water
For each series, start with two cuvettes of blank water. Also include a further two blanks after 10 samples. The mean number of RLUs of the blank water must be < 20 RLUs. If it is not, repeat the measurement of the blank water. Perform operations, for example, use another batch of water or measure the blank LuminEX and Blank LuminATE(PM) again. Replace the chemicals when previously described criteria are not met and thoroughly rinse the hose system.

6.4 Positive checks
In the case of each measurement series, include two standard solutions. After every 10 samples, include a further two standard solutions. Enter the mean of the RLU values measured before the sample series on the relevant Shewhart charts. If it turns out that the 3S limit is exceeded, repeat the measurement. If the 3S limit is again exceeded, try to trace the cause. The cause may be reagents that are either contaminated or too old. If the 2S limit is exceeded twice running, try to identify the cause. If eleven measurements in succession are on the same side of the 2S limit, a fresh standard solution should be
produced. The concentration of the standard solution may have decreased during storage.

Check whether the other RLU values of the standard solutions are within the 3S limits of the control charts. If this is not the case, the ten samples measured before the standard solutions should be measured again. Draw up a new control chart for new standard solutions. Before Shewhart charts are produced for new standard solutions, these solutions should first be measured ten times. Measure this standard solution preferably at the same time as the old standard solution.

6.5 Calibration curve
Twice a year, on five (consecutive) days, produce a calibration curve as described in Annex 1. The mean of the factor of these calibration curves must not diverge by more than 10% from the factor used for calculation purposes (11.1). A calibration curve should also be produced if eleven measurements in succession from the standard solutions are on the same side of the mean on the control chart or if the profile of the standard solutions exhibits a sharp rise or fall ("break").

7 Reagents and additional compounds

Allow all reagents to reach room temperature before use (about 15 minutes).

7.1 ATP standard solutions

7.1.1 Composition
ATP-standard (Celsis) 1 bottle
LuminATE buffer (Celsis) 10,000 g

7.1.2 Preparation
Weigh the LuminATE buffer in the ATP standard bottle.
Dissolve the ATP standard by swinging carefully (concentration 10⁴ ng ATP/l).
ALLOW THIS SOLUTION TO STAND FOR 1 HOUR BEFORE USING FURTHER!!

7.1.2.1 Standard solution 100 ng ATP/l
Produce a 1:100 dilution (final concentration: 10² ng ATP/l) from the standard solution in 7.1.2 in the following way:

LuminATE-buffer (Celsis) 9.9 g
ATP standard solution (7.1.2) 100 µl

Rinse the pipette tip.
Prepare another 1:100 dilution (final concentration: 10² ng ATP/l) from this dilution in the same way.
Transfer 0.3 ml of this dilution to deep-freeze tubes and store these at -70°C. This standard is stable for one month.

7.1.2.2 Standard solution 2 ng ATP/l
Prepare dilutions in the following way from the standard solution referred to in 7.1.2.1 (10² ng ATP/l):

LuminATE-buffer (Celsis) 9.0 g
ATP standard solution 1000 µl

Produce the following standard solution from this dilution:
LuminATE-buffer (Celsis) 12 g
ATP standard solution 3 ml

Transfer 0.3 ml of this dilution (2 ng ATP/l) to deep-freeze tubes and store these at -70°C. This standard is stable for one month.

*ATP is not stable at room temperature when not dissolved. However, the frozen tubes remain stable for longer periods at -70°C. The solution must not be refrozen after thawing.*

7.2 LuminATE (PM) enzyme
7.2.1 Composition
LuminATE (PM) (Celsis) 1 bottle
LuminATE buffer (Celsis) 7 ml

7.2.2 Preparation
Add the LuminATE buffer to LuminATE (PM). Dissolve the luminATE (PM) by swinging carefully. ALLOW THIS SOLUTION TO STAND FOR 15 MINUTES BEFORE USING FURTHER!!
Use this solution within 4 hours.

7.3 LuminEX (B)7.3 NRB-reagens

7.4 Wash & Rinse Kit
Bactowash (Celsis)

7.5 Sterile water
Sterile (autoclaved) drinking water with a low copper concentration and a low ATP content (< 20 RLUs).

8 Equipment

8.1 Equipment
8.1.1 Autoclave for sterilizing dilution water and glassware
8.1.2 Desiccant (150°C) for sterilizing glassware
8.1.3 System with PC and printer
8.1.4 Vortex
8.1.5 Balance with a measuring accuracy of 1 mg
8.1.6 Deep-freezer with a temperature of at least -70°C

8.2 Sterile glassware
8.2.1 Sealed 30 ml bottles (brown), sterilized

8.3 Miscellaneous
8.3.1 Sterile plastic 10 ml pipettes
8.3.2 Pipetman (P1000)
8.3.3 Pipetman (P2000)
8.3.4 Pipette tips
8.3.5 Polystyrene cuvettes
8.3.6 Small tanks for housing cuvettes
8.3.7 Deep-freeze tubes

9 Sample treatment
Start the investigation preferably immediately after sampling. If this is not possible, the sample can be stored in an uncooled state for a maximum of 6 hours; if cooled over the range 0 - 4°C, the sample must be used within 24 hours.

10 Method

10.1 Preparation of the system for the measurements
- Start up the system and the PC.
- Log in under your own name
- Click on the system icon; the program is started
- Log in as follows: xxxxx
- Password: xxxxx ....
The program can be used.

Start-up
- Click on maintenance – injectors – startup
- Follow the computer instructions. Under select reagent, select injector positions: 2 and 3.

10.2 Measurement of the controls
10.2.1 Blank water
Fill 2 cuvettes with 100 µl of sterile tap water using a micropipet.

10.2.2 Blank sample
When determining the ATP content of materials or filter sand, one blank sample is included per sample series. This blank sample consists of sterile tap water (in the case of materials) or sterile MilliQ water (in the case of filter sand) from the same batch as that in which pretreatment of the samples took place. For samples from a Biofilm Monitor, use is made of a procedure blank from a sterile glass cylinder which is treated in the same way as the samples, and with water from the same batch. Measure the blanks in the same series as the standard solutions:
- Thaw 1 tube of standard solution 100 ng ATP/l and 1 tube of standard solution 2 ng ATP/l by placing the tubes in a water bath at max. 37°C for 2 minutes.

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- For each standard solution, fill 2 cuvettes with 100 μl using a micropipet.
- An ID FILE ("start up .wkl") exists for the standard solutions.

   Open the file strat up.wkl. Fill the system with 2 cuvettes for blank cuvette, skip one space, then two cuvettes for blank reagents, skip one space again and then the standards (2 ng/l, 100 ng/l and blank water).

Press start. The System will scan the number of batches. A program can be selected per batch. The programs have already been set for start-up. The next batch is accessed by clicking on next. You then run through all batches in this way until the computer indicates how many reagents will be used. Then click on finish. The System starts measuring.

Record the results of the standards on the relevant Shewhart charts.
Store the data in an Excel file (start up C + date).

10.3 Building up of measurement serie
- Start with two cuvettes of blank water
- Place no more than 10 samples in the sample changer (see 10.5).
- Then place a blank, a standard of 2 and a standard of 100 ng (all in duplicate) in the sample changer.
- Then again place no more than 10 samples in the changer.
- etc.

10.4 Measurement of the samples
- Transfer 100 μl of sample to 2 cuvettes using a micropipet.
- Place the samples in the system.
- Click on file – new – workload – start (the system scans the chain). The file is produced.
- Click next, select the program that you wish to use via the arrow in the top block; the rest of the data are then automatically input. Click next again and then finish. Before the system starts measuring, the file must first be saved (name.wkl). The sample codes can then be entered. Click twice in the column sample-id and the code of the samples can be typed in.
- Print out the results and save the file as an Excel file (name + date).

10.5 Finishing
Click on maintenance – injector – shutdown.
Follow the computer instructions. Under select, select injectors positions: 2 and 3. 2.5 ml Rinsing solution and 5 ml Washing solution, in that order, are then set on the pumps.
Then switch off the system and the computer.
11 Identification and quantification

11.1 Calculation of the ATP content of the samples

11.1.1 Water samples

\[ \text{Total ATP} = \frac{\text{mean RLU - mean blank water}}{\text{factor}} \]

where:

- **Total ATP** = The ATP content in ng/l of sample or in pg/ml of sample.
- **Mean RLU** = The arithmetic mean of the two RLU values of the samples.
- **Mean blank water** = The arithmetic mean of the four RLU values of the blank water (two before the samples and two after the samples).
- **Factor** = A value laid down on the basis of historic data. This value is the mean gradient of calibration curves and is 4.75.

11.1.2 Samples from materials and filter sand

Calculate the ATP content using the following formula:

\[ \text{Total ATP} = \frac{\text{mean RLU - mean blank sampling}}{\text{factor}} \]

where:

- **Total ATP** = The ATP content in ng/l of sample on in pg/ml of sample.
- **Mean RLU** = The arithmetic mean of the two RLU values of the samples.
- **Mean blank sample** = The arithmetic mean of the four RLU values of the blank sample or of the procedure blank (two before the samples and two after the samples).
- **Factor** = A value laid down on the basis of historic data. This value is the mean gradient of calibration curves and is 4.75.

Calculation of the ATP content per cm² of material or per ml of material is described in Kiwa internal procedure LMB-010.

12 Characteristic values

12.1 Detection limit

The detection limit for the ATP determination is 1 ng ATP/l sample.
12.2 Repeatability

The variation coefficient (vc) for repeatability is:

<table>
<thead>
<tr>
<th>Results (RLUs)</th>
<th>vc (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 - 100</td>
<td>30</td>
</tr>
<tr>
<td>&lt; 100</td>
<td>20</td>
</tr>
</tbody>
</table>

12.3 Variation coefficient (vc) for in-lab reproducibility

The variation coefficient for in-lab reproducibility is:

<table>
<thead>
<tr>
<th>ATP level (ng ATP/l)</th>
<th>vc (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>20</td>
</tr>
<tr>
<td>100</td>
<td>10</td>
</tr>
</tbody>
</table>

13 Report

Specify:
- The details needed to identify the sample.
- The method adopted: in accordance with Kiwa internal procedures LMB-002.
- The ATP level found in ng ATP/l sample, rounded in accordance with the table below.
- Any special circumstances observed during determination.
- All operations not described in the procedure that may have influenced the result.

Rounding table

<table>
<thead>
<tr>
<th>Total ATP (ng/l or pg/ml)</th>
<th>Round to</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-10</td>
<td>0.1</td>
</tr>
<tr>
<td>10-100</td>
<td>1</td>
</tr>
<tr>
<td>100-1000</td>
<td>10</td>
</tr>
<tr>
<td>&gt;1000</td>
<td>100</td>
</tr>
</tbody>
</table>

14 Remarks

None.
15 Annexes

13.1 Annex 1: Production of a calibration curve (2 pages)
Annex 1, page 1

Production of a calibration curve

Make up an ATP standard solution in the following way

Composition
ATP standard 1 bottle
Tap water (ATP < 20 RLUs) 10,000 g

Preparation
Weigh the tap water in the ATP standard bottle.
Dissolve the ATP standard by swinging carefully.

- Prepare the calibration curve in cuvettes in accordance with the diagram described in the figure.
- Always properly mix the content of the cuvettes on the vortex, using a new pipette tip for producing each new dilution.
- Using a micropipet, transfer 100 μl to 2 cuvettes (total ATP) for each dilution to be measured.
  Two cuvettes are to be filled with each standard.
II Design of harmonised test

Title: Assessment of the Biomass Production Potential of Construction Products in Contact with Drinking Water (CPDW)

1 OBJECTIVE AND SCOPE

This method assesses the ability of construction products in contact with water intended for human consumption to promote growth of microorganisms. The procedure is applicable to all types of products with a solid surface and which do not release compounds, which are toxic to microorganisms. Such products include natural and synthetic organic products and inorganic products (with or without the addition of organic components).

Note: Products in contact with other water types (swimming water, industrial water types), products used in medical equipment and products used in contact with foods (drinks) can also be tested with the procedure described.

2 DEFINITIONS

Biomass Production Potential (BPP): the average concentration of active micro-organisms on the product surface and in water, measured as adenosinetriphosphate (ATP) per cm² of the product, after 56, 84 and 112 days of incubation in test water under defined conditions.

3 PRINCIPLE

Representative samples of the product to be tested are incubated in tap water containing specified inorganic "nutrients" and inoculated with a mixture of naturally occurring micro-organisms derived from river water. These product samples are incubated for a period up to 16 weeks at a constant surface to volume ratio of 0.16 cm⁻¹. Thoroughly cleaned glass rings serve as the negative control to measure the growth-promoting effects of water. PVC-P is used as positive control. The test water is replaced at a frequency of once a week. Formation of biomass on the product surface (biofilm) and in the water is determined with adenosinetriphosphate (ATP) measurements after 8, 12 and 16 weeks of incubation. Product samples are collected periodically and biomass is detached with ultrasound. The ATP concentration is used as a measure for the presence of active microbial biomass and the biomass production per unit surface is calculated from the concentration of attached and suspended biomass. Validation of the results is achieved by including glass controls and reference materials in parallel with the materials under test. The test is schematically presented in Fig. 1.

4 APPARATUS
4.1 Equipment

4.1.1 Oven for heat sterilisation at 150 to 175 °C
4.1.2 Oven for heat treatment of pipettes at 250 °C
4.1.3 Oven for heat treatment at 550 °C of glass samples.
4.1.4 Incubator 30 (± 1) °C
4.1.5 Analytical balance with an accuracy of 0.1 mg
4.1.6 Balance with an accuracy of 10 mg
4.1.7 Vortex mixer
4.1.8 Equipment for membrane filtration of water samples, including a vacuum pump
4.1.9 pH meter
4.1.10 Ultrasonic water bath (frequency 40 Hz)
4.1.11 Thermometer (0-30°C), divided in units of 0.1°C
4.1.12 Thermometer (100°C), accuracy 0.1°C
4.1.13 Equipment for ATP analysis and required chemicals

4.2 Glassware
Appropriate glassware must be able to resist frequent heating at temperatures up to 250 °C or 550 °C (borosilicate glass)

4.2.1 Glass jars with a volume of 1000 ml and a neck width of 57 mm, provided with a lid with PTFE inlay
4.2.2 Beakers with a volume of 100 ml and 500 ml
4.2.3 AOC-free glass pipettes (1 ml) divided in 0.01 ml
4.2.4 Culture tubes, ø 16-18 mm, 160 mm, with autoclavable cap.
4.2.5 Culture tubes, ø 21-24 mm, 200 mm (Scott Duran, with autoclavable cap).
4.2.6 Cylinder glass (h=45 cm, d=15 cm) with a glass siphon for washing pipettes.
4.2.7 Dispenser, resistant to wet sterilisation, for volumes up to 10.0 ml
4.2.8 Measuring cylinder, 250 ml, divided in units of 10 ml
4.2.9 Glass vessels, 250 ml
4.2.10 Beakers of 500 ml
4.2.11 Glass vessels, length 17 cm; width 8 cm and height 19 cm, with cover
4.2.12 Sterile pipettes of 10 ml, divided in 0.05 ml.

4.3 Various materials

4.3.1 Flowing cold tap water (drinking water quality)
4.3.2 Demineralised water
4.3.3 Stainless Steel cylinders, with a seal cap, for heat sterilisation of the pipettes
4.3.4 Tube racks, for large and normal culture tubes.
4.3.5 Pairs of tweezers with rounded corners for handling of the membrane filters, the materials and the Stainless Steel hooks
4.3.6 Membrane filters, pore size 1.2 µm
4.3.7 Callipers, accuracy 0.01 cm.

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4.3.8 Pair of scissors, craft knife and/or saw and pincers (all grease free) for preparing the material samples

4.3.9 Reference materials:
- plasticised PVC (PVC-P) tubing of defined characteristics and known not to contain growth-inhibiting agents;
- borosilicate glass rings (outer diameter 18 mm, wall thickness 2 mm; length 15 mm) with a total external surface of about 17 cm²;
- glass rings (outer diameter 18 mm, wall thickness 2 mm; length 7 mm) with a total external surface of about 8 cm² and Stainless Steel wire (Ø 0.8 mm; DIN 125 A quality) to increase weight of material samples.

4.3.10 Melting ice

5 REAGENTS

Use analytical grade reagent dissolved in demineralised water as required.

5.1 Reagents

5.1.1 Potassium-dihydrogen phosphate solution

Composition
KH₂PO₄  0.79 g
Demineralised water  100 ml

Preparation
Dissolve the potassium dihydrogen phosphate in demineralised water contained in a thoroughly cleaned flask and autoclave this solution at (121 ± 10) °C for 15 min. Store in a refrigerator.

5.1.2 Potassium-nitrate solution

Composition
KNO₃  3.24 g
Demineralised water  100 ml

Preparation
Dissolve the potassium nitrate in demineralised water in a thoroughly cleaned flask and autoclave this solution at (121 ± 10) °C for 15 min. Store in a refrigerator.

5.1.3 Dilution water
Autoclaved tap water free from toxic effects on bacteria with toxic properties for bacteria (including chemical disinfectants) and:
- a copper concentration < 50 µg/l,
- a pH value between 6.5 and 8.5

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Table 1 Quality criteria for test water

<table>
<thead>
<tr>
<th>Quality parameter</th>
<th>Concentration (range)</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>6.5 - 8.5</td>
<td>Preferably between 7.0 and 8.5</td>
</tr>
<tr>
<td>Oxygen (mg/l)</td>
<td>&gt; 6.5</td>
<td>Oxygen depletion is not acceptable</td>
</tr>
<tr>
<td>PO₄-P (mg/l)</td>
<td>2.0 - 6.7</td>
<td>Addition of 2 mg P/l</td>
</tr>
<tr>
<td>Nitrate-N</td>
<td>5 - 11.3</td>
<td>Addition of 5 mg N/l</td>
</tr>
<tr>
<td>Ammonia-N (mg/l)</td>
<td>&lt; 0.05</td>
<td></td>
</tr>
<tr>
<td>Disinfectant residual</td>
<td>Absent</td>
<td></td>
</tr>
<tr>
<td>Copper (mg/l)</td>
<td>&lt; 0.05</td>
<td></td>
</tr>
<tr>
<td>Silver (mg/l)</td>
<td>&lt; 0.01</td>
<td></td>
</tr>
<tr>
<td>Hardness</td>
<td>Not defined</td>
<td></td>
</tr>
<tr>
<td>Total dissolved organic</td>
<td>&lt; 3</td>
<td></td>
</tr>
<tr>
<td>carbon (mg/l)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biostability (BP, ng ATP/l)</td>
<td>&lt; 10</td>
<td>This degree of biostability corresponds with</td>
</tr>
<tr>
<td></td>
<td></td>
<td>an AOC value &lt; 10 µg C/l</td>
</tr>
<tr>
<td>Micro-organisms</td>
<td>Indigenous</td>
<td>River water after membrane filtration (1.2</td>
</tr>
<tr>
<td></td>
<td>bacteria + 1 %</td>
<td>µm pores)</td>
</tr>
<tr>
<td></td>
<td>river water</td>
<td></td>
</tr>
</tbody>
</table>

5.1.4 Test water
Tap water free from toxic effects on bacteria, a high degree of biological stability, sufficient inorganic nutrients (see below), a microbial community supplement with aquatic bacteria as present in river water.

Addition of inorganic nutrients:

Composition
Tap water 900 ml
Potassium dihydrogen phosphate solution (5.1.1) 1.00 ml
Potassium nitrate solution (5.1.2) 1.00 ml

Preparation of the test water
Determine the weight of the cleaned empty test jars flasks. Fill the marked flasks with about 900 ml of the selected tap water using a flowing tap, close the flasks with the lids and transport to the laboratory for direct further use. Determine the weight of the flasks filled with water and adjust the water volume to 900 ± 20 ml (from a spare flask when needed). Add the solutions of potassium nitrate and potassium dihydrogenphosphate aseptically using AOC-free pipettes. Assess the pH of the test water in a sample taken from one of the flasks.

5.2 Inoculum

Composition

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Freshly collected river water, without floating substances such as algae (500 ml)

*Preparation*
Place the filter house (4.1.8) with the membrane filter (4.3.7) on the vacuum vessel and connect with the vacuum pump. Flush the membrane filter with about 250 ml of demineralised water (not sterile) to remove DOC from the membrane. The filtrate is collected in the sterile flask and is ready for use.

6 CLEANING PROCEDURES

6.1 Glassware

6.1.1 Test jugs
Place the test jugs, the lids and inlays in a washing machine, applying a routine washing procedure. After washing, flush with demineralised water, with hot tap water and place the flasks up-side down to drain.

6.1.2 Tubes for dilution water
Wash culture tubes in the racks in a washing machine normally used for cleaning glassware from the laboratory. Cover the tubes with caps. Heat the tubes at a temperature of 150 to 175 °C for 4 hours.

7 TEST PROCEDURE

7.1 Handling of products

7.1.1 Nature of samples
The test product samples should originate from a normal production batch, which has not received any special treatment, and represent the product used in contact with drinking water. Products such as washers and O-rings can be tested as such provided that these materials are of suitable size. Large products (e.g. pipes) are cut to sample pieces with the desired surface area and size.

7.1.2 Storage of products
Products must be protected from contamination by dust, grease, oil etc. during storage and transportation. Protection is also needed against heat, sunlight and volatile chemicals. The products can be stored in the dark at 20 ± 3 °C, unless otherwise prescribed by the manufacturer.

NOTE: Do not test products marked with ink and/or pencil, or label glue etc. Do no allow skin contact with the test products.

7.2 Preparation of test samples and controls
7.2.1 **Size and number of sample pieces**
Two jars each containing three sample pieces, each with an external surface area of 50 cm², are prepared. Of each product, 8 pieces are needed (includes spare pieces). If the test product/material has a specific gravity of less than 1, and therefore floats, connect a glass ring (8 cm²) with Stainless Steel wire to the test sample (4.3.10) (use clean pincers). Coating samples are prepared by the manufacturer on Stainless Steel coupons with an appropriate surface area following normal procedures. The coating should cover all sides of the plate. For each coating one coupon is prepared. Cementitious materials are tested using cubes with an external surface of at least 50 cm².

NOTE: prevent direct contacts between skin and test samples by using polyethylene gloves and pincers. Use separate gloves for each type of material. Limit contact between gloves and test products as much as possible by using pincers.

7.2.2 **Sample cleaning**
Flush the product samples for 1 hour in a 500 ml glass beaker with cold running tap water on the day before the test is to be started; store the samples in water overnight, and flush again for 1 hour prior to testing.

7.2.3 **Glass rings, stainless steel (SS) wire and pincers**
Place the glass rings and wire, to be used to increase the weight of pieces of testing materials, in a glass tray and heat at 550°C for 4 hours. Clean pincers by heating in a brightly burning gas flame.

7.3 **Incubation**
Add the product samples to the jars, three pieces to each test jar. For each material type two jars (A and B) are used. Add pieces of the reference products (glass and biocide-free PVC-P) to additional jars with test water. Use nine glass cylinders (of 17 cm² each). Inoculate the flasks with 9 ml of membrane-filtered river water (5.3). Incubate the jars for 16 weeks in the dark in an incubator at (30 ± 2) °C without shaking. Replace the water weekly using fresh tap water supplemented with inorganic nutrients (P,N).

Ensure that the air in the incubator or hot room is free from volatile organic compounds.

NOTE: Volatile compounds may cause microbial growth in the test water masking the growth caused by the material.

7.4 **Analysis**
The concentration of biomass on the materials and in the water is assessed with adenosinetriphosphate (ATP) measurements, using the appropriate reagents and a luminometer, in accordance with the manufacturer’s instructions.

7.4.1 **Product samples**
Determine the biomass concentration (pg ATP/cm²) on the surface of the product samples as ATP on days 56, 84 and 112 respectively. Remove one sample from flask A and one from flask B, on each of the test days. Collect these samples using cleaned and heat-treated tweezers. Subsequently, place each sample in 50 ml of sterile tap water in a 100 ml glass beaker and place this beaker in an ultrasonic cleaning bath with demineralised water. The water in the bath should be at the same level as that in the beakers. Apply ultrasound for 2 minutes. Transfer the water from the beaker to a sterile container and place this container on melting ice. Add 50 ml of fresh sterile water to the beaker with the material and sonicate again. Repeat this procedure so that each material sample has received six sonications (glass need three treatments). Check the temperature of the water in the ultrasonic cleaner. Add ice when this temperature exceeds 25°C. Determine the ATP concentration of the total water volume obtained (which is 300 ml for materials with six sonications and 150 ml for glass). This ATP concentration is used to calculate the biomass concentration (pg ATP/cm²) of the material sample.

7.4.2 Water
Gently swirl the jar by hand prior to collecting a water sample of 50 ml from each the flasks A and B on the days 56, 84 and 112. Pour 50 ml of water from each flask into sterile graduated tubes. Determine the Suspended Biomass (SB) concentration (SB) in these water samples with ATP measurements. Water samples must be taken prior to water replacement.

7.4.3 Product sample size
Product samples are collected in the course of the investigation. Accurately determine the external surface area of each sample piece after analysis and calculate the biofilm concentration (pg ATP/cm²).

8 CALCULATIONS

8.1 Biomass Production (BP)
Calculate the BP value for each material from the ATP concentrations as observed on the product sample and in the test water. The average BP value is the biomass concentration on the material (pg ATP/cm²) and the biomass concentration in the test water × V/S after 56, 84 and 112 days of incubation. Also determine the standard deviation (SD) of the mean of the BP values.

8.2 Biomass Production Potential (BPP)
The Biomass Production Potential (BPP) is calculated as the average value of the BP values observed on days 56, 84 and 112 respectively, minus the average BP value as observed with the glass control. The BPP value is also expressed as pg ATP/cm².
Include the following information in the report:
- information needed to identify the product;
- the BP values on days 56, 84, and 112 as observed with the product samples, the average BP value and the standard deviation;
- the BP values of glass control on days 56, 84 and 112 and the average BP value;
- the BP values of the positive control (PVC-P) on days 56, 84 and 112 and the average BP value;
- the BPP value of the product(s) tested;
- special observations, during preparation of the samples and/or during testing;
- all treatments/procedures used which are not described in the standard, and which may have affected the results.
Biofilm Formation Potential (BPP) Test (Fig. 1)

Test Samples
6 samples, each
50 cm² Surface Area

Rinse Test Samples
1 hour in flowing tap water, etc

Inoculum
(9 ml membrane-filtered river water)

Test water
900 ml volume
KH₂PO₄ & KNO₃ additives

Test flasks (x 2)
1 litre jars

Incubate jars at
30 ± 1 °C in the dark for
8 weeks with weekly water replacement

Validation Materials
Glass
PVC-p tubing

Biomass Concentration on materials in both jars
Remove one test piece
Sonicate and measure ATP

Incubate jars at
30 ±1 °C in the dark for
a further 4 weeks with weekly water

Biomass concentration in Water of both jars
Remove 300 ml of water and measure ATP

Biomass concentration on materials in both jars
Remove one test piece
Sonicate and measure ATP

Incubate jars at
30 ±1 °C in the dark for
a further 4 weeks with weekly water replacement

Biomass concentration in Water of both jars
Remove 300 ml of water and measure ATP

Biomass Concentration on materials in both jars
Remove one test piece
Sonicate and measure ATP

End of test

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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.