A uniform bacterial growth potential assay for different water types

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Abstract

The bacterial growth potential is important to understand and manage bacterial regrowth-related water quality concerns. Bacterial growth potential depends on growth promoting/limiting compounds, therefore, nutrient availability is the key factor governing bacterial growth potential. Selecting proper tools for bacterial growth measurement is essential for routine implementation of the growth potential measurement.

This study proposes a growth potential assay that is universal and can be used for different water types and soil extract without restrictions of pure culture or cultivability of the bacterial strain. The proposed assay measures the sample bacterial growth potential by using the indigenous community as inocula. Flow cytometry (FCM) and adenosine tri-phosphate (ATP) were used to evaluate the growth potential of six different microbial communities indigenous to the sample being analyzed, with increasing carbon concentrations. Bottled mineral water, non-chlorinated tap water, seawater, river water, wastewater effluent and a soil organic carbon extract were analyzed.

Results showed that indigenous bacterial communities followed normal batch growth kinetics when grown on naturally present organic carbon. Indigenous bacterial growth could detect spiked organic carbon concentrations as low as 10 µg/L. The indigenous community in all samples responded proportionally to the increase in acetate-carbon and proportional growth could be measured with both FCM and ATP. Bacterial growth was proportional to the carbon concentration but not the same proportion factor for the different water samples tested. The effect of inoculating the same water with different indigenous microbial communities on the growth potential was also examined. The FCM results showed that the highest increase in total bacterial cell concentration was obtained with bacteria indigenous to the water sample.

The growth potential assay using indigenous bacterial community revealed consistent results of bacterial growth in all the different samples tested and therefore providing a fast, more stable, and accurate approach for monitoring the biological stability of waters compared to the previously

46 developed assays. The growth potential assay can be used to aid in detecting growth limitations by
47 compounds other than organic carbon.
48 **Keywords**: Assimilable organic carbon **(**AOC); Adenosine tri-phosphate (ATP); flow cytometry
49 (FCM); seawater.
50

1. Introduction

Bacterial growth potential is the quantification of the extent of bacterial growth that can occur in a sample under defined conditions. Nutrient availability, mainly organic carbon and other growth-promoting/limiting compounds (e.g., nitrogen, phosphorus and iron), govern bacterial growth potential (Prest et al. 2016a, Nescerecka et al. 2018). Numerous methods to determine the bacterial growth potential and growth promoting properties of water have been developed throughout the last three decades (Van der Kooij et al. 1982, Servais et al. 1989, Hu et al. 1999, Ross et al. 2013, Prest et al. 2016a). The first developed methods for bacterial growth potential determination focused on the biodegradable organic carbon. The assimilable organic carbon (AOC) notion, initially proposed by Van der Kooij et al. (1982), is used to describe the portion of dissolved organic carbon (DOC) that is rapidly used by microorganisms to grow. AOC is viewed as an important parameter to assess the biological stability of water and the microbial growth potential during treatment and distribution (Srinivasan and Harrington 2007, Baghoth et al. 2009, Hammes et al. 2010a, Weinrich et al. 2010, Kim et al. 2017). Unlike chemical methods to determine and characterize total organic carbon (TOC) or DOC, AOC explicitly targets a wide range of biologically available low molecular weight organic carbon compounds, generally present in low concentrations in water. The AOC bioassay is based on the linear relationship between the AOC concentration and maximum bacterial growth (i.e., maximum crop). For AOC calculations, a numerical yield factor (Y) is derived from the slope of a standard linear curve and is used to calculate the AOC concentration using the maximum bacterial growth of test bacteria. Determining the bacterial growth potential through the conventional AOC bioassay such as the assay by Van der Kooij et al. (1982) usually assumes organic carbon limitation which is not the case for all water samples. Several studies revealed that bacterial regrowth in drinking water in some regions was predominantly inhibited by inorganic phosphorous limitation (Sathasivan et al. 1997, Miettinen et al. 1999, Nescerecka et al. 2018). In these cases, determination of microbially available phosphorus (MAP), phosphorus that is readily assimilated by microorganisms, is more

77 crucial than the AOC (Lehtola et al. 1999). In such waters, MAP is linearly correlated to bacterial
78 growth potential, and a minor variation in the phosphorus concentration can have a major effect on
79 the growth of bacteria. Therefore, growth potential bioassays were developed focusing on other
80 possible microbial growth controlling substances which in some cases might be more crucial to
81 describe and understand the bacterial growth potential rather than mainly organic carbon as the single
82 growth-limiting substrate (States et al. 1985, Miettinen et al. 1997, Lehtola et al. 1999, Prest et al.
83 2016b, Nescerecka et al. 2018).

84 Numerous studies contributed to constantly optimize the bioassay with a main focus on three
85 aspects: the selection of inoculum, the optimization of inoculation and incubation, and the evolution
86 of bacterial growth measurements (LeChevallier et al. 1993, Sathasivan and Ohgaki 1999, Wang et
87 al. 2014, Van der Kooij et al. 2017). The conventional bioassays to measure the bacterial growth
88 potential use selected pure cultures mainly *Pseudomonas fluorescens* P17 (P17) and Spirillum sp.
89 NOX (NOX) as test strains primarily due to their abundance in water distribution systems and their
90 ability to utilize organic carbon in low concentrations (Van der Kooij et al. 1982, Kaplan et al. 1993,
91 LeChevallier et al. 1993). P17 and NOX require a simple nitrogen source and no growth-stimulating
92 substances, such as vitamins. A major drawback of using pure cultures is the inability of some pure
93 strains to universally grow in different water types (e.g., NOX does not grow in seawater) and to
94 assimilate all the AOC present in the water. Moreover, the selection and use of specific single
95 bacterial strains does not ensure similar results when different sample types are tested and changing
96 the bacterial strains according to the sample type leads to results that are hard to compare. Therefore,
97 the inoculum selection has been a point of focus in many studies and a principal alteration to the
98 initial bacterial growth potential methods (Kemmy et al. 1989, Sathasivan and Ohgaki 1999, Haddix
99 et al. 2004, Weinrich et al. 2011); from using a single bacterial strain to a mix of two or more bacterial
100 strains or the indigenous bacterial community. Some commercial assays and studies (Weinrich et al.
101 2011) used *Vibrio harveyi* bacteria instead of P17 and/or NOX to assess the growth potential of
102 seawater which could not necessarily be used for freshwater. Indigenous bacteria demonstrated the

ability to completely utilize the available AOC enabling a better estimate of the bacterial growth potential (Werner and Hambsch 1986, Sathasivan and Ohgaki 1999, Hammes and Egli 2005, Prest et al. 2016a).

Shifting from cultivation dependent quantification methods to cultivation independent methods was another primary variation to the initial growth potential methods (LeChevallier et al. 1993, Hammes and Egli 2005, Abushaban et al. 2017). Bacterial growth measurements evolved from the use of plate counting (Van der Kooij et al. 1982, Escobar and Randall 2000) and turbidity measurements (Werner and Hambsch 1986) to the use of adenosine tri-phosphate (ATP) luminescence method (LeChevallier et al. 1993, Van der Wielen and Van der Kooij 2010, Van der Kooij et al. 2017), bioluminescence method (Weinrich et al. 2011), and total cell count with fluorescence staining and flow cytometry method (FCM) (Hammes and Egli 2005, Gillespie et al. 2014, Wen et al. 2016). Flow cytometry (FCM) is a rapid bacterial cell counting tool for the assessment and evaluation of bacterial water quality. Adenosine tri-phosphate (ATP) dependent luminescence analysis is also viewed as a quick method for the measurement of viable microorganisms. The previously published FCM based AOC bioassay showed to be fast, reliable and reproducible (Hammes and Egli 2005). With this approach, all bacteria in a water sample, including inactive or unculturable bacteria, can be quantified using total nucleic acid fluorescence staining of bacterial cells and FCM. The bioassay (Hammes and Egli 2005) showed that with the application of an indigenous microbial community and incubation at 30 °C the stationary phase could be reached within 30-40 h following inoculation significantly reducing the time needed for AOC measurements.

This study aimed to provide an easy and uniform bacterial growth potential assay where different sample types are inoculated with their own bacterial community and growth is measured using less tedious and timesaving techniques: FCM and/or ATP. The suitability of the bioassay was investigated by evaluating the growth potential of six different microbial communities indigenous to the water being analyzed with increasing carbon concentrations. The effect of inoculating the same water with different indigenous microbial communities on the growth potential measurement was also examined.

Measuring bacterial growth using total ATP luminescence was compared to FCM total cell counts for the different sample types for assessment of the suitability of the proposed methods under different sample types. A universal assay that is suitable to different sample type eases the implementation of the bacterial growth potential measurement, allows further understanding of bacterial growth dynamics in different sample types and facilitates comparison of results between different studies.

2. Materials and Methods

2.1. Preparation of carbon-free materials

Carbon-free bottles (Schott, Mainz, Germany) and vials (Supelco, Bellefonte, PA, USA) were prepared as described previously (Hammes and Egli 2005). In short, all glassware was soaked overnight in 0.2 N HCl and subsequently rinsed properly with deionized water. After air-drying, the bottles and vials were baked in a Muffel furnace (500 °C; 6 hours). Teflon-coated screw caps for the glassware were soaked overnight in acid (0.2 N HCl), thereafter in a 10% sodium persulfate solution (60 °C, 1 hour), thereafter rinsed three times with deionized water, and finally air-dried. Plastic filter units were autoclaved (120 °C, 1 bar, 20 minutes), and rinsed with deionized water (300 mL) before use.

2.2. Water samples

Six different water types were used for the experiments described below, namely (1) commercially available bottled mineral water (Evian, France), (2) non-chlorinated tap water (Dübendorf, Switzerland), (3) seawater (Terneuzen, The Netherlands), (4) river water (Chriesbach creek, Dübendorf, Switzerland), (5) wastewater effluent (Dübendorf, Switzerland) and (6) a soil organic carbon extract. The soil extract was prepared as follow: 10 g (dry weight) of garden soil was suspended in 100 mL filtered bottled water (Evian, France), slowly stirred for 1 hour and then filtered (0.2 µm pore size) to remove particles and bacteria. All water samples were collect in 1 L glass bottles and closed with Teflon-lined caps. The wastewater and soil extract samples were furthermore pre-filtered (3 µm pore size; Sartorium Stedim, cellulose nitrate, 47 mm ø) to minimize excessive clogging of the smaller pore-size filter during subsequent processing. All samples were characterized for total cell concentration and adenosine tri-phosphate (ATP) prior to the experiments.

2.3. Sample preparation

All water samples were filtered (0.2 µm pore size Whatman PC MB, 47 mm ø, Whatman, Ireland). All filters were pre-flushed with approximately 300 mL of deionized water to remove organic carbon contamination. Approximately 100 mL of each raw water sample was reserved before filtration for analysis and to serve as inoculum. Additionally, 2 L of filtered bottled water was pasteurized (60°C for 30 minutes) to be used as dilution medium in selected experiments. A working solution of sodium-acetate (100 mg-C.L^{-1}) was prepared with deionized water in a carbon-free bottle and closed with a carbon-free Teflon cap and later diluted to the needed concentrations. A solution of trace elements was prepared as described previously (Prest et al. 2016a).

2.4. Experiment descriptions

This growth potential assay intends to serve as a uniform protocol for measuring sample growth potential. The use of the bacteria indigenous to the sample for measuring the growth potential is the primary emphasis of the assay. The below experiments were formulated to evaluate the applicability of the assay for different sample types.

Experiment 1: batch growth curves on naturally available carbon

A filtered river water sample was aliquoted (200 mL) into three separate AOC-free Schott bottles. Each sample was subsequently inoculated with approximately 10 µL/L of an indigenous bacterial community originating from the same river water at a final concentration of approximately 2×10^4 cells/mL without any external addition of carbon. Filtration of the river water was mainly done to remove the protozoa present, and a small inoculation volume was used (≈ 10µL) so that the statistical chance of re-inoculating protozoa is low. The inoculated samples were incubated (30 °C, 35 hours) in a water bath with continuous magnetic stirring. Each sample was fitted with a sterile sampling tube connected to an automated online staining robot similar to the system described previously (Hammes et al. 2012). The online system was set up in such a manner that discreet samples were collected and processed every 15 minutes, meaning that each bottle was sampled and processed every 45 minutes. For each measurement, 200 µL of the sample was stained with 200 µL of SYBR Green I (diluted

5000 fold from the stock) and incubated at 40 °C for 10 minutes. The stained sample was subsequently measured automatically with an Accuri C6 flow cytometer (BD Biosciences) for 30 seconds at a speed of 66 µL/min with the threshold set on green fluorescence (FL1; 1500 a.u.). For additional measurement details, see Van Nevel et al. (2013). The bacteria were gated on the green/red fluorescence density plot for determination of the total cell concentration, as described previously (Hammes et al. 2012, Van Nevel et al. 2013). Growth rate throughout the incubation was calculated based on the change in cell concentration during five subsequent measurements (Berney et al. 2006). Relative cell size was measured as the intensity of forward scattered (FSC) light by each particle (Wang et al. 2009). A schematic of the growth potential measurement method is shown in figure 1.

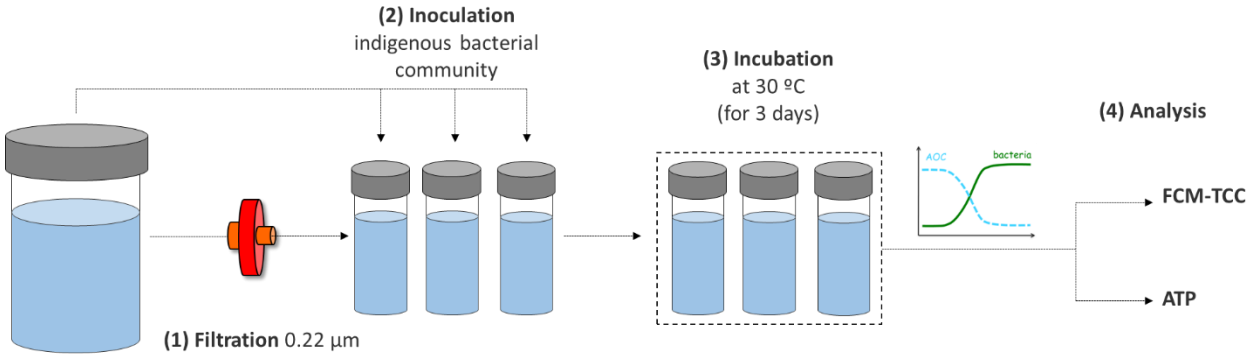

Figure 1. Schematic of the growth potential measurement method procedure.

Experiment 2: response to acetate addition

A lower range of acetate-carbon concentration (0-50 µg-C/L) was tested first. Filtered bottled water was supplemented with the trace element solution (Prest et al. 2016a) and re-inoculated with its initial community to a final concentration of approximately 1 x 10^4 cells/mL. Water samples of 20 mL were distributed in 40 mL AOC-free vials. Acetate was added at a final concentration of 10,20,30,40 and 50 µg-C/L. All samples were done in triplicates. Three vials containing 20 mL of the water were used without any other addition. All vials and bottles were then closed and incubated at 30°C for 3 days.

Subsequently, a higher range of acetate-carbon concentration (0-300 µg-C/L) was used for different water samples. Filtered bottled water, tap water, river water and seawater (all 1 L) were

supplemented with the trace element solution (Prest et al. 2016a) and re-inoculated with its initial community to a final concentration of approximately 1 x 10^4 cells/mL. Each water was then distributed into six 40 mL AOC-free vials (20 mL of sample per vial) and in three 100 mL AOC-free bottles (100 mL per bottle). Acetate was added at a final concentration of 100 µg-C/L in three vials, and 300 µg-C/L in the three bottles. The remaining three vials were used without any other addition. All vials and bottles were then closed and incubated at 30°C for 3 days.

Experiment 3: growth on dilutions of indigenous AOC

The wastewater and soil extract (1 L each) were supplemented with trace elements. The wastewater sample was then diluted with 0.2 µm filtered, pasteurized bottled water at 0%, 10% and 30% of the final wastewater sample volume into triplicate 40 mL AOC-free glass vials (20 mL per vial). The 100% samples were split into three 100 mL AOC-free glass bottles. The same procedure was applied to the soil extract sample, with dilutions at 0%, 1%, 10% and 100% of the final sample volume. All water samples were re-inoculated with their initial community to a final concentration of approximately 1 x 10^4 cells/mL. All vials and bottles were closed and incubated at 30°C for 3 days.

Experiment 4: impact of different inocula

Wastewater and bottled water were filtered as described above into a 1L AOC-free glass bottles and amended with trace elements. The bottled water was additionally amended with 300 µg-C/L acetate. Each water was then split into multiple 100 mL AOC-free glass bottles. Each triplicate set of bottles was then inoculated with a different inoculum: bottled water, tap water, river water, seawater, wastewater and soil extract (final concentration of approximately 1 x 10^4 cells/mL). All bottles were closed and incubated at 30°C for 3 days.

2.5. Measurements

The FCM total cell concentration and total ATP concentrations were measured in all samples after 3 days of incubation. All the samples prepared in 100 mL bottles (i.e. 300 µg-C/L acetate

concentration in experiment 1, 100% sample in experiment 2 and all samples in experiment 3) were further analyzed for heterotrophic plate counts and filtered for microbial community analysis.

Flow cytometric measurements (FCM)

FCM was used to determine the total cell concentration, as described previously (Van Nevel et al. 2013). In short, 2 µL aliquot of SYBR Green I (Molecular Probes, Basel, Switzerland), diluted 100 times in dimethyl sulfoxide (Fluka Chemie AG, Buchs, Switzerland), was added to 200 µL of each sample and incubated for 10 minutes at room temperature in the dark before analysis.

ATP determination

To determine ATP concentrations, 1 mL of the water sample was mixed with 10 µL $MgCl_2$ (1 M) and pre-heated at 38°C for 4 minutes. Thereafter, 750 µL of the pre-heated water sample were mixed thoroughly with 50 µL BacTiter-Glo™ reagent (Promega Corporation, Madison, WI, USA) and incubated at 38 °C for 20 seconds. Luminescence of the sample was measured in a luminometer (Glomax, Turner Biosystems, Sunnyvale, CA, USA) over a period of 10 s. The data were collected as relative light units (RLU) and converted to ATP (nM) by means of a calibration curve prepared with a known ATP standard (Promega). Details of the method can be found elsewhere (Hammes et al. 2010a, Hammes et al. 2010b)

3. Results

Batch growth of an indigenous bacterial community

River water indigenous bacterial communities followed normal batch growth kinetics when grown on naturally present organic matter without any carbon dosage. The batch growth of an indigenous river water bacterial community was determined using online FCM. The growth of the bacteria was relatively quick, with a lag phase of about 2 hours and reaching the stationary phase in about 30 hours (Figure 2A). Exponential growth was recorded in all samples from as early as 5 hours after inoculation. Figure 2B displays the bacterial growth rate in time and illustrates that most of the growth occurred during the first 15 hours and afterwards the bacterial growth rate is the lowest where approximately no growth is occurring. The maximum specific growth rate was about 0.5 h^{-1} in all replicates, recorded at c.a. 7 hours after inoculation. An increase in the relative bacterial cell size was observed during the 30 hour period with maximum relative cell size observed concurrent with the highest growth rate. However, no significant differences in cell size between the inoculum and those cells that have entered the stationary phase after all AOC was consumed were identified (Figure 2C). Reproducible results were seen from the triplicate samples.

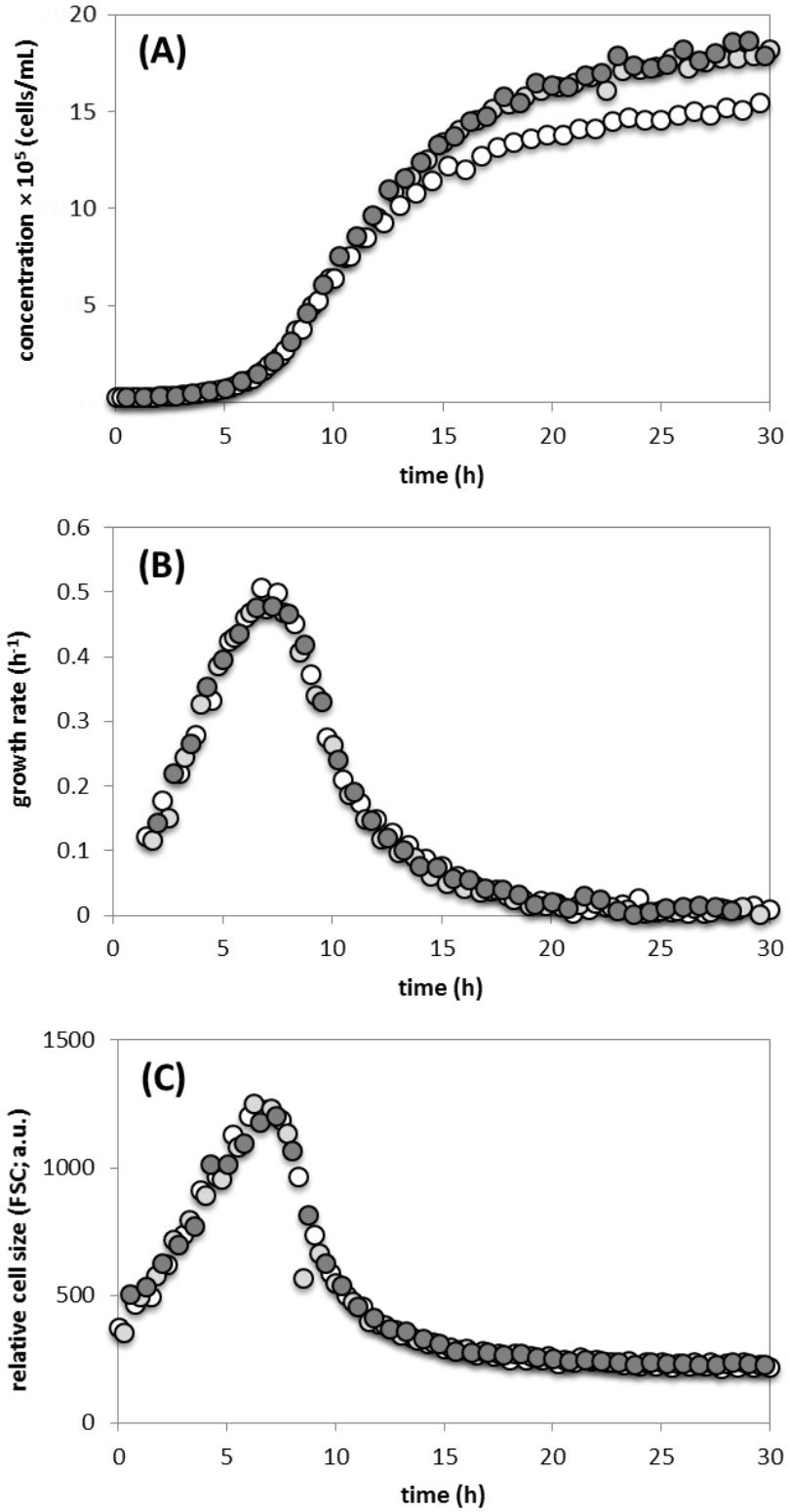

Figure 2. Batch growth (30 °C; 30h) of an indigenous bacterial community inoculum in filtered river water, measured with online FCM (measurement frequency: 45 minutes per replicate) (A) Total cell concentrations, (B) specific growth rate, and (C) relative cell size. Different colors indicate the samples from the three different bottles.

Sensitivity of the growth potential assay

Indigenous bacterial growth could detect spiked acetate-AOC concentrations as low as 10 µg/L. The sensitivity of the AOC assay was first tested using bottled water amended with a trace element solution and a lower range of acetate-carbon concentration (0-50 µg-C/L); where a linear trend (R^2=0.94) was observed between the increase in total cell concentration in response to the increase in acetate carbon concentration (Figure S1 - supplementary material). The corresponding numerical cell yield calculated from this data is 10^7 cells/µg acetate.

Bacterial growth was proportional to the acetate carbon concentration but not the same proportion factor for the different water samples tested. In addition to bottled water, the growth of indigenous bacteria in river water, seawater, and tap water at three acetate-carbon concentrations (0, 100, and 300 µg/L) was measured (Figure 3). The increase in cell concentration proportional to the increase in acetate carbon concentration was linear for the indigenous bacterial community for all the four water types. The numerical cell yield (cells/µg acetate) for the different waters were 5×10^6 for sea water and tap water, 6×10^6 for river water and 1×10^7 for bottled water. In addition to FCM quantification, the samples were measured with ATP. In all samples the ATP concentrations also exhibited a linear increase with the increase of acetate-carbon. The yield coefficient based on ATP (µg of ATP per µg Acetate carbon) for the different waters was 0.0004 for sea water and river water, 0.0006 for tap water, and 0.0010 for bottled water. The high growth potential of bottled water was intriguing and may be attributed to different bacteria dominating in bottled water, where these bacteria mainly uses the acetate to multiply. Therefore, bacterial physiology and the dominating community composition will affect the growth potential measurement. The results demonstrated that different quantification methods (FCM and ATP) can be used with the same approach. The total bacterial cell count and the ATP concentration corresponding to 0 µg/L acetate-carbon concentration were a result of the naturally present AOC present in each water type.

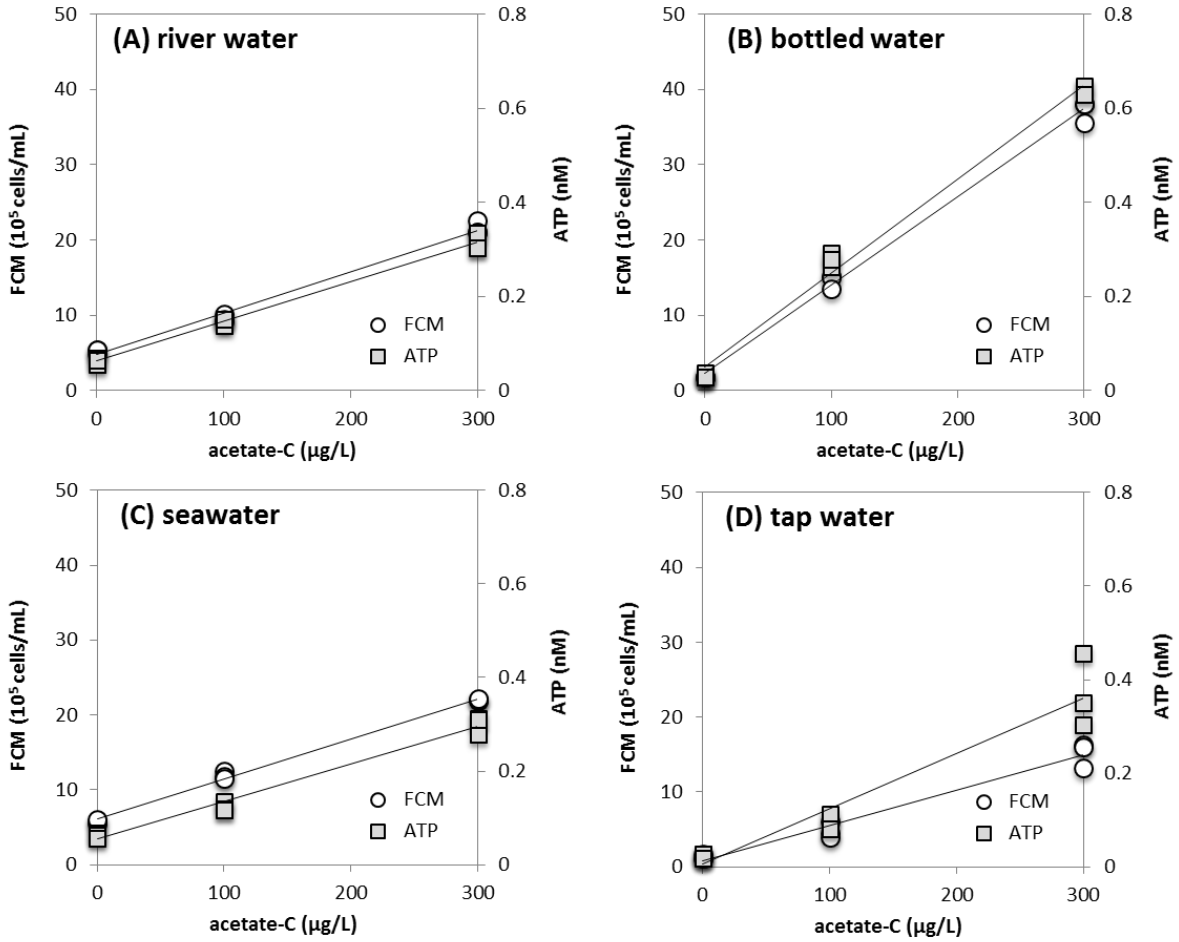

Figure 3. Growth of indigenous bacteria (30 °C; 3 days) from four different water samples spiked with either 0, 100 or 300 µg/L acetate-carbon, measured with FCM (open circles) and ATP (grey squares). (A) River water, (B) bottled mineral water, (C) seawater and (D) non-chlorinated tap water. The separate markers indicate triplicate samples for each treatment.

Bacterial growth in wastewater and soil extract samples was proportional to the naturally available carbon source present in these samples. The growth of indigenous bacteria in diluted samples of soil extract and wastewater effluent was also assessed with increasing dilutions of indigenous AOC from these samples. The ATP and the cell concentration of soil extract bacteria increased linearly with the increase in soil organic carbon extract. Similarly, the growth of indigenous bacteria in wastewater effluent showed a linear increase when the wastewater organic carbon content increased. For the soil extract and wastewater samples the proportionality of increase in bacterial growth, measured either as cell concentration or ATP concentration, was achieved by supplying the naturally present carbon source to the bacteria. The proportionality was observed irrespective of the carbon source type as the natural AOC present in these samples consists of several compounds indicating the ability of the

indigenous bacterial community to consume all the AOC present and that no limitations other than carbon occurred (Figure 4). This is important because using the mixture of naturally available carbon types will better estimate the growth potential of the bacteria present in the wastewater or soil extract.

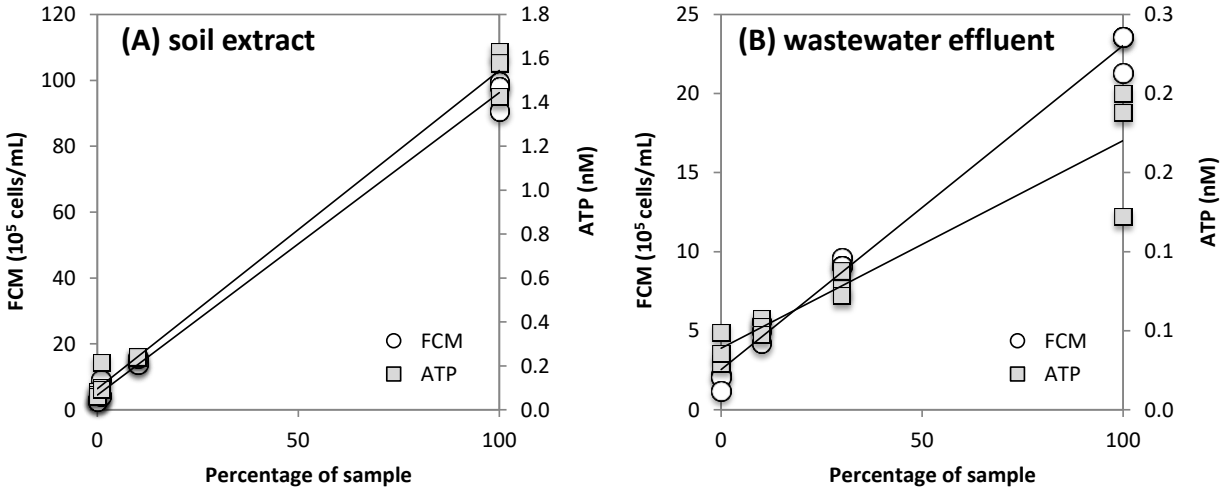

Figure 4. Growth of indigenous bacteria (30 °C; 3 days) from two different water samples that were diluted with bottled mineral water, measured with FCM (open circles) and ATP (grey squares). (A) Soil organic carbon extract (0, 1, 10 and 100%) and (B) wastewater effluent (0, 10, 30 and 100%). The separate markers indicate triplicate samples for each treatment.

When different inocula to the same sample were compared, the highest final cell concentration was detected for the bacteria indigenous to the water sample tested. The growth potential assay was used to evaluate the growth of six different indigenous bacterial inocula in two water types: wastewater with indigenous AOC and bottled water amended with 300 µg/L acetate-carbon. For the wastewater sample, bacterial inocula from wastewater, river water, and bottled water showed the highest increase in cell concentration. A similar trend was observed with ATP concentration measurements. On the contrary, only the bottled water inocula exhibited the highest increase in cell concentration in the bottled water samples amended with acetate-carbon. All the other bacterial inocula types had a lower increase in cell concentration. The seawater sample had a high cell concentration standard deviation between the triplicate samples which might be attributed to delay in growth. The ATP concentration of bottled water inocula was the highest as well when compared to the other inocula types.

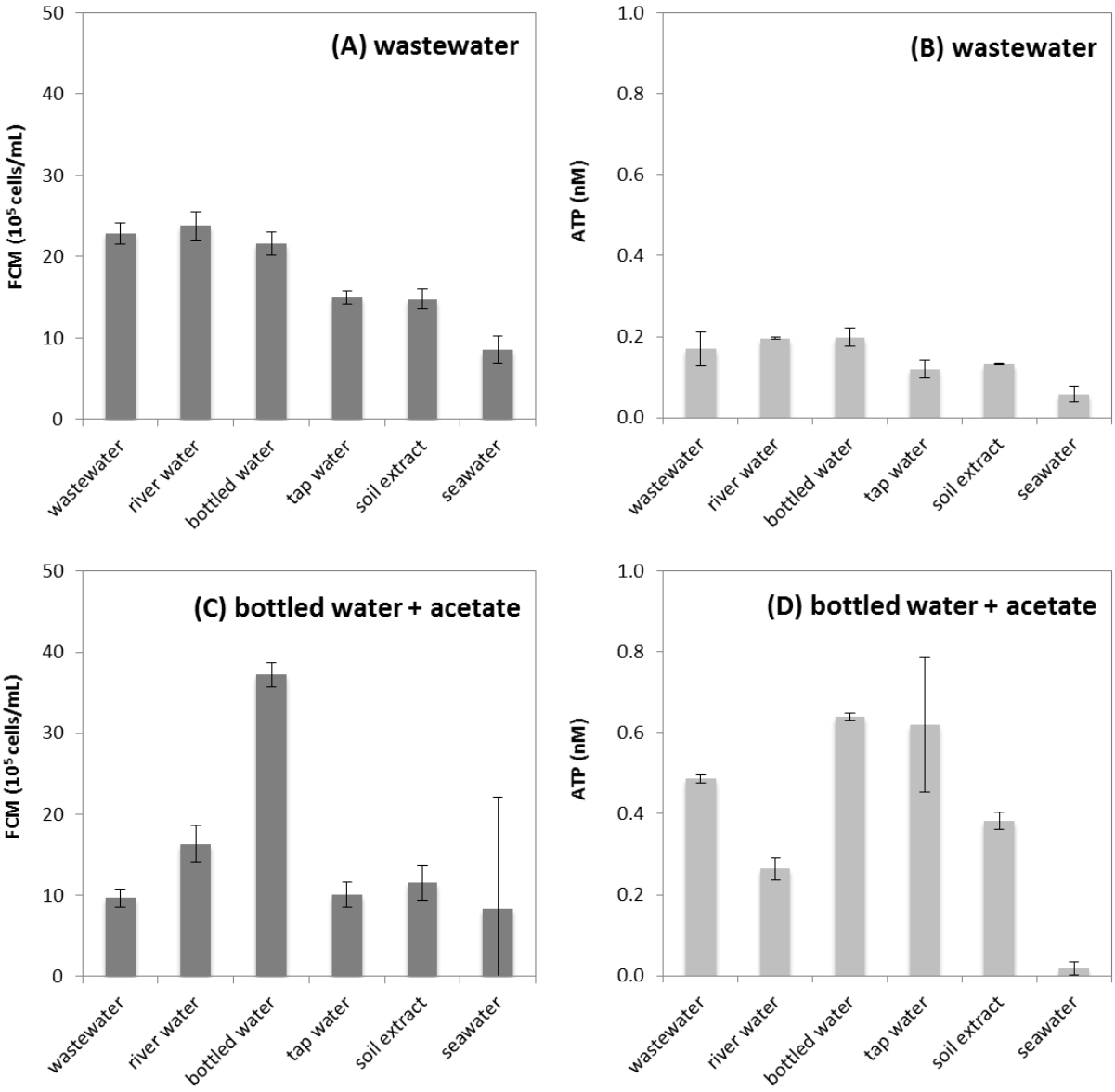

Figure 5. Growth (30 °C; 3 days) of six different indigenous bacterial inocula on two different water samples that were pre-filtered (0.22 µm), measured with FCM and ATP. **(A, B)** wastewater and **(C, D)** bottled water amended with 300 µg/L acetate-carbon. Error bars indicate standard deviation on triplicate samples.

4. Discussion

Growth potential test universal to all sample types

The uniform growth potential assay intends to provide a unified procedure that can be implemented for different sample types. The assay can measure the growth potential of different samples irrespective of the type of growth-limiting compounds. The use of indigenous bacterial community as inoculum is a focal point of the assay. Using the indigenous bacterial community present in the water tested as an inoculum demonstrated the ability to utilize a broader and diverse range of assimilable substrates compared to specific strain bacteria; thereby, offering a more realistic interpretation of the actual growth potential when naturally available substrates are assayed as the indigenous bacteria are more adapted to the types of AOC present in specific samples (Prest et al. 2016a, Long et al. 2017). The reduction of sample handling steps in this unified assay is a major advantage compared to existing growth potential assays such as AOC, BDOC (Van der Kooij 2000, Schneider et al. 2005), minimizing both labor and risk of sample contamination with external nutrients.

Monitoring the kinetics of indigenous bacterial growth enabled monitoring all the phases of the bacterial growth reaching the stationary phase with information about both cell number and relative cell size. Our results show that the tested river water indigenous bacterial communities followed normal batch growth kinetics when grown on naturally present organic matter (Figure 2). The relative bacterial cell size obtained using FCM was maximum concurrent with the highest growth rate. Nevertheless, the relative cell size of cells that have entered the stationary phase was not significantly different compared to the inoculum cell size (Figure 2). Microbial cells sizes and carbon content differ immensely between different water types/samples as well as the amount of carbon being assimilated by the cells (Linton and Stephenson 1978). Therefore, information on the size of the cells is important to interpret the growth potential results and is also essential when converting the growth on natural complex AOC to carbon equivalents. Expressing the growth potential as a measurement of growth,

either as cells/mL or pg ATP/mL, is a better approach as the conversion to AOC has inevitable limitations (Hammes and Egli 2005).

Indigenous bacterial growth was then monitored under varying ranges of acetate carbon concentration. Bottled water amended with acetate was spiked with a low range of acetate concentration (Figure S1), and the indigenous bacteria demonstrated proportionality in growth indicating that the growth was not limited by any compound other than carbon at any of the tested concentrations. The range of carbon concentration tested in figure S1 was considered low in context to the relatively high range of carbon concentrations present in the different samples tested in this study. Therefore, figure S1 shows that the growth potential assay is sensitive and accurate and can detect spiked AOC at 10 µg/L. A higher range of spiked acetate carbon was then tested to assess the suitability of the assay under a broader range relevant to the different samples (river, bottled, tap, and seawater) (Figure 3). Wastewater and soil extract were not included in the spiked acetate experiment as the growth potential in these samples was too high and therefore much higher carbon concentrations were needed before any growth could be detected. Instead, these samples were diluted to identify the minimum amount of sample where growth could be detected. Using this approach, the suitability of the assay to detect natural AOC was demonstrated (Figure 4). To sum up, all the indigenous bacterial communities in the samples tested showed a proportional increase with increasing concentrations of acetate carbon or natural organic matter dilutions. Similarly, Van der Kooij et al. (2017) found the use of indigenous bacterial community essential for the utilization of the slowly biodegradable, maintenance-supporting, compounds. Observing proportionality in bacterial growth is important as it signals to no limitations other than carbon for the range of carbon tested. When limitations other than carbon exist such as phosphate or inorganics, proportionality in growth would not be achieved. The proposed growth potential assay can be used to test for possible limitations in different samples, the order in which they are limiting, and the extent of microbial growth at which they become limiting. Adding different suspected growth limiting compounds and

measuring the bacterial growth potential using the proposed assay aids in identifying the limiting compound (Prest et al. 2016a, Nescerecka et al. 2018).

Observing linearity in the growth of indigenous bacteria in all the samples led to testing the degree to which different indigenous microbial communities would grow in the same water type. The results confirmed that the microbial community indigenous to the sample grows best in its own sample and emphasized on the variations in the growth of bacterial communities that are not indigenous to the sample when grown in the two water types tested. The bottled water bacteria showed a significantly higher growth measured as cell count when compared to all other indigenous microbial communities when grown in bottle water with acetate. Alternatively, wastewater, river water, and bottled water bacteria had a significantly high growth in wastewater samples which can mainly be attributed to the wide range of carbon sources these indigenous communities are adapted to.

Growth detection methods: FCM and ATP

FCM and ATP luminescence analysis can be implemented as rapid and more stable methods for bacterial growth potential measurement and AOC determination in water (Hammes and Egli 2005, Elhadidy et al. 2016, Li et al. 2017, Van der Kooij et al. 2017). Compared to conventional plate counting, FCM and ATP luminescence are fast, more reproducible and less vulnerable to variations in culturability of the cells. ATP analysis enables an accurate quantification of changes in the active-biomass concentration as a measure of the utilization of energy sources during incubation (Van der Kooij et al. 2017). In this study, the proportional increase in bacterial growth corresponding to the increase in organic carbon in all the samples was detected with both FCM and ATP luminescence. The linearity between ATP luminescence and the added acetate carbon concentration illustrated the applicability of monitoring bacteria by luminescence method as shown recently by Li et al. (2017), Van der Kooij et al. (2017). However, when testing the degree to which different indigenous microbial communities would grow in the same water type, the ATP did not show the same trend as the cell count. The authors attribute the discrepancy between the ATP results and the FCM results to the nature of the ATP measurement and possibility of missing the peak ATP value as recent studies

using online ATP measurements illustrated a rapid increase and collapse in ATP especially when simple carbon types like acetate are added (data not shown). The same observation has been previously seen in (Nescerecka et al. 2014). Accordingly, offline ATP measurements can possibly miss the peak in ATP value providing an inaccurate endpoint interpretation of the growth. Moreover, as seen in (Nescerecka et al. 2016), ATP luminescence remains as a cell associated parameter that can vary according to the growth stage of each cell (Nescerecka et al. 2016). As a result, compared to ATP analysis, FCM investigates specifically the numerical growth of a culture, which is the fundamental principle of the bacterial growth potential concept. Therefore, the definition of growth, in terms of cell multiplication or biomass formation, dictates what method evaluates growth better. Both methods are accurate and reliable, and when used together information such as ATP/cell gives more insights about the growth potential.

Possible modifications to the uniform assay

The main advantage from this uniform bacterial growth potential method is that the assay is straightforward, easy to adapt, universal, and can be applied to any sample type. This method enables one coherent framework for comparison of different samples and expands the application field of bacterial growth potential assays from freshwaters to seawater or any other sample type irrespective of the water characteristics (e.g. salinity, pH, available nutrients, etc.). Therefore, when used this method reduces the test time, allows further understanding of bacterial growth dynamics and establishment of complete growth curves for an indigenous microbial consortium growing on AOC facilitating comparison of results between different studies. Moreover, the method can be extended to investigate bacterial growth limitations, by additions of individual compounds, as proposed by (Prest et al. 2016a, Nescerecka et al. 2018). However, some alterations to the assay might deem necessary for some samples such as water samples after disinfection where concentration of viable bacteria can be very low. Some options for assay adjustment can be considered when disinfection is used depending on the type of disinfection applied. In case of chlorination, sodium bisulfite or nitrite can be added to remove residual chlorine and the water can be inoculated with the indigenous

445 community or the indigenous community before chlorination. Similarly, UV and ozone disinfected
446 samples can be inoculated with the indigenous bacteria before disinfection. Disinfected samples were
447 not evaluated in this study but shall be essentially addressed in future research.
448

5. Conclusions

The indigenous community bacterial growth potential assay showed consistent and reproducible bacterial growth results for all sample types tested providing clearer insights into the actual growth potential of different water types. The bioassay using FCM and ATP is fast, easy, and reliable therefore allowing the routine implementation of the growth potential measurement and providing a unified framework for different samples. The results showed:

- Indigenous bacterial growth could detect low spiked AOC concentrations.
- Proportional growth in indigenous bacterial community with increase in carbon concentration could be measured with both FCM and ATP.
- Bacteria indigenous to the sample grows best in its own sample.

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