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A report on a bio-assay to estimate the growth potential of pathogenic bacteria in drinking water

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Colophon

Title
A report on a bio-assay to estimate the pathogen
growth potential

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Summary

A bio-assay estimating the pathogen growth potential (PGP) in a given water sample is presented in this report. The principle is based on the Eawag AOC assay presented earlier in the Techneau project (Deliverable 3. 3. 1.) and as an enhancement specially focuses on the growth potential of pathogenic bacteria. At the beginning of the procedure the water sample of interest is first sterilized by a sequential pasteurization / filtration procedure. Then, three different pathogenic bacteria - *Escherichia coli* O157, *Vibrio cholerae* and *Pseudomonas aeruginosa* are separately inoculated in a definite cell count to the sterile water and finally grown into stationary phase. After that the final cell concentrations of the different pathogenic bacteria are determined using nucleic acid staining in combination with flow cytometry. The obtained cell counts yield information on the potential growth of an individual pathogen in a given sample. Finally, the data is evaluated together with AOC (assimilable organic carbon) and DOC (dissolved organic carbon) concentrations for each sample.

For the assay development and internal validation, several pre-experiments were performed with individual pathogenic bacteria in different water samples. After that, the assay was applied as a first validation on drinking water samples in several steps of a drinking water treatment pilot plant in Zürich (Switzerland). The obtained results indicate that AOC concentration as sole parameter is insufficient to describe the growth potential of pathogenic bacteria in water samples. Therefore, the individual growth potential of each pathogen needs to be monitored as a single parameter to investigate if a certain water sample contains the nutrients to promote growth of this bacterium. During the analysis of the water samples from the Zürich pilot plan clear differences between the growth potential of the three pathogens (*Escherichia coli* O157, *Vibrio cholerae* and *Pseudomonas aeruginosa)* and at the different treatment steps could be detected. Furthermore, it was observed that the growth potential of each pathogen distinctly changes during treatment. In comparison to the raw water entering the plant with high pathogen growth potential, the finished water after treatment had a very low pathogen growth potential. For further validation the PGP assay will be applied on different other full scale drinking water treatment plants from several Techneau collaborators in order to get more comparative data.

The presented bio-assay is a further step to estimate the risk of potential pathogen growth during drinking water treatment and distribution. It should advance the risk assessment associated with growth of pathogenic bacteria in drinking water to limit and prevent waterborne diseases.

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1 Introduction

1.1 Background

Waterborne infections due to the consumption of contaminated drinking water are estimated to cause around one billion cases of disease and account for more than three million deaths worldwide each year (OECD, 2003). The majority of these occur in developing countries, but both the health and economic effects associated with waterborne disease are also considerable for industrialised societies (Payment, 1997). Most studies focus on the survival and die-off of pathogenic bacteria in the environment (Morita, 1997; Rozen & Belkin). As a result, the common view established that pathogens deriving from faecal sources, mostly members of the Enterobacteriaceae such as *Escherichia coli, Salmonella* spp. *or Campylobacter* spp., are not able to grow at low nutrient concentrations as present in drinking water. A new understanding about bacterial growth at low nutrient concentration based on the Eawag AOC method (presented in Deliverable 3. 3. 1.), together with new analytical technologies as flow cytometry, enabled us to illustrate that members of these pathogenic bacteria are in fact able to multiply under conditions characterized by low nutrient concentrations (Vital *et al.,* 2007; 2008; Deliverable 3. 3. 9.). Furthermore, *in situ* as well as laboratory studies from other collaborators of the Techneau framework indicate that *E. coli* is in fact able to grow in drinking water under certain circumstances (Juhna *et al.,* 2007; Deliverable 3. 6. 8. 1.).

Among pathogens from faecal sources, there are so-called "environmental (opportunistic) pathogens" like *Pseudomonas aeruginosa, Aeromonas hydrophila* or *Legionella pneumophila,* for whom environmental reservoirs could be detected (Steinert *et al.,* 2002; Torvinen *et al.,* 2004). It is commonly accepted that these pathogens can multiply in drinking water under low nutrient concentrations (Szewzyk *et al.,* 2000). To limit and prevent waterborne infections, information on the growth of pathogenic bacteria in drinking water is therefore essential for both, "pathogens from faecal sources" as well as for "environmental pathogens".

Assimilable organic carbon (AOC) is a collective term describing the fraction of labile dissolved organic carbon (DOC) that is readily assimilated by microorganisms (Hammes & Egli, 2005). Measuring of AOC provides information on the general microbial growth potential of a certain drinking water. It is regarded as a critical parameter for drinking water treatment and distribution processes. AOC consits of many different organic carbon molecules such as sugars, organic acids and amino acids (Meylan *et al.,* 2007; Hammes *et al.,* 2007; Hammes *et al.,* 2006). Furthermore, AOC was directly linked to microbial regrowth and biofilm formation in drinking water distribution systems (Escobar *et al.,* 2001; Van der Kooij, 1992; Van der Kooij, 2002; Hammes *et al.,* 2008). Altough AOC was also shown to correlate with the presence or even growth of pathogenic bacteria in drinking water

(LeChevallier *et al.,* 1996; Torvinen *et al.,* 2004) the parameter is often not an adequate value to estimate the potential risk regarding growth of pathogenic bacteria in a system as it does not consider the actual composition of organic carbon molecules present. Unfortunately, it is not possible to decode the bulk DOC into individual substrates in order to gain information on the presence of certain substrates available for individual microorganisms. The parameter AOC is thus an indirect method determined by growing either a mixture of many different bacterial species (Hammes & Egli, 2005) or one specific bacterial species (Van der Kooij *et al.,* 1982) in a water sample. The final growth rate is than correlated with to the original amount of substrates available for the growth of microorganisms. Since every bacterial species harbours a certain specific enzyme pattern in order to take up and metabolize nutrients, the ability of a bacterium to grow in a certain water type is strongly determined by the organic carbon composition. Whether the substrates, which are potentially available for the bacteria used for the AOC determination are also available for pathogenic bacteria has to be investigated. We therefore developed an bio-assay, based on the principle of the Eawag AOC assay, in order to gain more information wether the present AOC quality enables proliferation of several different pathogenic bacteria. The assay should advance the risk assessment for drinking water in order to prevent the spreading of disease associated with pathogenic bacteria.

1.2 Goal of this work

This report provides the technical aspects of the PGP bio-assay and some examples of data collected during a first validation test on water samples from a drinking water treatment pilot plant. For more information see also D. 3. 3. 9, D. 3. 3. 1 and Vital *et al.,* 2007; 2008.

2 The Method

2.1 General overview

The method consists of **1.)** sterilizing a water sample, **2.)** making aliquots, **3.)** separately inoculating the aliquots with different pathogenic bacteria **4.)** incubating at 30 °C until the bacteria have reached stationary phase, **5.)** measuring the final growth using flow cytometry and finally **6.)** analyzing the obtained data. A schematic overview is given in Figure 1 . The three pathogens *Escherichia coli* O157, *Vibrio cholerae* O1 and *Pseudomonas aeruginosa* are used. In addition, to get information on the general bacterial growth potential, every water sample is also analyzed with the Eawag-AOC method presented in Deliverable 3. 3. 1.

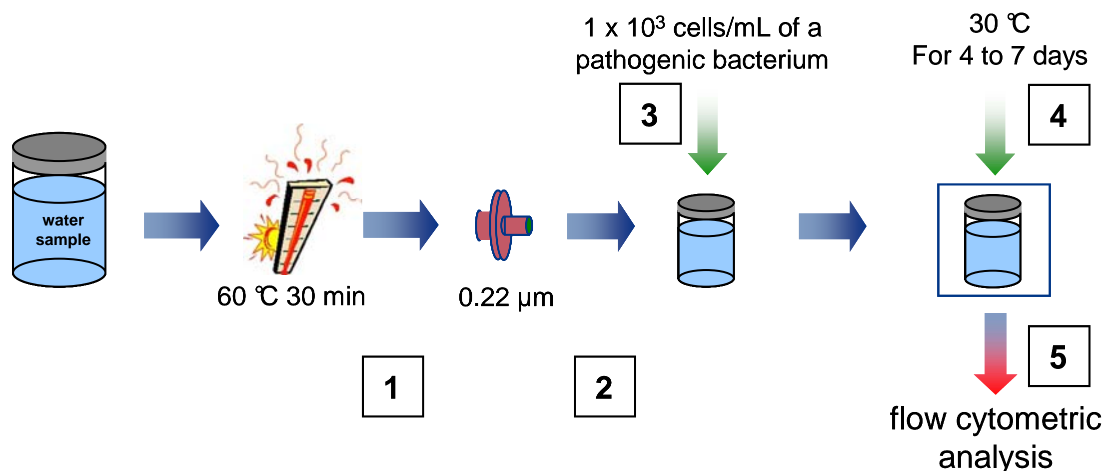

Figure 1. *Schematic presentation of the pathogen growth potential method.*

2.2 Step by step

1.) Sterilization of the water sample

500 mL of a given sample is first pasteurized (60 °C for 30 min), cooled down to room temperature and then filtered using a 0.22 µm syringe filter.

2.) Aliquotation

For each pathogen, 3 sub-samples of the sterilized water (à 15 mL aliquots) are transferred into 20 mL AOC-free and sterile glass vials.

3.) Pre-cultivation of the bacteria and inoculation

For preparing the initial inoculum solution, a tip of an agar plate colony of each pathogen is transferred individually into ten times

diluted LB medium and incubated overnight. Then, the grown bacteria are transferred into AOC free and sterile glass vials containing 10.000 times diluted LB medium (sterile AOC free mineral water is used as the diluter; AOC of the solution ~ 400 µg L^{-1}) at an initial concentration of 5 x 10^3 cells mL^{-1}. Then the solutions are incubated for four days at 30 °C (late stationary phase). The grown cells are then used as an inoculum for the actual PGP assay. Every pathogen is monitored in triplicate samples. Therefore, three vials for each water sample and each bacterium are then inoculated at an initial concentration of 1 x 10^3 cells mL^{-1}. In addition, three vials containing AOC free sterile mineral water are inoculated as a negative control.

4.) Incubation

The solutions are incubated at 30 °C for minimum four up to seven days till the bacteria reached stationary phase.

5.) Measurement by flow cytometry

1 mL of each sample is stained with 10 µl of SYBR Green I (100x diluted in DMSO) together with EDTA (0.5 mM final concentration), incubated for 15 minutes in the dark and then analyzed by flow cytometry as described in Hammes *et al* (2008). For *P. aeruginosa* the vials are put for 10 minutes into a sonication bath in order to release bacteria which might have attached to the vial into the suspension.

6.) Analysis

The data is displayed as presented below. Next to the final cell concentration of the individual pathogens also the number of doublings is calculated.

2.3 Apparatus needed

a. *Sampling bottles*: organic-carbon-free glass bottles (500 mL) with glass stoppers (preferably) or with TFE-lined silicone screw caps (see later for preparation).

b. *Incubation vials*: organic-carbon-free glass vials (20 mL) with TFE-lined silicone septa (see later for preparation).

c. *water bath:* in order to pasteurize the samples.

d. *sterile bench:* in order to process the samples in a sterile way.

e. *Syringe and syringe filter*: a pre-rinsed 50 mL plastic syringe and a pre-rinsed 0.22 µm pore-size PES syringe filter (see later for pre-rinsing instructions).

f. *Incubator:* for incubation at 30 °C

g. *sonication bath:* in order to release *P. aeruginosa* cells which might have attached to the vial during incubation into the suspension.

h. *Flow cytometer:* equipped with a 488 nm blue laser with a minimum power of 15 mW; also a 520 nm bandpass filter for green fluorescence detection and a 610 nm bandpass or longpass filter for red fluorescence detection. Volumetric counting hardware is inclusive to some instruments and preferable, though this can be

compensated for by the use of commercially available bead count standards.

i. *S2 laboratory*: Note that for working with pathogenic bacteria used in this study a special laboratory (biosafty level 2) is needed.

2.4 Reagents

a. *Bottled mineral water*: Commercially available natural mineral water containing no carbon dioxide and no chemical treatment

b. *Sodium persulphate solution*: Dissolve 100 g $Na_2S_2O_8$ in 1 L deionised water (according to Greenberg et al., 1993).

c. *Fluorochrome*: SYBR® Green I, diluted 1:100 from original stock solution in DMSO, and stored at -20 °C until use

d. *EDTA:* stock solution of 500 mM (pH = 7.2) (see Berney et al., 2007).

e. *Inocula*: Individual pathogens as described above.

f. *LB medium:* 10 g L^{-1} Tryptone, 5 g L^{-1} Yeast extract and 10 g L^{-1} NaCl

2.5 Additional information

a. *General care and handling*: For all AOC work, use gloves, but do not use sterilization solution (e.g. ethanol or isopropanol) in order to not contaminate the samples with organic carbon. Use AOC-free glassware for all steps handling the samples. After pasteurization all following steps have to be performed under a sterile bench in order to prevent contamination. For inoculation, incubation and final analysis by flow cytometry the use of a S2 laboratory is mandatory. Ideally, use a laboratory which is free of volatile organic carbon compounds (e.g. agar, liquid medium, ethanol, etc…).

b. *Preparation of glassware and caps*: All glassware are first washed with a common detergent, and thereafter rinsed with deionised water. These were then submerged overnight in 0.1 N HCl, and thereafter again rinsed with deionised water and air-dried. All glassware are then capped with aluminium foil and heated in a Muffel furnace at 500 °C for 5 h. Teflon-coated caps for the glassware were washed and rinsed in the same manner, and thereafter soaked in a 10 % sodium persulphate solution at 60 °C for at least 1 h, rinsed twice with deionised water and finally air-dried (Greenberg *et al.*, 1993; Hammes and Egli, 2005). For sterilization the vials were caped and heat-treated for 2 h at 90 °C.

c. *Preparation of filters and syringes*: The plastic syringes are washed 3 times with deionised water. The syringe filters are pre-rinsed with 200 mL of deionised AOC-free water.

d. *Collection and preparation of water sample*: Water is sampled in AOC-free glassware, transported under cooled conditions to the laboratory, and pasteurized within 2-6 hours after sampling. For all following steps see above.

e. *Counting with flow cytometry*: For the establishment of complete growth curves, incubated vials can be sampled at regular intervals (2 – 3 h) (Hammes and Egli, 2005; Vital et al., 2007, 2008; deliverable 3.3.9.), or only after four to seven days for end-point measurements.

f. *Ozone or chlorine quenching*: When treatment processes such as ozonation or chlorination are studied, or when residual chlorine is suspected in the water, it is required to quench the disinfectant before starting with the PGP assay. This is done using nitrite (double molar to oxidant).

2.6 Calculation

a. The negative control for each individual pathogen, which measures carbon contamination during the processing of the assay, is subtracted from all corresponding data points.

b. *Conversion of final cell concentration to number of doublings*:

$$N°_{doublings} = (\log(\text{final cell concentration})/1000) / \log 2 \qquad \text{(Eq. 1)}$$

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3 Results

3.1 Pre-experiments on LB medium and riverwater.

In order to monitor the growth of different pathogens (*E. coli* O157, *V. cholerae and P. aeruginosa*) at low nutrient concentrations, several pre-experiments were performed. Diluted LB medium and undiluted river water were inoculated individually with each pathogen and analyzed for the resulting growth.

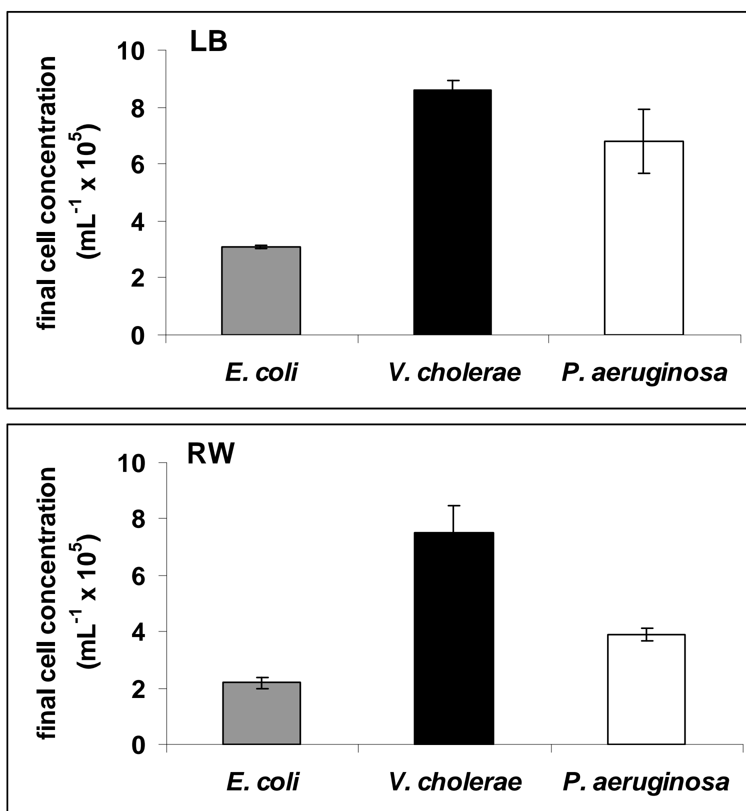

Figure 2. *Growth of the individual pathogens in diluted LB medium containing 350 µg AOC L^{-1} (LB) and river water characterized by 660 µg AOC L^{-1} (RW). Error bars indicate the standard deviation on triplicate samples.*

The general growth pattern/behaviour between the different bacteria is nearly the same in both, diluted LB medium and river water. *V. cholerae* showed the highest growth, followed by *P. aeruginosa*, whereas *E. coli* O157 formed the lowest amount of cells. The degree of difference in the final cell counts is however varying between the three bacteria. Although, the diluted LB medium contained only half of the AOC concentration compared to the river water, the pathogens grew to higher final cell concentrations then in river water, suggesting that the diluted LB medium has a bigger potential for pathogenic growth. Furthermore the difference between *P. aeruginosa* and *V.*

cholerae is varying. In river water *V. cholerae* formed clearly more cells than *P. aeruginosa*, whereas in diluted LB medium the final cell concentration between the two bacterial species are more similar.

In order to test the growth of pathogens also under conditions charcterized by low AOC concentrations as often found in drinking water, the LB medium and river water were diluted to different concentrations (till AOC around 10 µg L^{-1}). The resulting growth of all three pathogens is presented in Figure 3.

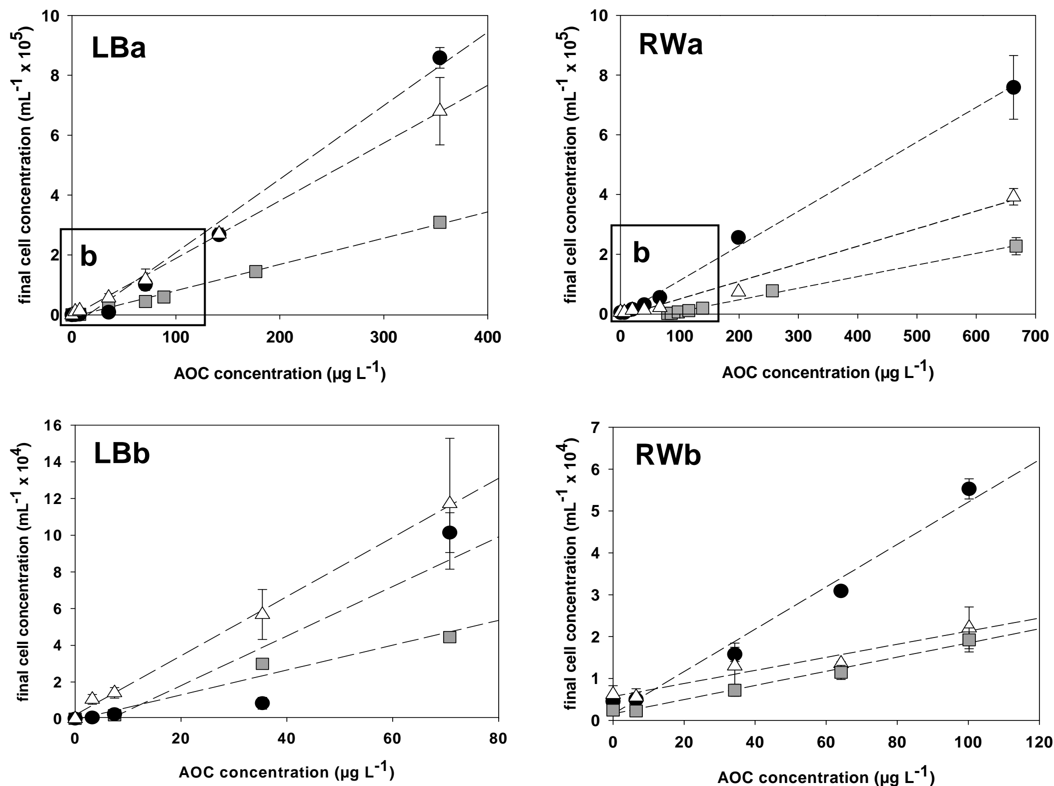

Figure 3. *Growth of the individual pathogens in diluted LB medium (LB a,b) and river water (RW a, b). E. coli O157 (squares), V. cholerae (dots) and P. aeruginosa (triangles). Error bars indicate the standard deviation on triplicate samples. This figure is adapted from Vital et al., (in preparation).*

A correlation between the final growth of the individual pathogens and the available AOC concentration in the sample (harbouring the same composition of organic carbon substrates) could be established. The pathogens grew in all dilutions, also those characterized by a little amount of AOC (10 - 50 µg L^{-1}).

Additional information on the growth kinetics of *E. coli* O157 and *V. cholerae* at low nutrient concentrations can be found in deliverable 3. 3. 9.

3.2 Application of the assay in a drinking water treatment pilot plant.

After the presented pre-tests, water from a drinking water pilot plant was taken at several treatment steps and analyzed with the pathogen growth potential bio-assay (PGP assay). A schematic representation of the pilot plant is given in Figure 4. For more information on the pilot plant see Hammes *et al.,* 2008.

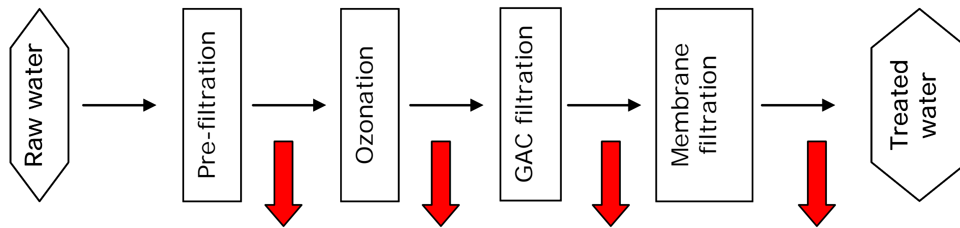

Figure 4. *Schematic representation of the pilot plant. The red arrows indicate the locations, where samples were taken for analysis.*

The obtained results are presented in Figure 5.

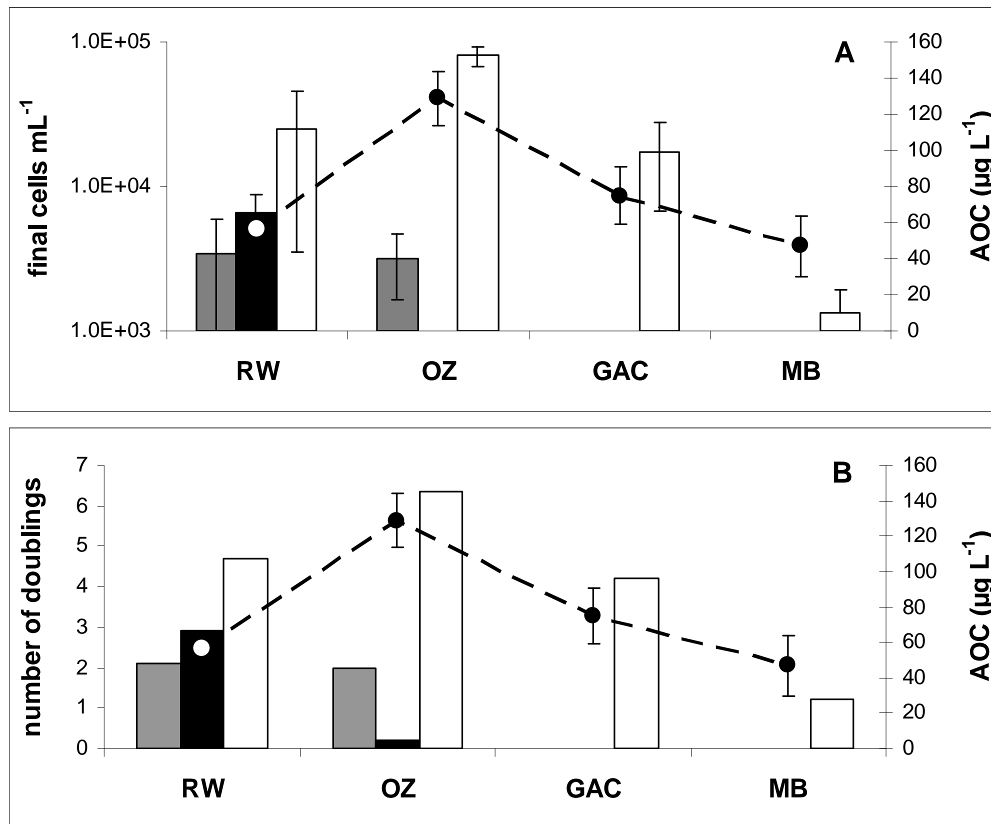

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Figure 5. *Application of the PGP assay on samples from a drinking water treatment pilot plant. Three different pathogens (E. coli O157 (grey bars), V. cholerae (black bars) and P. aeruginosa (white bars)) were grown in different water samples. Picture A displays the result as final cell concentration, whereas in picture B the number of doublings of the individual pathogens have been calculated. The AOC concentration is illustrated as black dots. RW - raw water; OZ - ozonation; GAC - granluar active carbon filtration; MB - membrane filtration. Error bars indicate the standard deviation on triplicate samples.*

The overall pathogenic growth potential is changing during the treatment process at the different treatment steps. The finished water, however, seems to have very little potential for the growth of pathogens; only *P. aeruginosa* proliferated (to a very little extend).

The differences between the tested pathogens in the individual water samples can be clearly seen. *P. aeruginosa* grew in all samples and also formed in direct comparison to the other pathogens the highest final cell concentrations. Whereas, *E. coli* only multiplied in the raw water and after ozonation but not after activated carbon filtration or in the finished drinking water. *V. cholerae* only grew in the raw water. Furthermore, the AOC concentration is only correlating with the growth of *P. aeruginosa* but not with the growth of the other two pathogens. This can be most clearly demonstrated by comparing the raw water sample with the sample taken after ozonation. During ozonation a breakdown of DOC yield an increase in AOC (Hammes *et al.,* 2008) meaning that the natural bacteria (flora isolated from drinking water) using to determine the AOC concentration grow better after the ozonation treatment. This is also the case in this result. *P. aeruginosa* followed this pattern, whereas for *E. coli* O157 the final growth in the two water samples (RW, OZ) is identical. Interestingly, *V. cholerae* was behaving completely different and did not grow (or only very little) after ozonation. The results suggest that next to the amount also the composition of the AOC is a key factor promoting growth of the used pathogenic bacteria in the analyzed samples.

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4 Discussion

The presented PGP assay is a first try to estimate the risk of a certain pathogen to grow in a given water sample. The assay was developed as enhancement to the existing AOC assay and aims to contribute to the understanding of the nutrient fluxes and composition during drinking water treatment and distribution with special focus on substrates available for pathogenic bacteria. Clearly the assay only yields estimations on potential growth and can not give a definite answer weather the conditions in the analyzed water system can promote the growth of pathogens or not. Many other factors like competition for nutrients, grazing or viral lysis play major roles in the growth of pathogenic bacteria. Furthermore, abiotic factors such as oxygen availability, temperature or pH are critical aspects controlling bacterial growth, too. With the presented results we could however show that 1.) pathogens are able to grow with nutrient concentrations present during drinking water treatment process, also in the relatively nutrient limited treatment system of Zürich (Switzerland). Furthermore 2.) that there is a need to test different pathogens individually as AOC concentration as a sole parameter seems often not to correlate with the growth of pathogenic bacteria. We would like to mention that the presented assay can in theory be adapted to any pathogenic bacteria, which is interesting for the user. However, certain pre-experiments as presented above should be performed previously in order to ensure a reliable result.

As a next step, the assay will be applied via 3. 3. 6. (application of methods) in several different drinking water treatment systems of other Techneau collaborators. The results should give a broader overview on the growth potential of individual pathogens in different water treatment systems all over Europe.

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