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A Report on the Growth of Pathogenic Bacteria on Natural Assimilable Organic Carbon

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Colophon

Title
A Report on the Growth of Pathogenic Bacteria on
Natural Assimilable Organic Carbon

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Summary

In contrast to the common view we could clearly demonstrate the growth of three different *E. coli* strains (a pathogenic, an environmental isolate and a lab strain) and *V. cholerae* O1 in natural freshwater on natural assimilable organic carbon (AOC). A new approach on the batch growth of pure cultures in sterile freshwater is given, which overcomes several shortcomings connected to previous studies. Different types of freshwater were sterilized using a combined pasteurization-filtration step. Initial bacterial concentrations were low (1 - 5 x 10^3 mL^{-1}) and enumeration was achieved by a culture independent flow cytometric based method. Furthermore ATP as a sum parameter was used to follow bacterial proliferation. The growth curves obtained were similar to those of bacteria growing on synthetic complex media at high nutrient concentrations. After an initial lag phase, an exponential growth phase was recorded with a continuously decreasing specific growth rate towards the stationary phase. Final cell concentrations were between 0.82 - 4.07 x 10^5 cells mL^{-1} for *E. coli* and of 0.29 - 1.55 x 10^6 cells mL^{-1} for *V. cholerae*. Furthermore, a correlation between the AOC concentration and the final cell numbers (R^2 of 0.85 for *E. coli* and R^2 of 0.36 for *V. cholerae*) could be established for both pathogens.

The presented results give new insights into the behaviour of enteric pathogens in natural freshwater. In the future it is important to get more information on different factors governing growth of these bacteria on natural AOC at low concentrations and to include the parameter growth into microbial risk assessment in order to get more reliable estimations.

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1 Introduction

Poor water quality and lack of sanitation and hygiene account for some 1.7 million deaths per year, and mostly children are affected. 99,8% of these occur in developing countries, where several factors like malnutrition, poor hygiene/sanitation and immunodeficiencies come together with unsafe water consumption (Ashbolt, 2004). Nevertheless, waterborne diseases are not restricted to developing countries. Morris and Levine (1995) estimated the annual waterborne disease burden in the US and indicated, that 560.000 people per year may suffer from a moderate to severe waterborne infection and that 7.1 million suffer from a mild to moderate one (OECD, 2003). Thus, both the health and economic effects are considerable even for an industrialised society (Payment, 1997).

It is therefore of great interest to study the behaviour of enteric bacterial pathogens in the environment in order to control and prevent the spreading of disease associated with them. Different natural microbial habitats differ greatly in parameters controlling microbial growth like nutrient concentrations, pH, salinity, temperature and biotic factors like viral lysis or grazing. Based on the substrate concentrations, available for the bacterial metabolism, the environment is divided into two major parts: oligotrophic and copiotrophic habitats. Oligotrophic environments are characterised by low substrate concentrations and are hence including the majority of aquatic systems, whereas other habitats, like the animal intestine are normally rich in nutrient concentrations and therefore defined as copiotrophic systems (Koch, 2001). Enteric pathogens can be found in both of these different environments, whereas the ability to multiply in these environments differs between species. A lot of the "classical enteric pathogens" (e.g pathogenic *E. coli* strains or *Salmonella spp*) are thought to be strictly copiotroph hence are not able to divide in environments where low nutrient concentrations prevail. Others like *Vibrio cholerae* or *Pseudomonas aeruginosa* have the ability to proliferate at low nutrient concentrations (Legnani *et al.,* 1999; Worden *et al.,*

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2006), but the factors governing multiplications in these environments are still poorly understood.

Looking at *E. coli,* it is the common view that the bacterium can only grow in its primary habitat the colon - and, once disposed into water, the bacterium enters a starvation state followed by die-off due to multiple environmental stresses (reviewed by Morita (1997) and Rozen & Belkin (2001)). This view, already established over 100 years ago (Schardinger, 1892) and was continuously supported by laboratory experiments throughout the time (Banning *et al.*, 2002; Bogosian *et al.*, 1996; Carlucci & Pramer, 1960; De Wet *et al.*, 1995; Gauthier *et al.*, 1987; Lim & Flint, 1989; Munro *et al.*, 1987; Perez-Rosas & Hazen, 1989; Ravva & Korn, 2007; Scheuerman, 1988). These observations resulted in the implementation of *E. coli* as an index parameter for faecal pollution for the monitoring of drinking water quality (WHO, 1996).

In contrast to *E. coli,* the general opinion on the growth of *Vibrio cholerae* is that the bacterium is able to adapt to both copiotrophic and oligotrophic environments. In water it can be found in both a free living state as well as attached to zooplankters and algaes (Lipp *et al.,* 2002). Previous studies have indicated the influence of salinity suggesting that the bacterium can only grow in waters characterized by moderate salinity (Miller et al., 1982; Singleton et al., 1982a; Singleton et al., 1982b). Indeed *V. cholerae* is often detected in these environments (Binsztein *et al.*, 2004; Louis *et al.*, 2003). Nevertheless the pathogen is also found in freshwater systems even in the absence of faecal index organisms indicating that *V. cholerae* can also grow in freshwater (Bourke et al., 1986; Jesudason et al., 2000).

Using new flow cytometric based methods for the enumeration of total bacterial cells (Hammes et al., 2008) and for the measurements of assimilable organic carbon (AOC) concentrations (Hammes & Egli, 2005) allowed us to get new insights into the growth of enteric pathogens on AOC at low concentrations. We could clearly demonstrate that *E. coli* O157 as well as *V. cholerae* O1 can grow in microcosms containing sterile freshwater (Vital *et al.*,

2007; Vital *et al.,* 2008). Furthermore, the final growth was correlated with the AOC concentration present in a sample for both pathogens. The results contribute to a better understanding on the behaviour of these pathogens in the environment and are crucial for risk assessment and the prevention of disease.

Goal of this study:

The goal of this study was to investigate the possibility of growth of *V. cholerae* O1 and *E. coli* O157 on natural AOC in pure culture using nucleic acid staining in combination with flow cytometry for bacterial enumeration.

Selected data from this study was published in:

Vital, M., Fuchslin, H. P., Hammes, F. & Egli, T. (2007). Growth of *Vibrio cholerae* O1 Ogawa Eltor in freshwater. *Microbiol* 153, 1993-2001.

Vital, M., Hammes, F. & Egli, T. (2008). *E. coli* O157 can grow in natural freshwater at low carbon concentrations. *Env Microbiol* (ahead of print).

2 The Approach to study bacterial growth in pure culture

Freshwater systems are typically characterized by low cell concentrations (total cell counts between $1x10^4$ - $5x10^6$ cells mL^{-1}; LeChevallier *et al.,* 1991) as well as low nutrient concentrations (10 - 800 μg AOC L^{-1}; Sanders *et al.,* 1992; Vital et al., 2007). Thus to study growth of bacteria in pure culture using microcosms, three basic points apply: (1) inoculum concentrations should be considerably lower than the carrying capacity of the water, (2) the sampled water has to be sterilized in order to remove all autochthonous bacteria, and (3) the experiment should be conducted in an AOC-free environment. Unfortunately, many of the above mentioned studies used very high initial bacterial concentrations (> 1 x 10^6 cells mL^{-1}), which resulted in a die-off of the bacteria, since nutrient concentrations in the water samples could not support growth of such high cell numbers. Furthermore microfiltration has often been used in order to sterilize sampled freshwater. However, it was recently shown that a big part of the autochthonous bacterial freshwater community can pass these filters and subsequently grow up using the available nutrients (Wang *et al.,* 2007). Hence, an unplanned competition experiment might be initiated in this way. In addition, microbiologists have found that some bacteria can enter a physiological state called viable-but-not-culturable (VBNC) meaning that bacteria are viable but do not form a colony on an agar plate. Such a state has been found for several pathogens inoculated into freshwater (Bjerbaek *et al.,* 2005; Chaiyanan *et al.,* 2001). Conventional cultivation-based detection methods are thus not always suitable to study growth of enteric pathogens on natural AOC.

In order to overcome the mentioned shortcomings (proper sterilization of the freshwater, low inoculum concentrations and detection of all bacterial cells) we used the following approach to study batch-growth of enteric pathogens on natural AOC (figure 1).

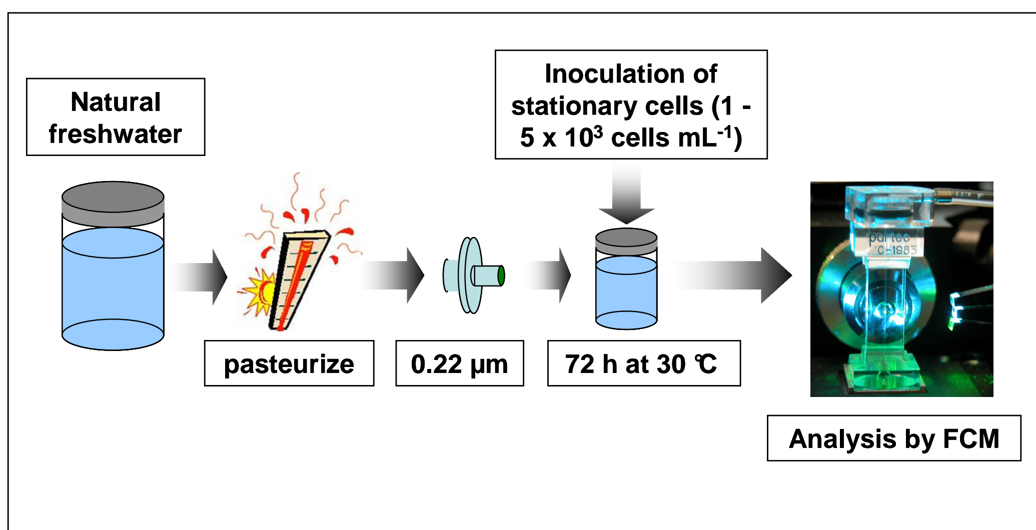

Figure 1. *Approach to study batch-growth of enteric pathogens on natural AOC. The three main points are 1.) Proper sterilization (combined pasteurization-filtration step) 2.) Low inoculum concentrations (1 - 5 x 10^3 cells mL^{-1}) and 3.) Enumeration of bacterial cells using SYBR Green I staining (or specific antibody staining) in combination with flow cytometry.*

Figure 2 shows the flow cytometric dot blots for the detection of pure cultures (*E. coli* O157 and *V. cholerae* O1) growing in freshwater using SYBR Green staining in combination with flow cytometry. This is a very accurate and sensitive method to count bacteria in a sample at low concentrations (Hammes *et al.*, 2008). The fluorescence signals resulting from stained bacteria are clearly separated from the background.

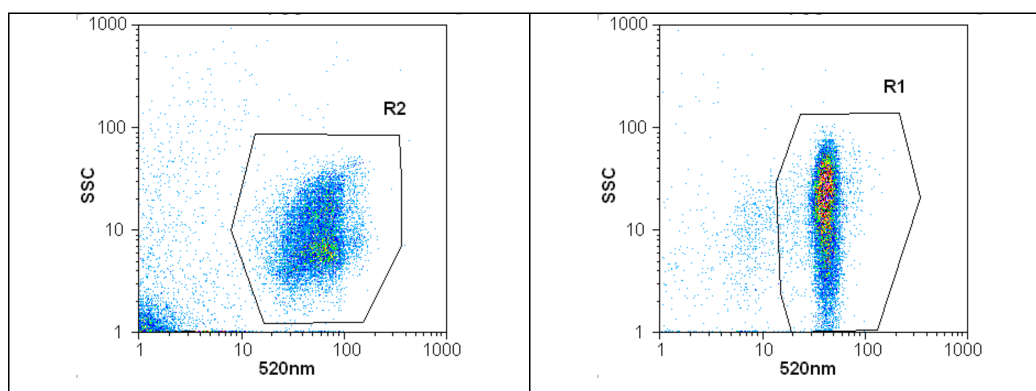

Figure 2. *Detection of E. coli O157 (A) and V. cholerae O1 (B) during growth in sterile freshwater using SYBR Green staining in combination with Flow Cytometry. SSC = sideward scatter, 520 = Green fluorescence intensity at 520 nm.*

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3 Materials & Methods

Strains and pre-cultivation

The verotoxin-negative *E. coli* O157 (N2540-04; provided by the reference laboratory for coliforms (NENT, Luzern, Switzerland)), *E. coli* K12 MG1655, *E. coli* WK8 (environmental isolate from surface water) and *Vibrio cholerae* O1, biovar Eltor, serotype Ogawa, strain Nent 720-95 were used. All strains were kept at -80 °C before use. The cryo-cultures were streaked onto TBX agar plates (Biorad, Reinach, Switzerland) and incubated for 24 h at 37 °C. A small tip of a colony was transferred into carbon-limited minimal medium (Ihssen & Egli, 2004) containing 10 mg glucose L^{-1} and the cells were grown into late stationary phase (3 days at 37 °C) before use.

Preparation of natural freshwater

Different types of freshwater were used: non-chlorinated tap water (DOC = 570 µg L^{-1}; AOC = 10- 20 µg L^{-1}, pH = 7.6; conductivity = 476 µS; Dübendorf, Switzerland), water from a shallow stream (DOC = 2546 - 4136 µg L^{-1}; AOC = 157 - 270 µg L^{-1}, pH = 7.7 - 7.9; conductivity = 402 - 502 µS, Chriesbach stream, Dübendorf, Switzerland), river water (DOC = 3322 - 4048 µg L^{-1}; AOC = 320 - 820 µg L^{-1}, pH = 7.7 - 8.1; conductivity = 570 - 696 µS, Glatt river, Dübendorf, Switzerland), stagnant pond water (DOC = 7036 - 8168 µg L^{-1}; AOC = 290- 570 µg L^{-1}, pH = 7.9 - 8.1; conductivity = 680 - 832 µS, Dübendorf, Switzerland) and wastewater treatment plant (WWTP) effluent (DOC = 6733 - 7198 µg L^{-1}; AOC = 300 - 450 µg L^{-1}, pH = 7.4 - 7.5; conductivity = 1040 - 1130 µS, Dübendorf, Switzerland). The sterilization was achieved as mentioned above. Before experimentation all glassware and caps were treated in order to remove all AOC. For all growth curves obtained only water from the river Glatt was used. The correlation for AOC concentration and final cell numbers was established using all different types of water.

Enumeration of total cell concentration by flow cytometry

Enumeration was done according to Hammes *et al.,* 2008. In brief: To 1 mL of a sample 10 µL of a 100 x diluted SYBR Green staining solution was added. The suspension was keep for at least 15 min in the dark before measuring with the flow cytometer. The combined green/red fluorescence dot blot was used to obtain results.

<u>Adenosine triphosphate (ATP) determination</u>

Measurements were done according to Vital *et al.,* 2008. In brief: For total ATP measurements 100 µL of a sample was pre-warmed to 30 °C and 100 µL of ATP buffer was added. The resulting light was immediately measured using the BacTiterGlo™ System (Promega, Dübendorf, Switzerland). ATP concentrations were calculated using a calibration curve established with pure ATP. Furthermore, each sample was filtered (0.22 µm) and the resulting free ATP (which is not cell bound) was measured. The total cellular ATP was thus calculated substracting the free ATP from the total ATP. ATP per cell was calculated by dividing the total cellular ATP by the cell numbers obtained by flow cytometry (see above).

<u>Plating of *E. coli*</u>

Samples were diluted in decimal steps (10^{-1} up to 10^{-4}) with sterile saline solution (8.5 % NaCl). Following dilution, 1 mL of test solution was mixed with 7 ml of liquid PCA agar (Oxoid, UK) or liquid R2A agar (Oxoid), respectively (pour plate method). Both agars were kept at 45 °C prior to use. After 20 minutes, the solidified agar was covered with another 4 mL of the respective liquid agar. Plates were incubated for five days at 30 °C before analysis. All measurements were done in triplicate.

<u>Growth of pathogens in sterile natural freshwater</u>

Cells were harvested after three days of incubation at 37 °C (late stationary phase) in carbon-limited minimal medium (see above). The total cell concentration was determined (see below) and the cells were inoculated into carbon-free glass vials (20 mL and 40 mL respectively) containing sterile freshwater originating from different sources (see above) to an initial

concentration of 1 - 5 x 10^3 cells mL^{-1}. The suspensions were incubated at 30 °C for four days and the final cell concentrations in the late stationary phase were enumerated with flow cytometry as described above. For batch growth curve experiments bacterial samples (100 µL to 1 mL) were collected throughout the growth cycle at different time points until stationary phase was reached. All experiments were performed in triplicate and analysed immediately after sampling. The specific growth rate (µ) based on cell concentration increase was determined as follows:

$$\mu = [\ln (N_t) - \ln (N_0)] / \triangle t \qquad \text{(Eq. 1)}$$

where N_t, N_0 are the cell concentrations measured at two subsequent time points and $\triangle t$ is the expired time interval between these points.

AOC determination

AOC determination was done according to Hammes & Egli, 2005.

Dissolved organic carbon (DOC) analysis

Organic carbon was measured in the pasteurized and 0.1 µm-filtered freshwater samples by an infrared detector after complete oxidation of the natural organic matter (NOM) to CO_2 by a Graentzel Thin-Film Reactor (Huber & Frimmel, 1992).

4 Results

Using the experimental approach shown above we were able to demonstrate the growth of *E. coli* and *V. cholerae* in freshwater on natural AOC in the range of 100 to 800 μg L^{-1} from (Vital et al., 2007, 2008.).

<u>Growth of *E. coli* using flow cytometry</u>

All three *E. coli* strains used (a pathogenic *E. coli* strain (O157), an environmental isolate (WK8) and a lab strain (K12)) multiplied in sterile river water (river Glatt, Dübendorf, Switzerland). The batch growth was characterized by a short lag phase followed by linear exponential growth and decreasing growth rates towards the stationary phase (figure 3). The final cell numbers and maximum specific growth rates (μ_{max}) are given in table 1.

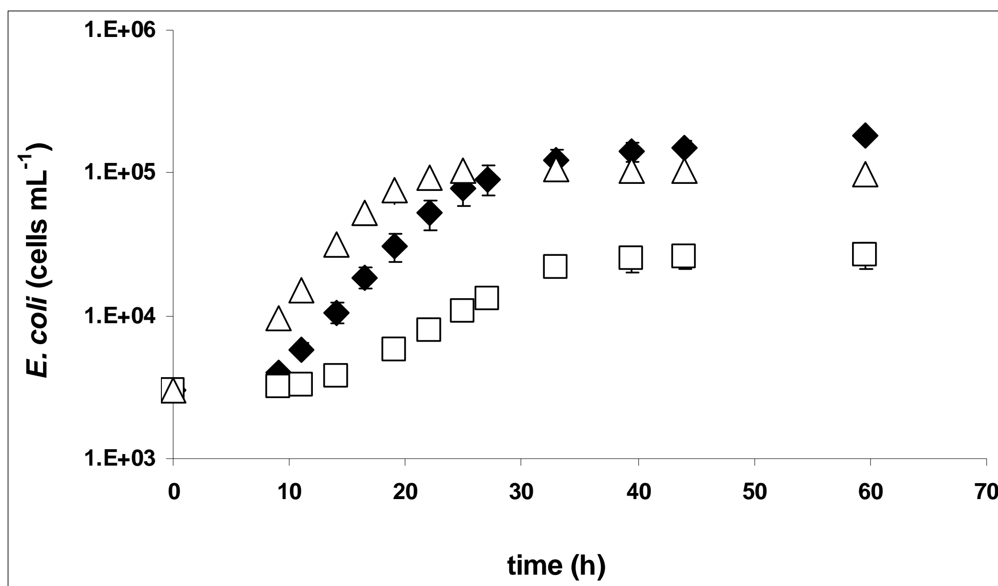

Figure 3. Batch-growth of three different *E. coli* strains (*E. coli* O157 (◆), an environmental isolate WK8 (Δ) and a lab strain K12 (□)) in sterile river water (adapted from Vital et al., 2008).

Table 1. Final cell numbers and maximum specific growth rates (μ_{max}) from three different *E. coli* strains growing in sterile river water.

strain	O157	WK8	K12
final cell concentration (cells ml^{-1} x 10^{5})	2.02 ± 0.10	1.49 ± 0.03	0.25 ± 0.04
μ_{max} (h^{-1})	0.20	0.23	0.11

Growth of *E. coli* using ATP analysis

Next to flow cytometry we also used adenosine triphosphate (ATP) measurements in order to characterize the growth of *E. coli* O157 in sterile riverwater. The total cellular ATP concentration showed a very similar pattern as the total cell concentration (Figure 4). After a short lag phase, a constant exponential increase was observed, followed by a deceleration towards the stationary phase. Within the first 27 h, the ATP concentration increased about 100-times (0.00268 nM to 0.2256 nM). In order to measure only the cellular ATP concentration the free ATP was subtracted from the total ATP measured. The calculated ATP content per cell varied during growth showing an increase during exponential growth phase followed by a steady decrease towards the stationary growth phase (Figure 5).

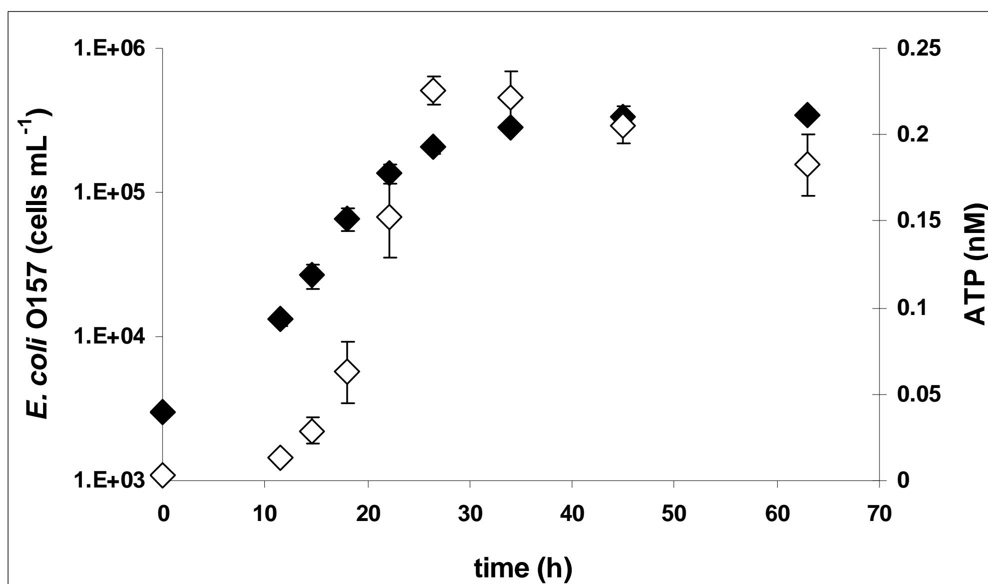

Figure 4. Batch growth of *E. coli* O157 in sterile riverwater analysed by flow cytometry (◆) and by cellular ATP (◇) measurements (adapted from Vital et al., 2008).

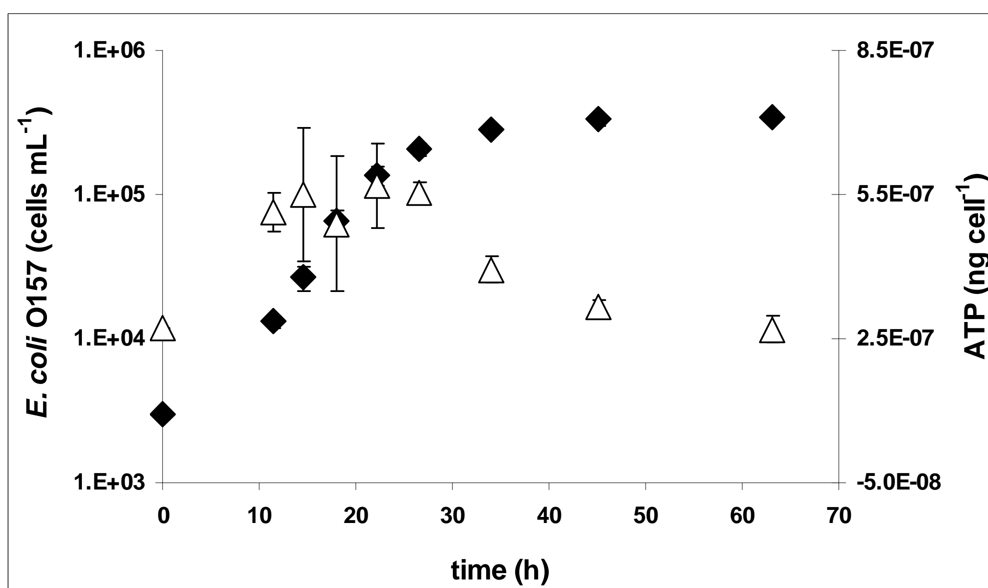

Figure 5. Batch growth of E. coli o157 in sterile riverwater. The total cell concentration (◆) and the ATP content per cell (◇) are given (adapted from Vital et al., 2008).

Following growth of *E. coli* using plating techniques

Next to FCM and ATP analysis also conventional cultivation based methods were used in order to describe the growth of *E. coli* O157. Two different types

of agar (R2A and PCA) were used for bacterial enumeration. The results are shown in Figure 6.

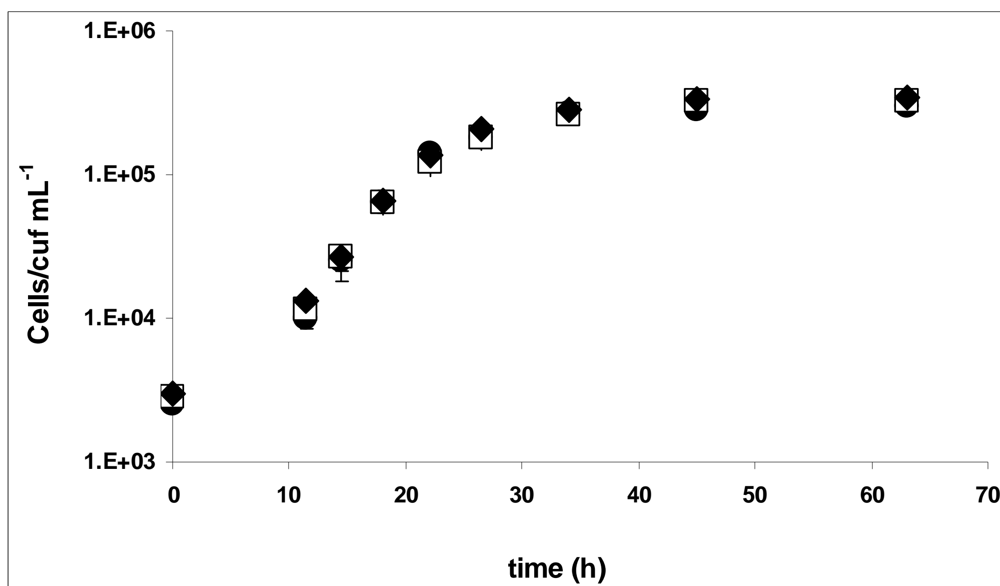

Figure 6. Following batch-growth of *E. coli* O157 in sterile river water using flow cytometry (◆), PCA (□) and R2A (●) agar (adapted from Vital et al., 2008).

Cultivation based methods confirmed the results obtained by flow cytometry and ATP measurements.

Growth of *Vibrio cholerae* in sterile freshwater

Also growth for *V. choloerae* in river water on natural AOC could be demonstrated. The batch growth curve (Figure 7) had the same pattern as for growth of *E. coli* (see above).

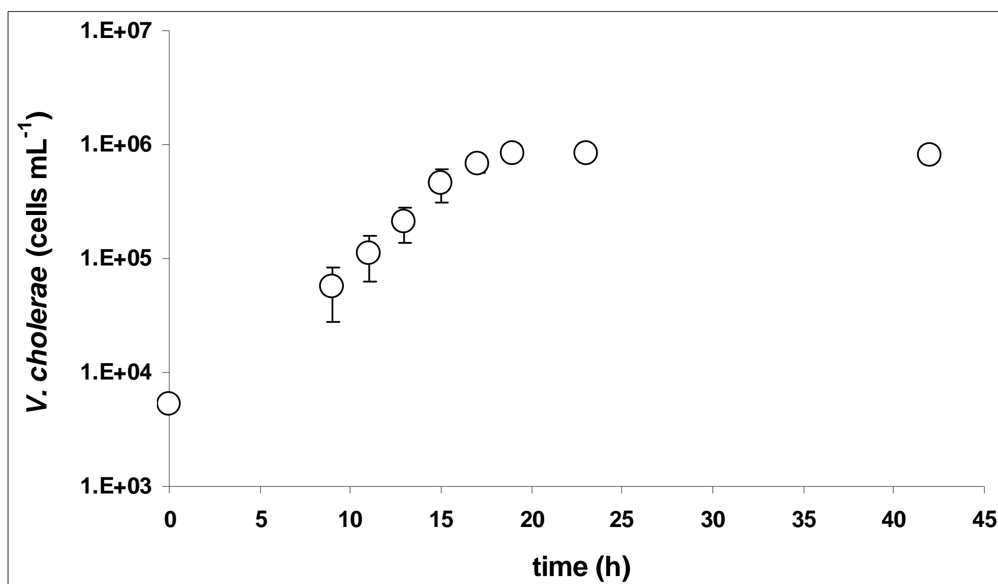

Figure 7. Batch growth of *V. cholerae* O1 in sterile riverwater analysed by flow cytometry.

The maximum specific growth rate of *V. cholerae* O1 growing in sterile river water at 30 °C was 0.45 h^{-1}.

<u>Correlation between AOC concentration and the final growth.</u>

A correlation between the AOC concentration of a sample and the final growth of *E. coli* O157 and *V. cholerae* O1 has been established (R^2 of 0.85 for *E. coli* and R^2 of 0.36 for *V. cholerae*) and are displayed in Figure 8 and 9. A positive impact of AOC on growth was observed for both pathogens. The final cell numbers were in the range of 0.82 - 4.07 x 10^5 cells mL^{-1} for *E. coli* O157 and of 0.29 - 1.55 x 10^6 cells mL^{-1} for *V. cholerae*. No growth was observed in tap water or bottled drinking water, both characterised by a low amount of AOC (10 - 20 µg L^{-1}).

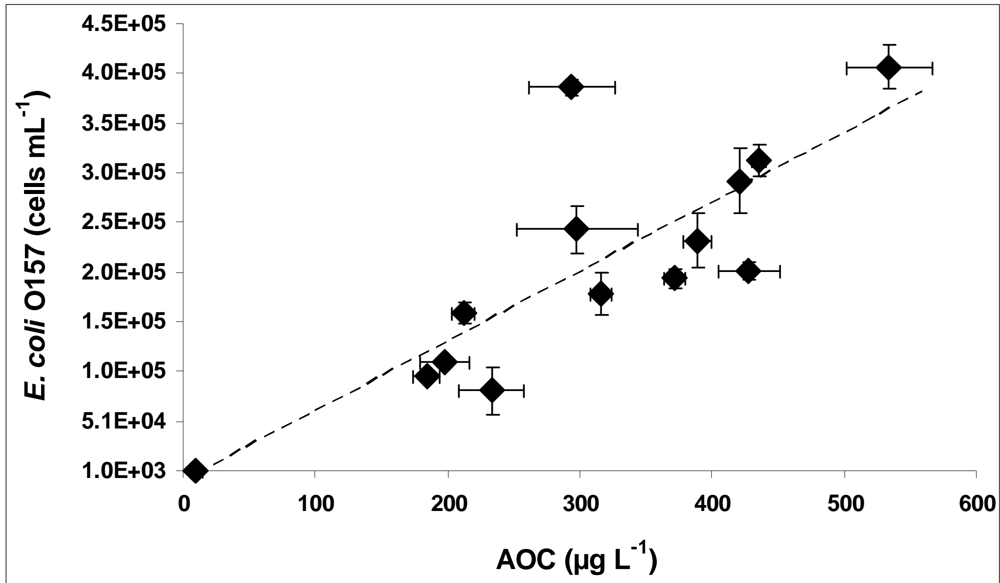

Figure 8. Correlation between AOC of a sample and the final cell number of *E. coli* O157 (adapted from Vital et al., 2008).

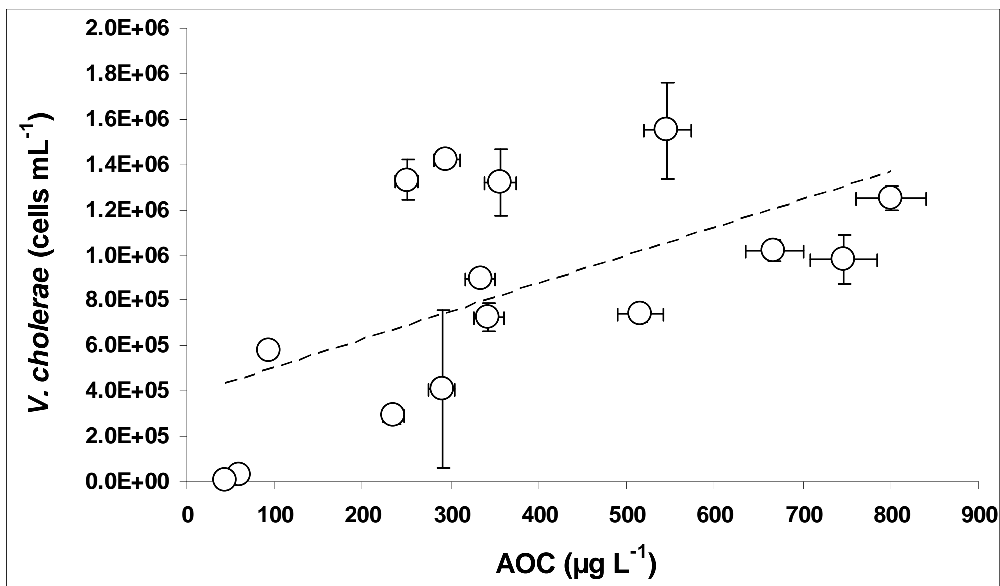

Figure 9. Correlation between AOC of a sample and the final cell number of *V. cholerae* O1 (adapted from Vital et al., 2007)..

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5 Discussion

With the approach given we could demonstrate that *E. coli* and *V. cholerae* are able to grow in sterile natural freshwater which is against the common view (reviewed by Morita, 1997). A correlation between the AOC concentration and the final cell yield could be established for both bacteria. Such a correlation was also reported for *Mycobacterium avium* (Torvinen et al., 2004). Furthermore, LeChevallier *et al.* (1996) reported a significant negative correlation between the occurrence of coliforms in drinking water systems and AOC concentrations lower than 100 µg L^{-1}. This together highlights the importance to measure AOC during drinking water processing and to take measures in order to keep nutrient concentrations low in order to minimize the potential of growth of pathogenic bacteria. One has to mention that the correlation between AOC concentration and final growth of *V. cholerae* was not as clear as for *E. coli*. This observation and the fact that final cell concentration was much higher for *V. cholerae* than for *E. coli* indicate that the substrate spectrum for available AOC compounds differs between the two pathogens. However the final cell yield of the AOC consortium used for AOC determination was even one order of magnitude higher than that of *E. coli* and still two to five times higher than that of *V. cholerae* shown in figure 8 and 9 (1µg AOC L^{-1} reflects 10^4 cells mL^{-1} of the natural AOC-test community. This means that for 100µg AOC L^{-1} 10^6 cells mL^{-1} are formed). The same pattern was observed for the maximum specific growth rates at 30 °C. *E. coli* O157 was growing with a μ_{max} of 0.2 h^{-1} followed by *V. cholerae* with 0.45 $^{h-1}$ and the natural lake water bacteria with 0.88 h^{-1} (Vital et al., 2007). One has to mention that not the same water was used for all experimentation which renders comparison more difficult since the AOC quality can be very different between samples. However, this seems to be a general trend, since the same pattern can be observed at highly diluted lysogeny browth (LB) medium (data not shown).

Growth in the environment does not happen in pure culture but with many different bacterial populations competing for the available nutrients. The results also clearly show the shortcomings on the growth of the investigated pathogens in natural freshwater, since μ_{max} and final cell concentrations are much lower in comparison to the natural bacteria. Competition experiments specifically focusing on this issue have to be carried out in order to get more information. Another important factor governing growth of heterotrophic bacteria in the environment is temperature. Future experiments investigating the influence of this factor on growth of pathogenic bacteria are therefore important. In addition , biofilms are important habitats for bacterial growth in the aquatic environment (Szewzyk *et al.*, 2000) and were not investigated in this study. Experiments on that topic should be carried out as well.

Using the described approach we were able to show the growth of pure cultures of enteric pathogenic bacteria in freshwater. In general, the presented findings govern a key issue for microbial risk assessment. They contribute to a better understanding of the behaviour of the investigated pathogens in the aquatic environment.

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