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‘Growth of pathogenic bacteria in freshwater and their competition with the autochthonous bacterial flora’

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Summary

The most common and widespread health risks associated with the consumption of water are infectious diseases caused by bacteria, viruses and protozoa. Thus, understanding the behavior of these organisms in aquatic ecosystems is of particular importance to prevent waterborne disease. With respect to pathogenic bacteria, many previous studies focused on their survival and/or die-off in water, whereas only little knowledge is available on factors governing their growth under environmental conditions, i.e. growth at low cell/nutrient concentrations with mixtures of substrates. The few existing data on this topic can be ascribed to technical limitations in the past and to a lack of know-how regarding to work under such environmental conditions *in vitro*. Therefore, the aim of this thesis was to study growth of different pathogens (*Escherichia coli* O157, *Vibrio cholerae* and *Pseudomonas aeruginosa*) in water under laboratory conditions and to elucidate the factors controlling their competition with autochthonous bacterial communities. The basic experimental methodology used in this work was based on principles developed previously in our group when investigating bacterial growth on natural assimilable organic carbon (AOC). However, the techniques were specifically optimized to enable work with pathogens in pure culture and to analyze bacterial competition in a controlled manner. Growth was monitored using several flow cytometry-based methods yielding multi-parametric data acquisition at the single cell level.

As a first step, growth of *V. cholerae* in freshwater on natural AOC (52 - 800 µg L^{-1}) was investigated. It was demonstrated for the first time that the bacterium is not only able to proliferate in brackish water ecosystems but that it can also multiply in freshwater *in vitro* (final cell concentrations from 2.9 x 10^5 - 1.6 x 10^6 cells mL^{-1}). Furthermore, *V. cholerae* was even able to grow in competition with a bacterial community derived from lake water. Although the kinetic properties of the pathogen were considerably lower than those of the community, *V. cholerae* accounted for a significant fraction (around 10 %) of the total cells formed at the end of competition experiments. These results illustrate that the frequent detection of this pathogen in surface waters might be due to its ability to grow in these ecosystems and suggest that even freshwater bodies not affected by recent fecal pollution can be a source for cholera infection.

It is a long-held common view that the classic enteric organism *E. coli* cannot grow at the low organic carbon concentrations found in the environment, and that its growth is restricted to the nutrient-rich intestinal tract of mammals and birds. However, this common view is based on reports using questionable experimental set-ups. In this thesis it was demonstrated that different strains of

E. coli were able to multiply in freshwater on natural AOC *in vitro* (final cell concentrations from 0.2 x 10^4 - 5 x 10^5 cells mL^{-1}). Furthermore, it was shown that temperature (from 15 to 37 °C) had a considerable influence on both, final cell numbers and μ of *E. coli* O157 indicating that proliferation of the pathogen is favored in regions with elevated annual water temperatures. Hence, the presented results question the use of *E. coli* as a reliable fecal indicator and suggest that the bacterium can be autochthonous in natural waters, especially in warmer regions.

E. coli O157, *V. cholerae* and *P. aeruginosa*, belong to distinctly different taxonomical groups and we wanted to know, whether this is also reflected by a different growth behavior of these organisms at low AOC concentrations. Therefore, several aspects of stoichiometry and kinetics of their growth were investigated in different nutrient-poor media and compared with those of a bacterial community derived from freshwater. It was possible to extract Monod constants (μ_{max} and K_s) using mathematical modelling. *P. aeruginosa* displayed better kinetic growth properties (μ_{max}: 0.58 $h^{-1}$and K_s: 57 μg L^{-1}) than *V. cholerae* (μ_{max}: 0.39 $h^{-1}$and K_s: 174 μg L^{-1}) and *E. coli* O157 (μ_{max}: 0.42 h^{-1} and K_s: 252 μg L^{-1}), whereas the stoichiometric parameters were similar for the three pathogens (2.5 - 6.7 x 10^6 cells (μg consumed DOC)$^{-1}$). The bacterial community performed best at all parameters investigated (μ_{max}: 0.66 $h^{-1}$and K_s: 27 μg L^{-1}; 7.7 - 13.3 x 10^6 cells (μg consumed DOC)$^{-1}$) illustrating the enormous competition challenges pathogens encounter during growth in the environment. The data contribute to the understanding of the distinctly different abundance of particular pathogens. On the one hand are the "classic enteric pathogens" such as *E. coli* O157 that exhibits weak kinetics which restrict its growth and, consequently, its abundance in the environment. On the other hand, the ability of the "environmental pathogen" *P. aeruginosa* to grow fast even at very low AOC concentrations can explain its ubiquitous detection, even in ecosystems characterized by minute substrate concentrations such as drinking water.

In the environment, pathogens constantly have to compete for substrates with autochthonous bacteria. Therefore, we took a closer look at the basic principles governing bacterial competition in the environment at low nutrient concentrations. *E. coli* O157 was grown in both batch and continuous culture in competition with a bacterial community derived from drinking water, using natural AOC (diluted wastewater) as the growth medium. A classical "opportunist" versus "gleaner" relationship was established, where *E. coli* O157 is the "opportunist", specialised for growth at high nutrient concentrations (μ_{max}: 0.87 h^{-1} and K_s: 490 μg L^{-1}), and the bacterial community is the "gleaner" adapted to nutrient-poor environments (μ_{max}: 0.33 h^{-1} and K_s: 7.4 μg L^{-1}). The influence of AOC concentration (36 - 3644 μg AOC L^{-1}), temperature (12 - 30 °C) and

dilution rate (D = 0.1, 0.2 and 0.3 h^{-1}) was investigated, demonstrating that all these parameters are positively influencing the competitive fitness of *E. coli* O157. It was possible to explain the observed results in competition experiments by the experimentally determined growth properties of the individual competitors in pure culture, namely (1) availability of AOC in batch and continuous culture (2) yield and (3) kinetic constants, μ_{max} and K_s. Many of the results could be modelled by conventional Monod kinetics. The study provides first insights into the principles governing bacterial competition in the environment. It specifically reveals new insights into competitive growth of the pathogen *E. coli* O157 with autochthonous communities.

The knowledge obtained from these studies was used to develop a bioassay which allows to estimate the pathogen growth potential (PGP) in water. In the batch growth assay, water samples are sterilized, inoculated with a specific pathogen (*E. coli* O157, *V. cholerae* and *P. aeruginosa*) and cell concentration is enumerated after growth into stationary phase. With this assay, a total of 22 water samples were analyzed, including the treatment trains of two drinking water plants and one wastewater treatment plant. Growth of the individual pathogens did not correlate with (DOC) concentrations of water, whereas the results suggest that AOC concentration can give indications on pathogen growth potential. However, correlations between pathogen growth and AOC concentration were weak (R^2 = 0.18 - 0.56) and each water sample exhibited a specific PGP pattern demonstrating that in addition to AOC concentration also AOC composition is governing growth of pathogens. The developed assay provides water utilities with an additional decision making tool for optimum design and operation of water treatment systems in order to minimize the risk of waterborne disease.

In conclusion, the results of this thesis shed considerable new light on pathogen growth and their competition with freshwater microbial communities at low nutrient concentrations. The thesis provides a first framework to include pathogen growth as a parameter within microbial risk assessment, which could help to limit the spreading of disease associated with them. Furthermore, the presented data add general understanding on the dynamics of microbial growth under nutrient-poor conditions.

Zusammenfassung

Infektionskrankheiten, verursacht durch Bakterien, Viren und Protozoen, sind die am häufigsten auftretenden und am meisten verbreiteten Gesundheitsrisiken im Zusammenhang mit Trinkwasser. Um das Ausbrechen von wasserbedingten Infektionskrankheiten zu minimieren ist es daher essentiell das Verhalten von diesen Krankheitserregern (Pathogenen) in der Umwelt zu verstehen. Viele Studien haben das Absterben und Überleben von pathogenen Bakterien im Wasser untersucht, jedoch gibt es nur wenige Daten über jene Faktoren, welche deren Wachstum unter „umweltrelevanten Bedingungen“ (d.h. bakterielles Wachstum mit einer Vielzahl von verschiedenen, gering konzentrierten Nährstoffen) regulieren. Dies ist teils auf bisherige methodische Limitationen und teils auf fehlendem „know how“, wie man im Labor unter solchen „umweltrelevanten Bedingungen“ arbeiten soll, zurückzuführen. Daher war das Ziel dieser Doktorarbeit das Wachstum von pathogenen Bakterien (*Escherichia coli* O157, *Vibrio cholerae* O1 und *Pseudomonas aeruginosa*) im Süsswasser, und deren Konkurrenzverhalten mit den dort lebenden Mikroorganismen, zu untersuchen. Der Versuchsaufbau basierte auf den Prinzipien, welche im Zusammenhang mit Wachstumsexperimenten von Bakterien auf natürlichem organischen Kohlenstoff (AOC) in der Gruppe entwickelt worden waren, wurde hier jedoch modifiziert und optimiert um speziell das Wachstum von pathogenen Bakterien in Reinkultur und unter kontrollierten Konkurrenzbedingungen mit natürlichen Süsswasserbakterien zu ermöglichen. Das Wachstum wurde dabei mit Hilfe der Durchflusszytometrie, welche das simultane Messen von verschiedenen Parameter von Einzelzellen erlaubt, untersucht.

Als erster Schritt wurde das Wachstum von *V. cholerae* im Süsswasser auf natürlichem AOC (52 - 800 µg L^{-1}) erforscht. Dabei konnte zum ersten Mal gezeigt werden, dass sich Cholerabakterien nicht nur in salzhaltigen Ökosystemen sonder auch im Süsswasser *in vitro* vermehren können (Endzellzahlkonzentration zwischen 2.9 x 10^5 - 1.6 x 10^6 mL^{-1}). Des Weiteren konnte *V. cholerae* sogar in Konkurrenz mit natürlichen Süsswasserbakterien wachsen. Obwohl *V. cholerae* erhebliche Nachteile in den gemessenen kinetischen Wachstumsparameter gegenüber den Süsswasserbakterien aufwies, machte der Anteil von *V. cholerae* am Ende der Kompetitionsexperimente einen beträchtlichen Anteil, nämlich 10 % der gesamten Bakterienzellzahl, aus. Die Resultate erklären die häufige Detektion von *V. cholerae* im Süsswasser und zeigen auf, dass auch Süsswasserökosysteme, welche nicht unter direktem Fäkalieneintrag stehen, Infektionsquellen für Cholera sein können.

Es ist allgemein anerkannt, dass klassische Darmbakterien, wie z.B. *E. coli*, in der Umwelt mit den vorherrschenden geringen Nährstoffkonzentrationen nicht wachsen können, sondern dass deren Vermehrung ausschliesslich im nährstoffreichen Darm von Säugetieren und Vögeln erfolgt. In dieser Doktorarbeit konnte jedoch gezeigt werden, dass sich diese allgemeine Meinung auf Resultate von Experimenten mit zweifelhaftem Versuchsaufbau stützt und dass *E. coli* tatsächlich im Süsswasser auf natürlichem AOC im Labor wachsen kann (Endzellzahlkonzentration zwischen 0.2×10^4 - 5×10^5 mL^{-1}). Dabei hatte die Temperatur einen grossen Einfluss auf das Wachstumsverhalten von *E. coli*, was darauf hinweisst, dass dessen Vermehrung in wärmeren Gewässern bevorzugt wird. Die gewonnen Resultate werfen Fragen über die Verwendung von *E. coli* als verlässlichen Fäkalindikator auf und indizieren, im Gegensatz zur allgemeinen Meinung, dass das Bakterium in natürlichen, vor allem wärmeren, Gewässern autochthon vorkommen kann.

E. coli O157, *V. cholerae* und *P. aeruginosa* sind taxonomisch fern verwandte Pathogene. Um zu untersuchen, ob sich diese Unterschiede auch in deren Wachstumseigenschaften unter geringen Nährstoffkonzentrationen widerspiegeln, wurden verschiedene Wachstumsaspekte in nährstoffarmen Medien analysiert und zusätzlich mit denen von natürlichen Süsswasserbakterien verglichen. Die Abhängigkeit der spezifischen Wachstumsrate von der AOC Konzentration konnte dabei mittels Monod Kinetik, welche auf der maximalen Wachstumsgeschwindigkeit (μ_{max}) und der Affinitätskonstanten (K_s) beruht, erklärt werden. *P. aeruginosa* zeigte eine bessere Wachstumskinetik (μ_{max}: 0.58 h^{-1}; K_s: 57 µg L^{-1}) als *V. cholerae* (μ_{max}: 0.39 h^{-1}; K_s: 174 µg L^{-1}) oder *E. coli* O157 (μ_{max}: 0.42 h^{-1}; K_s: 252 µg L^{-1}). Die Ausbeute war jedoch bei allen Pathogenen im selben Rahmen (2.5×10^6 - 6.7×10^6 produzierte Zellen (µg verbrauchten organisch gelösten Kohlenstoff (DOC))$^{-1}$). Die Süsswasserbakterien waren den Pathogenen in allen untersuchten Parametern überlegen (μ_{max}: 0.66 h^{-1}; K_s: 27 µg L^{-1}; 7.7×10^6 - 13.3×10^6 produzierte Zellen (µg verbrauchten DOC)$^{-1}$), was den ernormen Konkurrenzdruck, welchem pathogene Bakterien in der Umwelt ausgesetzt sind, aufzeigt. Die gewonnenen Resultate geben Aufschluss über das unterschiedliche Auftreten von unterschiedlichen Pathogenen in der Umwelt. Auf der einen Seite stehen die klassischen Enteropathogenen (z.B. *E. coli* O157), welche nur langsam in der Umwelt wachsen können und daher auch meistens in geringer Anzahl vorhanden sind. Auf der anderen Seite ermöglicht das schnelle Wachstum von sogenannten „Umweltpathogenen" (z.B. *P. aeruginosa*) deren weite Verbreitung in der Umwelt, auch bei extrem geringen Nährstoffkonzentrationen wie sie z.B. im Trinkwasser anzutreffen sind.

In der Umwelt wachsen pathogene Bakterien nicht in Reinkultur sondern müssen ständig mit anderen Bakterien um Nährstoffe konkurrieren. In dieser Studie wurden die Prinzipien, welche das Konkurrenzverhalten von Bakterien in der Umwelt lenken, näher betrachtet. *E. coli* O157 wurde dabei in Kompetition mit Trinkwasserbakterien sowohl in batch als auch in kontinuierlicher Kultur auf natürlichem AOC (verdünntem Abwasser) kultiviert. Eine sogenannte „opportunist" versus „gleaner" Beziehnung wurde beobachtet, wobei *E. coli* O157 als „opportunist" betrachtet werden kann, das für Wachstum unter hohen Nährstoffbedingungen spezialisiert ist (μ_{max}: 0.87 h^{-1} und K_s: 490 µg L^{-1}) und die Trinkwasserbakterien, oder „gleaner", auf nährstoffarme Ökosysteme adaptiert sind (μ_{max}: 0.33 h^{-1} and K_s: 7.4 µg L^{-1}). Der Einfluss der AOC Konzentration (36 - 3644 µg AOC L^{-1}), der Temperatur (12 - 30 °C) und der Durchflussrate (D = 0.1 h^{-1}, 0.2 h^{-1} und 0.3 h^{-1}) auf das Wachstum in Konkurrenz wurden analysiert, wobei erhöhte Werte aller drei Faktoren das Wachstum von *E. coli* O157 positiv beeinflussten. Die Resultate konnten mit Hilfe der zuvor gemessenen Wachstumseigenschaften in separaten Kulturen, nämlich (1) AOC Verfügbarkeit in Batch und kontinuierlicher Kultur, (2) Ausbeute und (3) kinetische Parameter (μ_{max} und K_s), erklärt werden. Ausserdem war es möglich die meisten Daten mittels Monod Kinetik zu modellieren. Die Studie liefert neue Erkenntnisse über das Konkurrenzverhalten von *E. coli* O157 in der Umwelt.

Basierend auf den gewonnen Erkenntnissen aus den vorhergehenden Kapiteln wurde ein Test entwickelt, mit dessen Hilfe man das Wachstumspotential von pathogenen Bakterien (PGP) im Wasser analysieren kann. Dabei wird die Wasserprobe entkeimt, separat mit verschiedenen Pathogenen beimpft und das resultierende Wachstum mittels Durchflusszytometrie gemessen. Mit dem entwickelten Test wurden Proben von zwei Trinkwasseraufbereitungsanlagen und einer Abwasserreinigungsanlage analysiert. Es wurde gezeigt, dass das Wachstum der pathogenen Bakterien nicht mit der Konzentration von DOC korreliert, wohingegen AOC Messungen erste Hinweise für Pathogenwachstum geben können. Die Korrelation zwischen AOC und dem Pathogenwachstum war jedoch gering (R^2 = 0.18 - 0.56) und jede Probe wies ein individuelles „PGP" auf, d.h. nicht nur die Konzentration sondern auch die Zusammen-setzung von AOC beeinflusst das Wachstum von Pathogenen. Der entwickelte Test bietet eine neue Möglichkeit Wasseraufbereitungssysteme optimal zu konstruieren, im Hinblick auf Pathogenwachstum, um somit wasserbedingte Krankheiten zu limitieren.

Die gewonnen Resultate von dieser Doktorarbeit geben neue Einblicke in das Wachstum von pathogenen Bakterien unter nährstoffarmen Bedingungen auch in Konkurrenz mit natürlichen Süsswasserbakterien was die mikrobielle Riskoanalyse verbessert und somit einen Beitrag zur

Minimierung von wasserbedingte Infektionskrankheiten leistet. Des Weiteren liefern die gewonnenen Daten und Konzepte Erkenntnisse über generelle Prinzipien des Bakterienwachstums in der Umwelt.

1. General Introduction

Waterborne disease

Infectious disease caused by pathogenic bacteria, viruses and parasites is the most common and widespread health risk associated with drinking water (WHO, 2008). It is estimated that globally around 1.1 billion people globally have to drink unsafe drinking water each day (Kindhauser, 2003). Poor water quality and lack of sanitation and hygiene account for some 1.7 million deaths per year, mostly among children under the age of five. More than 99 % of these deaths occur in low-income countries, where several factors like malnutrition, poor hygiene/sanitation and immunodeficiencies come together with unsafe water consumption (Ashbolt, 2004). However, waterborne diseases are not restricted to these countries. Morris and Levine (1995) assessed the annual waterborne disease burden in the US and estimated that 7.1 million people per year suffer from a mild to moderate waterborne infection and that 560,000 suffer from a moderate to severe one, sometimes with lethal consequence. Thus both the health and economic effects are considerable even for high-income nations (Payment, 1997; OECD/WHO, 2003).

Bacteria promoting waterborne diseases

Many bacterial species promoting waterborne diseases are known. The majority are enteric bacteria infecting the human gastro-intestinal tract, but also others are documented (Table 1.1). So called "(re-) emerging pathogens" have recently caught much attention. Emerging pathogens are those that have appeared in the human population for the first time, or have occurred previously but are increasing in incidence or expanding into areas where they previously have not been reported, usually over the last two decades. Re-emerging pathogens are microorganisms whose incidence is increasing due to long-term changes in their underlying epidemiology (Taylor *et al.,* 2001; WHO, 2003). Most of the species presented in Table 1.1 are classified as (re-)emerging pathogens. However, it has to be mentioned that statistics on the epidemiology of emerging pathogens are often directly linked with improved sensitivity for their detection and increased reporting thereof. Whereas it was previously often not possible to identify the disease causing agent, new molecular based detection methods considerably improved the success of identification. Therefore, certain organisms are classified as (re-)emerging pathogens due to improvements of methods enabling their detection and not based on their epidemiology (Woolhouse, 2002).

Table 1.1. Waterborne pathogens and their significance in water supplies (adopted from WHO 2003). * (re-)emerging pathogen according to Taylor *et al.* (2001); GI-tract: gastrointestinal tract.

Pathogen	Health significance	Site of infection	Persistence in water supplies
Burkholderia pseudomallei	High	GI - tract	May multiply
Campylobacter jejuni, C. Coli*	High	GI - tract	Moderate
E. coli – Pathogenic	High	GI - tract	Moderate
*E. coli – Enterohaemorrhagic**	High	GI - tract	Moderate
*Legionella spp.**	High	Respiratory	May multiply
Non-tuberculous mycobacteria*	Low	Respiratory	May multiply
*Pseudomonas aeruginosa**	Moderate	Various	May multiply
*Salmonella typhi**	High	GI - tract	Moderate
Other salmonellae*	High	GI - tract	May multiply
*Shigella spp.**	High	GI - tract	Short
*Vibrio cholera**	High	GI - tract	Short to long
*Yersinia enterocolitica**	Moderate	GI - tract	Long

Examples of (re-)emerging waterborne pathogens

A typical example of an emerging waterborne pathogen is *Legionella pneumophilia* causing severe lung infections called Legionnaires' disease. The pathogen was first described in 1979 and it was estimated that the bacterium affects 25,000 - 100,000 persons annually in the United States alone (Steinert *et al.,* 2002). Of all pneumoniae from North America and Western Europe, 1 – 13 % were associated with *L. pneumophilia*. Changes in human behaviour such as an increased usage of air conditioning, which is a possible transmission route for the pathogen, are attributed to its emerging properties.

Other examples are certain pathogenic *E. coli* strains, especially *E. coli* O157:H7, which was first recognized in 1982 and is, hence, classified as an emerging pathogen. Whereas, *E. coli* is a part of the normal bacterial flora of mammals and birds, certain strains have also been associated with disease. Thereby, enterohaemorrhaghic strains (EHEC), especially *E. coli* O157:H7, comprise the most important ones (Caprioli, 2005). *E. coli* O157:H7 has often been related to waterborne disease. It is estimated that this bacterium causes 73,000 illnesses in the United States annually and from 1982 – 2002, 350 outbreaks in 49 States were reported (Rangel *et al.,* 2005). Contaminated food was identified to be responsible for most of these outbreaks, but also consumption of contaminated water played a significant role (Figure 1.2). Although the pathogen is considered to be a specific problem in high-income countries, EHECs have been recently detected in less developed nations as well (Sharma *et al.,* 2003).

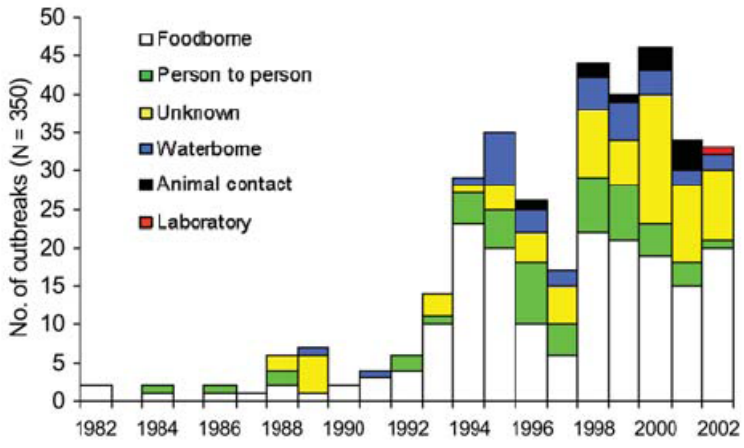

Figure 1.1. Overview of outbreaks and corresponding transmission routes of *E. coli* O157:H7 in the US from 1982 to 2002 (from Rangel et al., 2005).

Another well known waterborne pathogen is *Vibrio cholerae*, the causative agent of cholera disease. Although the devastating pandemics in the 19th century, affecting also Europe, occurred back in the past, *V. cholerae* is still very prominent in developing and transitional countries. Between 1998 - 2001 the WHO reported 578 infectious disease outbreaks in 132 countries, where acute diarrhoea was the fourth important category with cholera as the most frequent (Ashbolt, 2004). After an absence of over 100 years, the pathogen reached again South America in 1991, where 400,000 cases with 4000 deaths in 11 countries were reported**.** *V. cholera* is, hence, classified as a re-emerging pathogen. Figure 1.2 displays the global cholera incidents from 1995 - 2008, indicating the multitude of countries affected by *V. cholerae.*

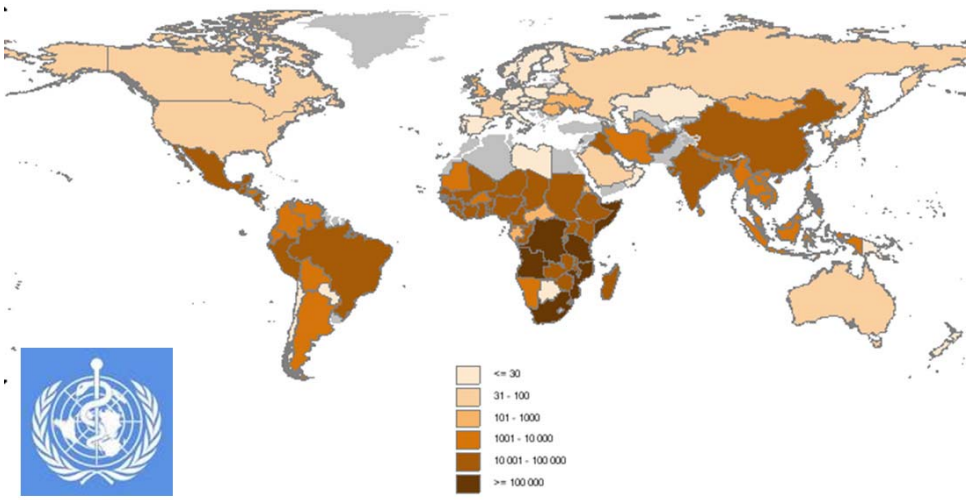

Figure 1.2. Global cholera incidents from 1995 to 2008 according to WHO (global health atlas).

The recent dramatic outbreaks in Zimbabwe further illustrate the severity of the disease; from August 2008 till May 2009 around 100,000 infections with 4,300 deaths were reported (WHO, 2009).

Cycling between two habitats

Many waterborne pathogenic bacteria cycle between two habitats: their host and the (open) environment, i.e. water and soil (Savageau, 1983; Figure 1.3). Whereas for some pathogens the human population represents their sole host, others are also able to infect a variety of warm-blooded animals or even insects and protists. For example, cattle are an important host for *E. coli* O157:H7 and it is estimated that up to 30 % of all cattle are asymptomatic carriers of the bacterium (Caprioli, 2005). The two habitats differ distinctly in their physicochemical conditions and the "cycling" pathogens usually encounter a so called "feast and famine" existence. Whereas the ecosystem of the host is characterized by rather high nutrient concentrations, nutrient-poor (oligotrophic) conditions prevail in the environment (Morita, 1997). Apart from low nutrient concentrations, pathogens are exposed to several additional stressors in the environment such as varying temperatures, high oxygen tension (for enteric pathogens), UV light and predation (Winfield & Groisman, 2003) and their fate, i.e. whether they die-off, survive or even grow, is considered to depend on species.

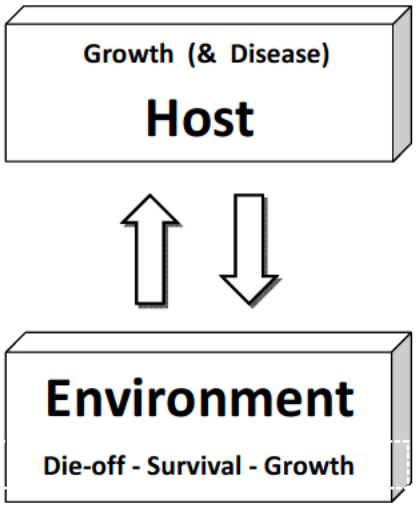

Figure 1.3. Pathogens continuously cycle between two habitats; their host(s) and the environment (water & soil). In the host(s), they grow and can promote disease. Their behaviour in the environment, die off, survive or even growth is considered to depend on species (see text.).

In the host: growth (and disease)

In non-human hosts, pathogenic bacteria are often autochthonous and also people may carry pathogens as part of their normal bacterial flora without disease; this is especially true for opportunistic pathogens, i.e. bacteria which can only cause disease in immunocompromised persons (Kayser *et al.,* 2001). If pathogens cause disease the pathogenesis (development of disease) is species dependent involving a number of virulence factors such as the ability to attach to and invade epithelial cells or the production and expression of toxins. For example, *V. cholerae* expresses two major virulence factors namely the cholera toxin (CT) and a colonization factor named toxin co-regulated pilus (TCP), which enables the bacterium to colonize the small intestine. Considerable scientific effort was performed in order to elucidate the regulating mechanisms and we have today a

sound understanding on the signal transduction cascades involved (Klose, 2001). A key element thereby is a protein called ToxT activating the transcription of the genes encoding CT (*ctx*) and TCP (*tcp*). Several environmental signals such as pH, temperature or osmolarity were identified that affect the transcription of *toxT in vitro* (Reidl & Klose, 2002). However, next to the expression of such "conventional virulence factors", pathogens also have to scavenge nutrients and grow inside the host in order to promote disease; a fact which is often neglected and rarely studied. For example, Merrell and colleagues (2002) performed a signature-tagged mutagenesis (STM) screen of *V. cholerae*. Infecting mice they were able to show that factors involved in the biosynthesis of amino acids, purines or fatty acids are required for colonization and, thus, essential to promote disease. Metabolic properties of pathogens are thus a key element for pathogenesis and it is an ongoing debate, whether the genes involved should be also classified as virulence factors (Merrell *et al.,* 2002).

E. coli O157:H7 colonizes the mucosa of the human intestinal tract and produces toxins, the shiga toxins, which represents the pathogen's main virulence factor. Serious clinical manifestations can occur, such as haemorrhagic colitis and the haemolytic uraemic syndrome. Although, the intestinal tract is considered to be nutrient rich, growth of bacteria is not unrestricted but carbon/energy-limited. The doubling time of *E. coli* in the human large intestine, the colon, was estimated to be around 40 h based on the mean transition time (Savageau, 1983). However, the bacterium is not equally distributed throughout the whole intestine; *E. coli* represents 0.1 % to 1 % of the total bacterial biomass in the colon (Bettelheim, 1992), but probably exhibits specialization for growth in the cecum, which is the first part of the large intestine (Gordon & Cowling, 2003). Thus, the actual growth rates differ probably distinctly between sites. In the colon, bacteria grow on undigested food components as well as on host derived substrates such as mucus (Macfarlane *et al.,* 1998). The substrate composition is complex including organic molecules such as different poly- and oligosaccharides, (oligo)peptides or free amino acids. Based on *in vitro* testing, we have a sound understanding on the potential substrate spectrum of *E. coli* and the catabolic pathways involved; the question "what the bacterium is eating *in vivo*" is, however, still unclear. Furthermore, the colonic ecosystem comprises hundreds of different phylotypes, most of them uncultured so far (Eckburg *et al.,* 2005). Hence, enteric pathogens invading the host have to compete with a multitude of different species, which are acting as "placeholders", for substrates. Again, only if the conditions allow a pathogen to access the nutrient pool and grow, it can promote disease. In this respect, the diet has caught specific attention since it was shown that its composition can have a significant impact on the shedding of *E. coli* O157:H7 by cattle (reviewed by Callaway *et al.,* 2008). It is

suggested that certain substrates can positively influence the population of *E. coli* O157:H7 by providing growth factors, whereas others decrease the concentration of the pathogen due to growth inhibitory effects. Furthermore, competitive exclusion of *E. coli* O157:H7 and of *Salmonella spp.* by a defined population of multiple non-O157:H7 *E. coli* was reported (Tabe *et al.,* 2008). Strains closely related to pathogens introduce high competition challenges, because they occupy a similar niche in the host. Thus, non-pathogenic bacteria can prevent disease by "eating away the substrates" required for pathogen growth. It has to be mentioned that the biological mechanisms underlying the link between the diet and growth of *E. coli* O157:H7 are often unclear, but this research field nicely demonstrates the important role of ecosystem characteristics for pathogenesis.

Growth of heterotrophic bacteria in the environment

In the environment, heterotrophic bacteria face mostly carbon/energy-limited oligotrophic (nutrient-poor) conditions (Morita, 1997). The total organic carbon (TOC) consists of a particulate (POC) and a dissolved (DOC) fraction, where specifically the latter is recognized to be relevant for growth of heterotrophic bacteria. The DOC pool is, however, not uniform but highly divers and usually divided into two distinct fractions, i. e., "assimilable organic carbon" (AOC; van der Kooij *et al.,* 1982), and "polymeric DOC" and each fraction differs in its availability for bacterial growth (Figure 1.4). The former, AOC, is composed of many different low molecular substrates including monomeric sugars or free amino acids, all present in the nano or low micro-g L^{-1} range and all readily available for microorganisms (Egli, 1995), whereas the latter consists of polymeric substances, which require degradation/hydrolysis before they can be taken up by a cell (Münster, 1993; Rosenstock *et al.,* 2005).

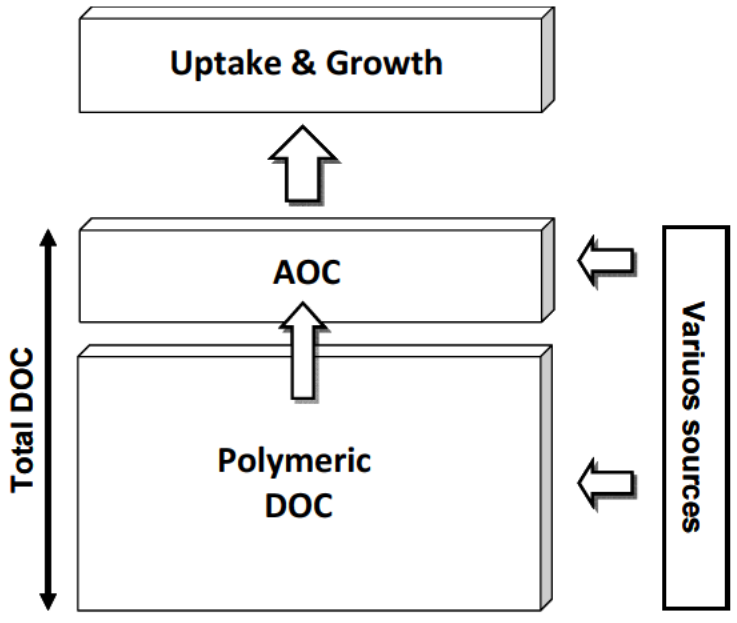

Figure 1.4. The total dissolved organic carbon (DOC) pool is composed of (1) assimilable organic carbon (AOC), which consists of low molecular weight compounds, and (2) a polymeric fraction. Only AOC is readily available for heterotrophic growth.

Therefore, not the total DOC, but the concentration of AOC is controlling growth and competition of heterotrophs. The AOC pool is directly fed by various sources such as primary producers or substances leaching from dying microorganisms as well as by the enzymatic and (photo)chemical hydrolysis of more complex DOC. In aquatic ecosystems, DOC concentrations are typically ranging from 0.1 mg L^{-1} to 10 mg L^{-1} (Morita, 1997), but only 0.1 to 10 per cent of this DOC is measured as AOC (Münster, 1993). It should be pointed out measured DOC/AOC cconcentrations are reflecting "quasi" steady-state concentrations resulting from continuous production and consumption. The calculated total turnover times of DOC range from several days up to months, whereas certain compounds contributing to AOC are reported to be turned-over much faster, often within a few hours (Münster, 1993; Rosenstock *et al.,* 2005). It was often argued that the minute substrate concentrations cannot support microbial growth and that the majority of bacteria are, hence, inactive (see Egli, 1995). Furthermore, only a small fraction of these bacteria, normally between 0.1 and 1 %, could be cultivated on conventional media, a phenomenon also known as "the great plate count anomaly" (Staley & Konopka, 1985). However, investigations using microautoradiographic methods demonstrated that the majority of cells were assimilating organic carbon substrates (e.g., Fuhrman & Azam, 1982) and actively dividing. By changing the cultivation approach mimicking environmental conditions, Kaeberlein and co-workers (2002) were able to increase the cultivability of marine bacteria up to 40 % (± 13 %). Hence, today there is no doubt that most of the bacteria present in water are alive and actively growing. Analysing the data of 57 previous studies using radioactive tracer methods, White and co-workers (1991) reported mean *in situ* growth rates of the total autochthonous community of 0.023 h^{-1} (from 0.0004 h^{-1} to 0.36 h^{-1}) in freshwater, 0.018 h^{-1} (from 0.00002 h^{-1} to 0.42 h^{-1}) in marine waters and 0.073 h^{-1} (from 0.0003 h^{-1} to 1.3 h^{-1}) in estuarine and coastal waters (temperature range from -2 °C to 38 °C). An important aspect hereby is the ability of bacteria to simultaneously take up and metabolize numerous substrates, also called "mixed substrate growth". This physiological capability was demonstrated for various species indicating its importance during growth, especially under oligotrophic conditions as found in most aquatic ecosystems. "Mixed substrate growth" confers to several advantages, namely the achievement of fairly high growth rates even at low AOC concentrations, metabolic flexibility and the circumvention of nutrient threshold concentrations for growth (reviewed by Egli, 1995).

Growth of pathogens in the environment

Research on the behaviour of pathogenic bacteria in the environment resulted in a crude separation between two groups. Those for which the environment serves as a reservoir are classified as "environmental pathogens" and comprise species such as *L. pneumophilia*, *V. cholerae,*

Pseudomonas aeruginosa or *Mycobacterium avium*. The second group includes and the "classic enteric pathogens" such as *Salmonella typhimurium* or pathogenic *E. coli,* which are considered not to multiply under oligotrophic conditions. This classification is also applied in microbial risk assessment; certain bacteria may multiply in drinking water supplies, whereas others only survive, either short-, moderate- or long-time (illustrated in Table 1.1).

Environmental pathogens

"Environmental pathogens" are frequently found in waters of different origin (Szewzyk, 2000). Many species allocated to the group of "environmental pathogens" are considered as opportunistic pathogens. Therefore, disease prevails in environments where older and/or immunocomprised people are living such as hospitals or retirement homes. For example, although the spectrum of intensive care unit (ICU)-associated infections changed significantly within the last two decades, *P. aeruginosa* holds an unchanged position; the organism is among the top five pathogens causing infections at distinct sites of the human body. Recently, molecular typing methods enabled source-tracking of the bacterium and it could be shown that between 14.2 % and 50 % of disease were due to genotypes found in ICU water systems (Trautmann *et al.,* 2005). These observations nicely demonstrate the link between water as an infection source and the incapability to prevent disease due to the lack of knowledge on pathogen growth. Many studies have investigated the survival of "environmental pathogens" in water (e.g., Pianetti *et al.,* 2005; Lehtola *et al.,* 2007), but proper investigations on the factors governing their growth under controlled conditions are rare. van der Kooij and coworkers (1982) demonstrated growth of *P. aeruginosa* in different sterilized tap waters at 15 °C with specific growth rates between 0.002 h^{-1} and 0.04 h^{-1}. In sterilized ozonated tap water and river water containing more AOC, the growth rates were significantly higher and reached values of 0.09 h^{-1} and 0.1 h^{-1}, respectively. Furthermore, a correlation between AOC concentration and the occurrence of *M. avium* in drinking water distribution systems was recently established (Torvinen *et al.*, 2004). In addition, Legnani and colleagues (1999) reported a high growth potential of *P. aeruginosa* in sterilized bottled mineral waters. However, the authors did not take specific care to avoid AOC contamination, a problem discussed below, and their results must therefore be questioned.

Also *V. cholerae* can grow in the environment, specifically in salt and brackish waters (Islam *et al.,* 1994). Many studies have focused on the factors controlling the occurrence of this pathogen in water, which makes *V. cholerae* one of the model organisms for studying the influence of ecosystem characteristics and the spreading of disease (Colwell, 1996; Lipp *et al.,* 2002). Cholera

prevalence in Bangladesh could be related to El Niño/Southern Oscillation (Rodo *et al.,* 2002). Furthermore, it was possible to predict cholera cases based on water temperature, conductivity, rainfall and cholera toxin (*ctx*) detection (Huq *et al.* 2005). Recently, even satellite images were used to forecast outbreaks of cholera disease (Ford *et al.,* 2009). The pathogen can be present in the water phase or attached to copepods, zooplankters and algae (Reidl & Klose, 2002; Huq *et al.,* 2005. It is assumed that high temperatures and moderate salinity provide the most favourable conditions for growth of *V. cholerae* (see also introduction (chapter 2)). However, as for the opportunistic pathogens, studies specifically investigating growth of *V. cholerae* in natural water under controlled conditions are scarce. A rare example provide Mouriño-Perez and colleagues (2003), who monitored growth of the pathogen in sterilized red tide waters off California and reported growth rates between 0.013 h^{-1} and 0.6 h^{-1} suggesting that nutrients released during phytoplankton blooms promote growth of *V. cholerae*.

Some properties are important in both habitats

Although, the physicochemical conditions differ considerably between the host and the environment, it was shown that certain properties of "environmental pathogens" are important in both habitats. An example is the ability of *P. aeruginosa* to form dense biofilms governing its growth and survival in both aquatic ecosystems and the respiratory system of humans affected by cystic fibrosis. Furthermore, the ability of *L. pneumophilia* to invade and grow inside Amoeba was directly linked to the promotion of disease in humans (Fields *et al.,* 2002). Another example is the protein GbpA found in *V. cholerae*. This protein mediates attachment of the bacterium to zooplankton as well as to human epithelial cells (Kirn *et al,* 2005). Hence, selection for certain abilities in one habitat can influence the behaviour of pathogens in its other habitat. Whether this is also true for metabolic properties, such as the ability to take-up and metabolize certain substrates present in both habitats, still needs to be investigated.

Classic enteric pathogens: no growth in the environment?

It is the common view that "classic enteric pathogens" cannot grow in the environment. The nutrient-rich intestinal tract of mammals and birds is considered to be their only habitat where they can proliferate, whereas it is thought that they die-off when shed into the (oligotrophic) environment. This is also reflected by the use of *E. coli* as the faecal indicator organism world-wide (Schardinger, 1892); our whole water hygiene concept is based on this organism and its inability to grow in the environments. Many studies investigating the growth and survival of enteric pathogens *in vitro* in various types of waters exist. In such experiments, a water sample is sterilized by heat

treatment or 0.22 µm filtration, the pathogens are then inoculated at concentrations of around 10^6 mL^{-1} or higher and their behaviour is monitored using different methods such as conventional cultivation or fluorescence "viability staining" (e.g., Munro *et al.,* 1987; Buswell *et al.,* 1998). In most cases the plateable or "viable" cell concentration did not increase but rather decrease over time, suggesting that the inoculated organism was unable to grow but died-off in the water tested. Often a small population persisted and was detectable even after days/weeks, suggesting that certain individuals were able to survive for a long time under the prevailing conditions. Furthermore, many authors correlate the shape of the die-off curve with the survival capability of a pathogen; a steep decline indicates bad survival, whereas well surviving strains are characterized by a flat curve (Roszak & Colwell, 1987). It should be noted that preceding growth conditions of the inoculum have a considerable influence on the outcome of such experiments (Gauthier *et al.,* 1987), a fact that is often neglected. All these experiments strengthened the common view that classic enteric bacteria cannot grow in the environment but the populations detected are maintained by supply from their primary habitats. However, there are a number of critical issues about the experimental set-ups used for investigating growth of pathogens in water, which are questioning the results obtained and conclusions drawn (see also chapter 3).

In contrast to this common view, several indications exist that *E. coli* can indeed grow in the environment. *In vitro* studies exist that demonstrate growth of the bacterium on natural AOC, specifically on excreted products of algae (McFeters *et al.,* 1978), as well as on highly diluted complex media (Camper *et al.,* 1991). Furthermore, the frequent detection of the bacterium *in vivo* from unpolluted tropical rivers, where annual water temperatures and nutrient concentrations are elevated, led to the speculation that *E. coli* is an active member of the microbial community in these ecosystems and, thus, not useful as an index organism (Winfield & Groisman, 2003). However, *E. coli* is also reported to be autochthonous in soil and sediments of Lake Superior, USA, suggesting that the bacterium can even grow in the cold climates of the Northern hemisphere (Ishii *et al.,* 2006, 2007).

Whereas the question whether *E. coli* can grow in the environment is vigorously debated, extensive research on its physiology during growth at low carbon concentrations was performed in carbon-limited chemostat cultures. Under such conditions, cells were able to immediately oxidize 43 different substrates (Ihssen & Egli, 2005) indicating that the bacterium is able to grow simultaneously with a multitude of different compounds - an important characteristic for growth in the environment. Furthermore, it was demonstrated that growth of *E. coli* in C-limited continuous

culture on a substrate mixture of six different sugars resulted in lower individual residual steady-state sugar concentrations in comparison to growth on single compounds alone (Lendenmann *et al.,* 1996). Hence, "mixed substrate growth" enables cells to sum up their growth rates form individual compounds leading to relatively fast growth even at very low individual substrate concentrations. Furthermore, long-term cultivation of *E. coli* K12 in glucose-limited continuous culture showed that steady-state glucose concentration considerably decreased from some 500 µg glucose L^{-1} to as low as 30 µg L^{-1} (at a dilution rate of 0.3 h^{-1}) indicating an extensive adaptation potential of the bacterium to grow at low substrate concentrations (Wick *et al.,* 2002). However, this observation was ascribed to the selection of mutants displaying high affinity uptake systems for the selected sugar and it is questionable whether such adaptation mechanisms are relevant for growth in the environment where many substrates limit growth simultaneously and the selection pressure of a single compound is, thus, considerably reduced. This is underpinned by the observation that natural *E. coli* isolates from drinking water display K_s values for glucose from 400 - 1500 µg L^{-1} which are very similar to the reported value of 500 µg L^{-1} for the "laboratory strain" *E. coli* K12 (Ihssen *et al.,* 2007). Many researchers focused on the molecular mechanisms determining the physiology of *E. coli* during slow carbon/energy-limited growth. They demonstrated that several main global regulators such as cyclic adenosine-monophosphate (cAMP) or the alternative sigma factor RpoS act on a broad scale. Whereas the former is promoting "catabolic flexibility" (Franchini, 2006), the latter is specifically relevant for the behaviour of the bacterium under starvation conditions (Lange & Hengge-Arnois, 1991). For example, it was shown that RpoS expression is negatively correlated with the specific growth rate (Ihssen & Egli, 2004) and that RpoS dependent promoter elements are dominating during the physiological response of *E. coli* inoculated into artificial seawater (Rozen *et al* 2001).

Methodology to study bacterial growth at low cell and low nutrient concentrations

Studying growth of bacteria in natural waters *in vitro* requires working with low cell and at low nutrient concentrations and, therefore, demands specific experimental set-ups. The basic principles were developed some 30 years ago with the bio-assay for quantifying AOC in drinking water (van der Kooij *et al.,* 1982). In this assay, a pure culture is batch-grown in pasteurized water samples and the produced cells are then correlated to the organic carbon consumed by the culture. The original assay was later modified and optimized in several ways by different research groups (e.g., van der Kooij, 1992, Hammes & Egli, 2005). However, the essential procedure was always the same and is based on the following points:

(1) Avoid AOC contamination
(2) Work sterile
(3) Start with low initial cell concentrations
(4) Use appropriate bacterial enumeration methods

Since water is contains low concentrations of AOC, experiments are prone to organic carbon contamination and specific controls are, hence, vital but frequently not performed (see Hammes *et al.,* 2010). Furthermore, inactivation or eliminating the indigenous microbial flora is essential when investigating specific factors governing pathogen growth in water. Often 0.22 µm filtration was used to remove autochthonous bacteria from a water sample (e.g., Rice *et al.,* 1990). However, this procedure is not sufficient to sterilize the water; up to 10 % of autochthonous bacteria can pass the filter and grow again on the AOC available (Wang *et al.,* 2007). Such bacteria do not grow on conventional agar plates, which might explain why they were not detected during experimentation. Hence, in such experiments either little or even no AOC was left for the growth of inoculated pathogens, or they encounter high competition from the filter-passing bacteria (Figure 1.5 A).

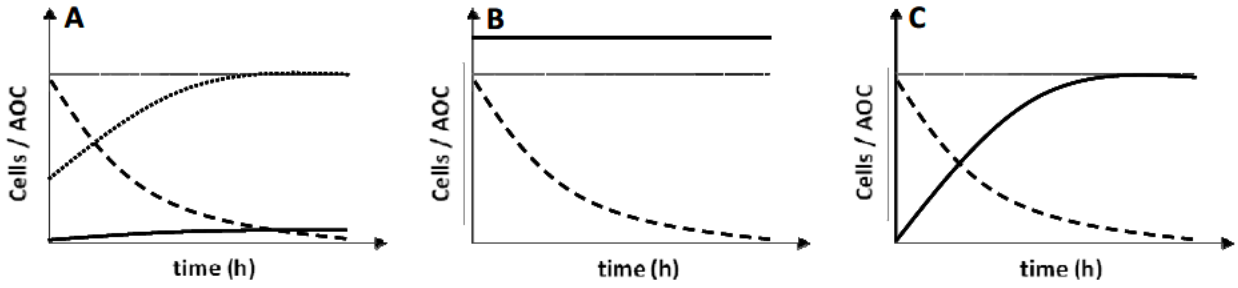

Figure 1.5. Different experimental set-ups, which were commonly used to study growth of pathogens (solid line) in water on natural assimilable organic carbon (AOC; dashed line). In the approach presented in Panel A the water was only 0.22 µm-filtered and the passing autochthonous cells (dotted line) were growing on the AOC available. Only little growth or even no growth of the inoculated pathogen was observed. In the experiments presented in Panel B the initial inoculum of pathogens was higher than the growth supporting nutrients and, thus, no growth (based on microscopic total counts) or even die-off (based on conventional plating methods) was recorded. Panel C illustrates the approach used in this study, where the water is appropriately sterilized and pathogens were inoculated at low cell concentrations. Growth, determined by the available AOC, can be observed. Thin dotted line: final theoretical growth of inoculated pathogen.

Moreover, the size of the inoculum has to be adjusted to the amount of available AOC. A concentration of 100 µg L^{-1} support growth of approximately 10^6 bacteria mL^{-1} (Hammes & Egli, 2005). Thus, initial bacterial concentrations below 10^4 mL^{-1} are appropriate for batch growth studies with natural waters; this fact was often overlooked in pathogen growth potential studies. Bacteria

were often inoculated 10 - 100 fold higher than the growth supporting nutrient concentration. Hence, no or only very little growth, based on microscopic total counts, or even die-off, when conventional cultivation based methods were used, was observed (e.g., Banning *et al.,* 2002; Ravva & Korn, 2007; Figure 1.5 B). After growth, bacteria have to be enumerated. For pure cultures, plating methods are commonly used. However, the procedure is time-consuming and increasingly replaced by cultivation-independent methods such as fluorescence-staining in combination with microscopic detection (e.g., Kirschner *et al.,* 2008). In this respect, flow cytometry-based methods are ideal because they enable counting of bacteria at a high throughput and allow multiparametric data acquisition of single cells. Flow cytometry is increasingly applied in aquatic microbial research in general (Vives-Rego *et al.,* 2000). For example, it was used to assess bacterial physiology *in situ* (Davey & Kell, 1996), revealed the existence of so called low nucleic acid (LNA) bacteria (Gasol *et al.,* 1999) and was furthermore applied to study growth rates of bacteria introduced into aquifers (Mailloux & Fuller, 2003). In addition, the possibility of cell sorting enables further investigations of individual bacterial populations (Czechowska *et al.,* 2008).

Objectives of the thesis

As outlined above, we are just at the beginning to understand the mechanisms governing growth of bacteria in water and the physiology involved for both microorganisms of hygienic interest such as pathogens, as well as for aquatic bacterial communities. Furthermore, previous results on pathogen growth were often prone by wrong experimental set-ups (illustrated in Figure 1.5). Hence, the separation of pathogens into two distinct groups (Table 1.1) with respect to their ability to grow in the environment, namely "environmental pathogens" and classic enteric pathogens, is doubtful. Therefore, in this thesis I intended to explore factors governing growth of pathogenic bacteria in water. The work should contribute to an improved microbial risk assessment, allowing to include pathogen growth in a quantitative manner. Furthermore, the obtained results and developed concepts should improve the knowledge on the mechanisms controlling microbial growth and competition in oligotrophic ecosystems in general.

To achieve this, selected pathogenic strains, namely *E. coli* O157, *V. cholerae* O1 and *P. aeruginosa*, as well as bacterial communities derived from different types of freshwater were grown on natural AOC *in vitro* under controlled conditions. The experimental set-up took care of the four critical points mentioned above and growth was monitored using various flow cytometry-based methods. The detailed questions I was asking were the following:

(1) Does the strict separation between two pathogenic groups, namely those which can grow in the environment and those lacking this ability hold true?

(2) Are the growth properties under oligotrophic conditions distinctly different for the pathogens selected?

(3) How do they perform in comparison to autochthonous bacterial communities?

(4) What are the dynamics during competitive growth between pathogens and autochthonous bacterial communities?

(5) Is it possible to mathematically explain the dynamics during growth and to predict competition outcome?

(6) Is it possible to integrate pathogen growth into microbial risk assessment by developing an assay for assessing water quality with respect to its pathogen growth potential?

2. Growth of *Vibrio cholerae* O1 Ogawa Eltor in freshwater

Abstract

Growth of *Vibrio cholerae* O1 Ogawa Eltor was studied with a growth assay in which autoclaved and filtered (0.22 µm) freshwater was inoculated at low cell density (5 x 10^3 cells mL^{-1}) and proliferation was followed with flow cytometry. Against the common view, *V. cholerae* was able to grow extensively in different kinds of freshwater. The bacterium multiplied in river water, lake water and effluent of a wastewater treatment plant up to a cell density of 1.55 x 10^6 cells mL^{-1}. In these samples, apparent assimilable organic carbon (AOC_{app}) concentrations ranged from 52 up to 800 µg L^{-1} and the results demonstrate a positive trend between the AOC_{app} concentration and final cell concentration suggesting that AOC was a key parameter governing growth of *V. cholerae*. No growth was observed in waters (tap- and bottled drinking water) containing less than approximately 60 µg AOC_{app} L^{-1}. When growing on identical lake water at different temperatures (20, 25 and 30 °C) the maximum specific growth rates (μ_{max}) of pure cultures of *V. cholerae* achieved were 0.23 h^{-1}, 0.32 h^{-1} and 0.39 h^{-1}, respectively. In addition, growth was characterised in lake water samples amended with different concentrations of NaCl. The highest μ_{max} of *V. cholerae* was recorded at moderate salinity levels (5 g NaCl L^{-1}, μ_{max} = 0.84 h^{-1}), whereas at 30 g NaCl L^{-1} (μ_{max} = 0.30 h^{-1}) or 0 g NaCl L^{-1} (μ_{max} = 0.40 h^{-1}) specific growth rates were significantly reduced. In the water tested here, μ_{max} of *V. cholerae* was always around 50 % of that exhibited by a freshwater community of indigenous bacteria enriched from the water sampling site. Direct batch competition experiments between *V. cholerae* and the lake water bacterial community were performed at different temperatures in which *V. cholerae* was enumerated in the total community using fluorescent-surface antibodies. In all cases *V. cholerae* was able to grow and contributed to around 10 % of the final total cell concentration of the community. No significant effect of temperature was observed on the outcome of the competition. Mathematical modelling of the competition at the different temperatures based on the calculated μ_{max} values confirmed these experimental observations. Our results demonstrate that *V. cholerae* is not only able to survive, but also able to grow in freshwater samples. In our experiments the bacterium was able to use a large fraction (12 % - 62 %) of the AOC_{app} available to the bacterial AOC-test community and has the ability to gain access to the substrates present in freshwater even in competition with an autochthonous bacterial lake water consortium.

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Introduction

Vibrio cholerae is able to adapt to both copiotrophic and oligotrophic environments: on the one hand, *V. cholerae* can promote disease by extensive growth in the human intestine, on the other hand it is also found amongst the autochthonous microbial flora of natural aquatic systems (West & Lee, 1982). In natural waters the bacterium can be present in both a free-living state (Worden *et al.,* 2006) or attached to copepods, zooplankters and algae (Lipp *et al.,* 2002; Reidl & Klose, 2002).
Previous laboratory studies have emphasized the influence of salinity on growth and, hence, the bacterium was considered to be naturally abundant in waters characterised by moderate or high salinity (Miller *et al.,* 1982; Singleton *et al.,* 1982a,b). Accordingly, *V. cholerae* O1 was frequently detected in estuary and coastal waters, and growth in sterile-filtered natural seawater under defined laboratory conditions has been reported (Binsztein *et al.,* 2004; Louis *et al.,* 2003; Mourino-Perez *et al.,* 2003; Worden *et al.,* 2006). Additionally, laboratory studies using artificial seawater demonstrated growth of *V. cholerae* O1 down to a salinity of 5 g L^{-1} (Singleton *et al.,* 1982a,b). However, *V. cholerae* O1 has been detected frequently in freshwater as well (Yamai *et al.,* 1996; Borroto, 1997), even in the absence of faecal index organisms (Bourke *et al.,* 1986; Jesudason *et al.,* 2000). Although freshwater systems are considered to be an environmental reservoir for *V. cholerae* (Shapiro *et al.,* 1999) there is, as far as we are aware, no information available demonstrating the growth of this bacterium in freshwater.

In addition to salinity, temperature is reported to be a second important parameter controlling growth of *V. cholerae* in estuarine environments. Laboratory experiments showed a positive correlation between temperature and the metabolic activity of *V. cholerae* O1 in defined sea salt solution in a temperature range from 10 °C to 25 °C (Singleton *et al.,* 1982a). Furthermore, a more frequent detection of *V. cholerae* O1 in estuary waters and a higher morbidity rate among people in Bangladesh was shown to be associated with increasing temperatures (Louis *et al.,* 2003; Rodo *et al.,* 2002).

Next to salinity and temperature, nutrients are considered to control growth of *V. cholerae* in aquatic systems (Colwell, 1996). In natural waters a key parameter governing microbial growth is assimilable organic carbon (AOC) (van der Kooij, 1992). AOC is the small fraction (0.1 % - 10 %) of the dissolved organic carbon, which is readily available for consumption by microorganisms, resulting in cell proliferation (Hammes & Egli, 2005). AOC is composed of low molecular weight compounds such as amino acids, sugars or organic acids (Egli, 1995; Münster, 1993). It is

constantly produced through photosynthesis, viral lysis of bacterial cells, biological and chemical hydrolysis of natural organic matter and it is continuously consumed by the microbial flora. Residual AOC concentrations between 1 µg L^{-1} up to 2000 µg L^{-1} can typically be observed in natural waters (LeChevallier *et al.,* 1991). AOC has been directly linked to bacterial regrowth in drinking water distribution systems (van der Kooij, 1992). A relationship between the occurrence of coliform bacteria and AOC concentrations has been also shown in a full scale study of drinking water systems (LeChevallier *et al.,* 1996). As most aquatic environments are usually oligotrophic and carbon/energy-limited (Morita, 1997) bacteria are continuously competing for AOC constituents. AOC is therefore an important factor in determining the microbial community composition in aquatic ecosystems.

Contaminated drinking water is one of the important transmission routes of the enteric pathogen *V. cholerae* and, therefore, data on the growth characteristics of this bacterium in freshwater are essential. The better the behaviour of *V. cholerae* in the natural environment is understood, the better hygienic measures can be taken in order to prevent the spreading of this disease. For this reason, the growth of *V. cholerae* in freshwater was investigated and the influence of several selected environmental factors (salinity, AOC concentration and temperature) was studied. Additionally, the growth of *V. cholerae* on nutrients in freshwater in competition with an autochthounous lake water bacterial community was investigated.

Materials and Methods

Strains

Vibrio cholerae O1 (Biovar Eltor, serotype Ogawa, strain Nent 720-95) and *Salmonella typhimurium* ATCC 14028 were used in this study.

Preparation of carbon-free materials

Carbon-free glassware (bottles and vials) was prepared as described in Greenberg *et al*. (1993). In short: all glassware was first washed with common detergent, and thereafter rinsed three times with deionised water. These were then submerged overnight in 0.2 N HCl and subsequently rinsed with deionised water again and finally air-dried. The bottles and vials were heated in a Muffel furnace at 550 °C for at least 6 h. Teflon coated screw caps for the glassware were similarly washed and treated with acid. Caps were thereafter soaked in a 10 % sodium persulphate solution at 60 °C for at least 1 h, rinsed three times with deionised water and finally air-dried.

Pre-cultivation of bacteria and growth in natural water

V. cholerae and *S. typhimurium* were cultivated in carbon-limited minimal medium (Ihssen & Egli, 2004) containing 10 mg glucose L^{-1}. Bacterial cells were sampled after 4 days of incubation during late stationary phase and directly inoculated to an initial concentration of 5 x 10^3 cells mL^{-1} into carbon-free glass vials (40 ml) that contained autoclaved and 0.22 µm-filtered freshwater (30 mL) originating from different sources. These vials were incubated at 30 °C and the total cell concentration was determined directly after inoculation (time 0) and at time 96 h when the stationary phase had been reached.

Preparation of natural water

Natural water was sampled with a 500 mL Duran® flask (Schott, Wertheim/Main, Germany) and autoclaved within 30 minutes. Subsequently, aliquots of 30 mL were filtered using a 50 mL Luer-Lok™ Syringe (BD, Franklin Lakes, N. J., USA) through a 0.22 µm Millex® syringe filter (Millipore, Billerica, MA, USA) into 40 mL carbon-free glass vials capped with a carbon-free PTFE/silicone septa cap (Supelco, Bellefonte, PA, USA). It should be pointed out that autoclaving (20 min at 121 °C) was used as the method for sterilization, since it has been shown that filtration processes allow the passage of a fraction of bacteria present in freshwater (Hahn, 2004). After sterilization, samples were filtered in order to remove crystalline particles formed during autoclaving, which can interfere with the flow cytometric analysis. Different types of freshwater

were used: tap water (community of Dübendorf, Switzerland), river water (Glatt River, Dübendorf, Switzerland), lake water (Lake Greifensee, Switzerland) and wastewater treatment plant effluent (WWTP - Glattbrugg, Switzerland). In addition, a dilution series of natural water (0, 10, 25, 50, 75 and 100 % of Lake Greifensee water) was prepared using filtered (0.22 µm Millex® filters) mineral water (Evian, France) as the diluent. These dilutions were subsequently autoclaved and again 0.22 µm–filtered.

AOC determination

AOC was determined with a batch growth assay as described previously (Hammes *et al.,* 2006; Hammes & Egli, 2005). In short: autoclaved and filtered water samples (30 mL) were inoculated with 10 µL (1 x 10^4 cells mL^{-1} final concentration) of a bacterial AOC-test community. These suspensions were then incubated at 30 °C for 96 h (until stationary phase was reached) and the resulting growth was measured with flow cytometry. The AOC inoculum originated from river water (Chriesbach river, Dübendorf, Switzerland) was prepared as described in Hammes and Egli (Hammes & Egli, 2005). The same bacterial community was used for all AOC determinations throughout the present study. As standard quality control prior to use, the performance of this inoculum was compared to bacterial AOC-test communities used in previous studies in our group (Hammes *et al.,* 2006), using different types of natural surface waters as media. A difference of less then ±10 % in the average AOC values was deemed acceptable for use. AOC (µg L^{-1}) is estimated from cell concentrations (cells mL^{-1}) using a theoretical conversion factor (Hammes *et al.,* 2006; van der Kooij, 2002), we will henceforth use the term "apparent AOC" (AOC_{app}) to express the data obtained with this method (Eq. 1). All assays were performed in triplicate. The detection limit of the method was 10 – 20 µg AOC_{app} L^{-1} and the average standard error was ± 10 %.

$$AOC_{app}\ [(\mu g\ C)\ L^{-1}] = \frac{(\text{net grown cells})\ L^{-1}}{1 \times 10^7\ \text{cells}\ (\mu g\ C)^{-1}} \qquad \text{(Eq. 1)}$$

Enumeration of total cell concentration

Absolute cell counting was performed with flow cytometry. 10 µL of SYBR®green (Molecular Probes, Basel, Switzerland), 100x diluted in dimethylsulfoxid (Fluka Chemie AG, Buchs, Switzerland), was added to 1 mL of a bacterial suspension and incubated in the dark at room temperature for 15 minutes before analysis. All samples were measured on a PASIII flow cytometer (Partec, Münster, Germany) equipped with a 25 mW argon laser emitting at a fixed wavelength of

488 nm and volumetric counting hardware. Green fluorescence signals were collected at 520 nm. The detection limit is 1000 cells mL^{-1} with an average standard error of ± 5 %.

Specific detection of *V. cholerae* O1 by flow cytometry with antibodies

A new method for specific detection of *V. cholerae* O1 with immunostaining was developed. Samples (1 ml) collected from growth experiments were UVC-irradiated in a collimated beam apparatus (Metanor AG, Regensdorf, Switzerland) with a fluence of 96 J m^{-2} to arrest growth. Then the sample was transferred into a flow cytometry vial (Sarstedt, Nürnbrecht, Germany) and 10 µL of the *V. cholerae* O1 DFA reagent (a fluorescent surface antibody) was added (New Horizons Diagnostics Corporation, Columbia, MD, USA). The bacteria/antibody suspension was incubated for 30 minutes at 35 °C to let the antibody pre-adsorb to the bacterial surface. Subsequently, suspensions were further incubated for 12 - 17 h at room temperature in the dark to obtain a strong fluorescent signal. Finally, the concentration of immunostained *V. cholerae* O1 was determined by flow cytometry (as described above). To evaluate the staining efficiency, autoclaved and filtered freshwater samples were spiked with different concentrations of *V. cholerae* in the range of 10^3 cells mL^{-1} to 10^5 cells mL^{-1} and the above procedure was applied immediately. Results were compared with cell counts obtained flow cytometrically with SYBR®green-staining. A linear correlation (R^2 = 0.99) was observed over the whole range of cell concentrations tested. As a negative control 10^6 cells mL^{-1} of the lake water bacterial community used for competition experiments were treated in exactly the same manner with the fluorescent antibody. No fluorescence was detected and thus it can be concluded that significant cross reaction of the antibody with the lake water bacterial community did not occur. Note that staining efficiency of all immunostained samples was evaluated with epifluorescence microscopy as well.

Growth of *V. cholerae* at different salt concentrations

For growth kinetic experiments at different salt concentrations, different volumes of an autoclaved 25 % NaCl stock solution were added to autoclaved and 0.22 µm-filtered freshwater samples of Lake Greifensee (Switzerland) to achieve the following final concentrations of NaCl: 0, 1, 5, 10, 20 and 30 g L^{-1}. The NaCl-amended water was subsequently inoculated with stationary phase *V. cholerae* bacteria at an initial concentration of about 5 x 10^3 cells mL^{-1} and incubated at 30 °C. Cells of *V. cholerae* for inoculation were pre-grown in autoclaved and 0.22 µm-filtered freshwater of Lake Greifensee (Switzerland) amended with the same NaCl concentration as used for each specific experiment. The growth of the indigenous lake water bacterial community in autoclaved and 0.22 µm-filtered freshwater was recorded by removing 1 ml samples at regular intervals and

total cell concentration was measured as described above. All assays were performed in triplicate samples. The specific growth rate (μ) based on cell concentration increase was determined as follows:

$$\mu = [\ln (N_t) - \ln (N_0)] / \triangle t \qquad \text{(Eq. 2)}$$

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. We have measured an increase in cell concentration and not in biomass, thus, growth could also be described using the cellular division rate (k), defined as the number of divisions per time unit. Since k is not often used and values differ from that of μ ($\mu = \ln 2 * k$), we decided to use the specific growth rate (Equation 2) throughout the whole study.

Competition experiments

For these experiments a lake water bacterial community of natural origin and *Vibrio cholerae* O1 grown on autoclaved and 0.22 μm-filtered lake water were used. The lake water bacterial community was sampled from the surface water (4.1 mg L^{-1} DOC; 22 °C, pH = 8.3) of the eutrophic Lake Greifensee (Switzerland) and pre-cultivated as follows: 30 mL of autoclaved and 0.22 μm-filtered lake water was inoculated with 100 μL of untreated lake water from the same original sample. Suspensions were incubated at 20, 25 and 30 °C, respectively, for one week and subsequently stored at 4 °C until further use. Culture density of the community was monitored with flow cytometry (see above).

Carbon-free 50 ml glass vials containing 30 ml of autoclaved and 0.22 μm-filtered freshwater from Lake Greifensee were inoculated with either *V. cholerae* or the lake water bacterial community at an initial concentration of about 5 x 10^3 cells mL^{-1}, each. The cells used as an inoculum were precultured on the same lake water at 30 °C and growth was monitored by recording the total cell concentration flow cytometrically at regular intervals. Cells were then sampled in the exponential growth phase and directly inoculated into competition assays at 5 x 10^3 cells mL^{-1} of each partner (thus 10^4 cells mL^{-1} initial total cell concentration). Assays were incubated at 30 °C and performed in triplicates. Samples of 2 mL were collected at different time points throughout the competition experiment. Of these samples 1 mL was used for determination of the total cell concentration and 1 mL for immunological determination of the cell concentration of *V. cholerae*.

For competition experiments performed at different temperatures the above pre-cultivation was performed at the relevant temperatures. Starting of the competition assays were performed as described above. Samples were incubated at 20, 25 and 30 °C, respectively, for 48 hours at which time the stationary phase had been reached. Subsequently, final concentrations of total cells and of *V. cholerae,* respectively, were determined as described above. Additionally, separate vials, containing the same autoclaved and 0.22 µm-filtered lake water as used for the competition experiments, were inoculated with exponential growth phase cells from the pre-cultures of either *V. cholerae* or the lake water bacterial community at an initial concentration of 5 x 10^3 cells mL^{-1}. The vials were then incubated at 20, 25 and 30 °C, respectively. Samples were taken at different time points from batch cultures and subsequently total cell concentrations were measured flow cytometrically. Specific growth rates (µ) were determined as described above (Eq. 2).

Relative cell number yield

The relative cell number yield for *V. cholerae*, $Y_{Vc/AOC}$, for growth on identical freshwater AOC is defined as Eq. 3. The total concentration of cells formed in freshwater samples was determined for *V. cholerae* and the bacterial AOC-test community in separate assays (as described above).

$$Y_{Vc/AOCapp} = \frac{\text{(cells formed of } V.\ cholerae\text{) } mL^{-1}}{\text{(cells formed of a bacterial AOC-test community) } mL^{-1}} \qquad \text{(Eq. 3)}$$

Simulation software

The simulation of growth and parameter estimation was carried out using AQUASIM software (Reichert, 1998). AQUASIM allows estimating parameters by fitting predicted model data to experimental results. This feature was used to estimate kinetic parameters, which resulted in the best fit of simulations (s_{pred}) and measurements (s_{obs}). The program estimated the best fit by minimising χ^2 values ($\chi^2=\sum((s_{obs}-s_{pred})/\sigma)^2$). For the estimation the standard deviation (σ) was set to 10 % (if not determined experimentally).

Results

Growth of *V. cholerae* in different types of freshwater

Stationary phase cells of *V. cholerae* were inoculated into autoclaved and 0.22 µm-filtered mineral, tap, river, lake, and effluent water from a wastewater treatment plant in order to investigate its growth potential in different types of freshwater. Total growth was measured after 96 h by enumerating total cell concentration with flow cytometry. With the exception of mineral and tap water, *V. cholerae* showed extensive growth in all the freshwater samples tested (reaching final cell concentrations from 2.9 x 10^5 mL^{-1} to 1.55 x 10^6 mL^{-1}). In addition, the growth of *Salmonella typhimurium* was also tested in the same waters. In contrast to *V. cholerae*, *S. typhimurium* was unable to grow in any of the natural waters tested.

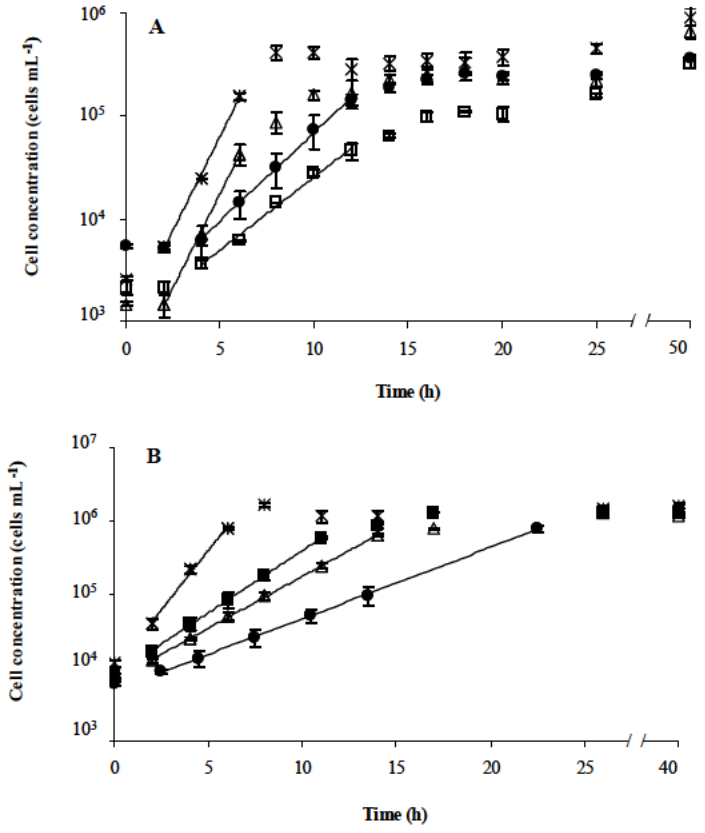

Figure 2.1. Selected growth curves of *V. cholerae* cultivated in autoclaved and 0.22 µm-filtered freshwater. Panel A shows growth of *Vibrio cholerae* in freshwater amended with different concentrations of NaCl (g L^{-1}); 0 (□); 5 (△); 30 (●) g NaCl L^{-1}. For comparison growth for the lake water bacterial community at 0 g NaCl L^{-1} is shown (×). Panel B shows growth of *V. cholerae* in lake water at different temperatures (°C); 20 (●), 25 (△), 30 (■). For comparison the growth curve for the lake water bacterial community at 30 °C is shown as well (×). The error bars indicate the standard deviation for triplicate samples. For the whole set of data see supplementary material.

Effect of salinity on the growth of *V. cholerae*

When *V. cholerae* cells were inoculated into autoclaved and 0.22 µm-filtered lake water amended with different concentrations of NaCl exponential growth of *V. cholerae* was observed after a short lag phase of about 2-4 hours (Figure 2.1. A). The growth pattern of *V. cholerae* in freshwater followed the typical pattern observed for pure and mixed microbial cultures growing on complex

media, consisting of an initial exponential growth phase at a constant μ, followed by a continuously decreasing specific growth rate until stationary phase is reached. A considerable fraction of the cells was formed in the exponential growth phase (16 - 80 %), where cells exhibited a constant μ. In the batches amended with NaCl, *V. cholerae* showed occasionally a prolonged decelerating phase.

In Figure 2.2 the maximum specific growth rates (μ_{max}) exhibited in the initial growth phase and the final cell concentration formed by *V. cholerae* are visualised as a function of NaCl concentration. The highest μ_{max} was achieved at moderate salinity levels (μ_{max} at 5 g L^{-1} NaCl was as high as that of the lake water bacterial community in freshwater), whereas at higher or lower salt concentrations, μ_{max} was significantly reduced (Figure 2.2). Furthermore, the maximum cell concentrations formed by *V. cholerae* followed a similar pattern as a function of the salt concentration as μ_{max}, with the highest cell concentration reached at moderate salinity (Figure 2.2).

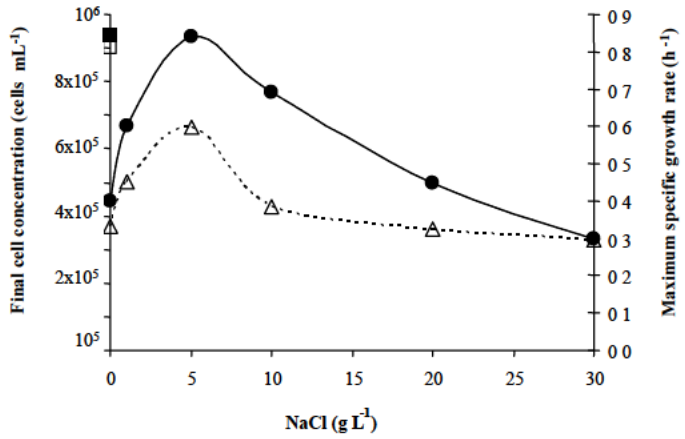

Figure 2.2. Growth of *V. cholerae* in autoclaved and 0.22 μm-filtered river water amended with different concentrations of NaCl (g L^{-1}). Maximum specific growth rates (h^{-1}) of *V. cholerae* (•) and the maximum cell concentration of *V. cholerae* (△) reached as a function of the NaCl concentration are shown. For comparison μ_{max} (■) and the final cell concentration (□) for the lake water bacterial community at 0 g NaCl L^{-1} in the same water are displayed as well. The maximum specific growth rate was determined from the linear part of the exponential growth curves shown in Figure 1 (and supplementary info).

Relationship between AOC_{app} and growth of *V. cholerae*

Growth can be impacted by both the quality and quantity of nutrients. To assess this we compared *V. cholerae* growth in a dilution series of Lake water (AOC quantity only varied) and in water from different sources (where both, AOC quantity and quality, vary). To assess the effect of AOC quantity water from Lake Greifensee was diluted with a water sample that did not support growth of *V. cholerae* (Evian, 52 AOC_{app} L^{-1}). This resulted in a dilution series that ranged from ca 52 to 300

AOC_{app} L^{-1}. A linear correlation ($r^2 = 0.98$) between AOC_{app} concentration originating from Lake Greifensee and final cell concentration of *V. cholerae* was observed (Figure 2.3 A).

In a subsequent experiment, water from different sources was sampled, the AOC_{app}-concentration was determined and the growth of *V. cholerae* was examined (Figure 2.3 B). Also here, a positive trend between AOC_{app} concentration and the total growth of *V. cholerae* was observed, although the correlation was much lower ($r^2 = 0.36$). Nevertheless, with increasing AOC_{app} concentrations higher final cell concentrations of *V. cholerae* were formed. The cell number yields of *V. cholerae* in relation to that of the bacterial AOC-test community (Eq. 2), were rather variable, ranging from $Y_{Vc/AOCapp}$ 0.12 to 0.62 with an average of 0.28. Waters containing very low amounts of AOC_{app} (tap water with 44 µg L^{-1}, and bottled drinking water with 52 µg L^{-1}) did not support growth of *V. cholerae*.

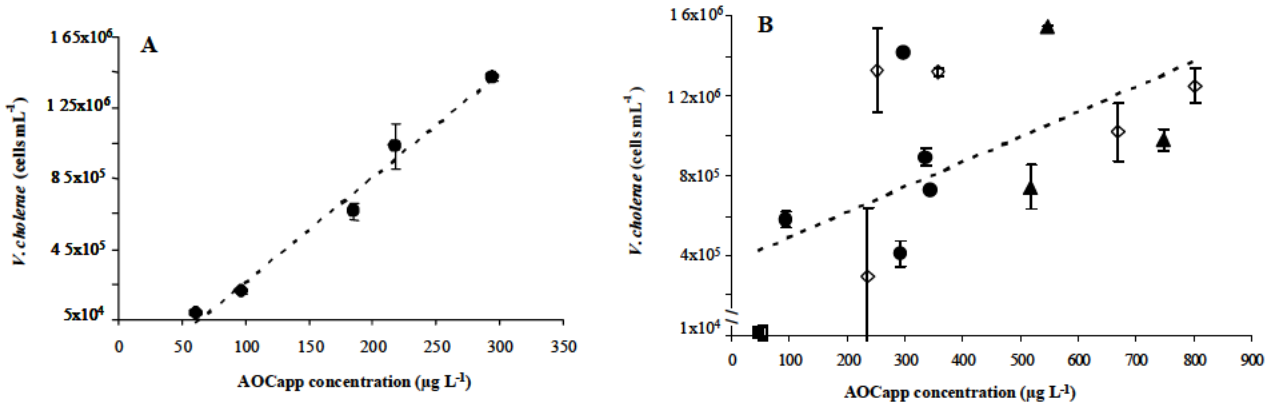

Figure 2.3. Correlation between AOC_{app} concentration and the final cell concentration of *V. cholera* reached in the stationary phase of batch cultures. The final cell yield of *V. cholerae* is dependent on both the AOC concentration and the composition or quality of the AOC present in a water body. Panel A shows the correlation between growth of *V. cholera* (•) and different concentrations of AOC_{app} of the same quality. Autoclaved, sterile-filtered lake water was used. The concentration of AOC_{app} resulting from the diluent (bottled mineral water) was subtracted in the individual dilutions, indicated in the x-axis. It is therefore not considered in the linear regression analysis ($r^2 = 0.98$). Panel B shows the correlation between AOC_{app} concentration and growth of *V. cholerae* on autoclaved sterile-filtered freshwater from different sources (i.e. with AOC of different quality). The different water samples (tap water Eawag, Dübendorf (■), bottled drinking water (□), river water (●), Lake Greifensee water (◊) and effluent of a sewage treatment plant (▲)) were collected on different days. Error bars indicate the standard deviation for triplicate samples. AOC_{app} concentration was determined according to Hammes & Egli (2005) with a standard bacterial AOC-test community as described in Materials and Methods.

Competition of *V. cholerae* with a lake water bacterial community

The ability of *V. cholerae* to compete in batch culture with a lake water bacterial community (LWBC) was tested in autoclaved and filtered lake water at 30 °C (Figure 2.4). The results show that *V. cholerae* was able to use a part of the available substrates for growth and could even grow when competing with the indigenous lake water bacterial community (starting from a similar initial concentration of 4.13×10^3 *V. cholerae* cells mL^{-1} and 2.84×10^3 cells mL^{-1} of a lake water bacterial community, respectively). A simultaneous exponential increase in the concentration of cells was observed for both *V. cholerae* and total bacterial cells. In these experiments a lag phase was successfully avoided by pre-culturing the two inocula in the same freshwater sample as used also in the competition experiment and by inoculating cells from the exponential growth phase. The average final cell concentration of *V. cholerae* after 22 hours (stationary phase) was $9.73 \times 10^4 \pm 1.31 \times 10^4$ cells mL^{-1}, which represents 9.7 % of the total bacterial concentration ($1 \times 10^6 \pm 3.50 \times 10^4$ cells mL^{-1}).

To test whether or not it is possible to predict the outcome of the competition with a simple growth model, the individual maximum specific growth rates of *V. cholerae* and of the lake water bacterial community were determined in a separate experiment during growth in sterilized, identical freshwater samples and were found to be 0.34 h^{-1} and 0.50 h^{-1} for *V. cholerae* and the lake water bacterial community, respectively. It was possible to simulate the population dynamics by applying the most simple growth model ($\mu=\mu_{max}$) using the previously determined μ_{max} of the pure cultures over the whole batch experiment until all the available nutrients were used up (Figure 2.4). This is a strong hint that this is a case of "pure and simple" competition for nutrients and that no other interaction was taking place between *V. cholerae* and the lake water bacterial community.

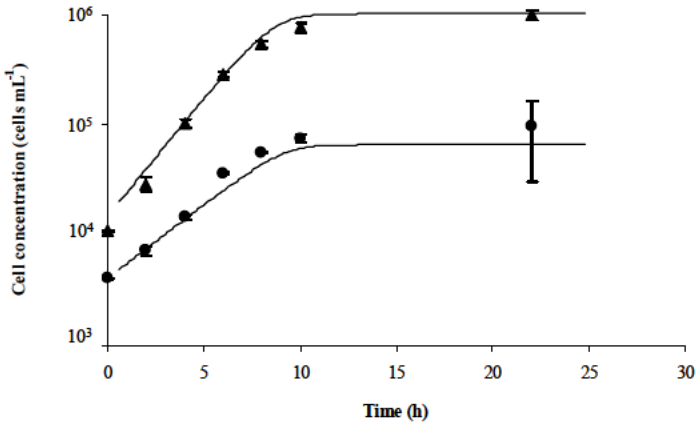

Figure 1.4. Growth of *V. cholerae* in autoclaved and 0.22 µm-filtered lake water in competition with a lake water bacterial community at 30 °C in batch culture. Experimental data of *V. cholerae* (●), total counts (▲) and mathematical simulations (solid line) using μ_{maxVc} = 0.34 h^{-1} and $\mu_{maxLWBC}$ = 0.50 h^{-1}. Error bars indicate the standard deviations for triplicate samples.

Effect of temperature on growth of *V. cholerae* and competition with the lake water bacterial community

To investigate the effect of temperature on growth and competition similar experiments were performed at 20, 25 and 30 °C. First, batches of autoclaved and 0.22 µm-filtered lake water were inoculated separately with either a lake water bacterial community or *V. cholerae*, respectively, and μ_{max} was determined. Selected corresponding growth curves are presented in Figure 2.1 B and the respective values for μ_{max} are listed in Table 2.1. In all these experiments an extended phase of exponential growth at μ_{max} was observed for both *V. cholerae* and the lake water bacterial community with only 50 % of the total cells formed in the second decelerating phase of growth.

The fact that the ratio of the maximum specific growth rates of *Vibrio cholerae* and the lake water bacterial community (μ_{maxVc} / $\mu_{maxLWBC}$) was virtually the same at all temperatures suggests little advantage or disadvantage of *V. cholerae* to compete for nutrients with the lake water bacterial community within the temperature range tested.

Table 2.1. Competition in batch culture of *V. cholerae* (Vc) and a lake water bacterial community (LWBC) for nutrients present in lake water at different temperatures. Autoclaved and 0.22 µm-filtered water from Lake Greifensee was inoculated with similar concentrations of *V. cholerae* and a lake water bacterial community (5 x 10^3 cells mL^{-1} each). The concentrations of total cells and of *V. cholerae*, respectively, were enumerated 48 h after inoculation when stationary phase had been reached. The predicted fraction of *V. cholerae* at the end of growth is based on a simulation with previously determined μ_{maxVc} and $\mu_{maxLWBC}$ on the same water. Maximum specific growth rates were determined in separate assays and corresponding growth curves for *V. cholerae* are shown in Figure 2.1 B (and supplementary info).

	20 °C	**25 °C**	**30 °C**
Bacterial concentration (total cells mL^{-1})	$1.74 \pm 0.22 \cdot 10^6$	$1.72 \pm 0.20 \cdot 10^6$	$1.89 \pm 0.13 \cdot 10^6$
***V. cholerae* (cells mL^{-1})**	$1.30 \pm 0.13 \cdot 10^5$	$1.76 \pm 0.30 \cdot 10^5$	$1.97 \pm 0.10 \cdot 10^5$
***V. cholerae* (% of total cell concentration)**	7.5 ± 0.6	10.2 ± 1.3	10.4 ± 1.0
Predicted fraction of *V. cholerae* (% of total cell concentration)	7.0	7.1	7.3
$\mu_{max Vc}$ (h^{-1})	0.218 ± 0.008	0.321 ± 0.004	0.449 ± 0.001
$\mu_{maxLWBC}$ (h^{-1})	0.418 ± 0.018	0.641 ± 0.006	0.884 ± 0.009
Ratio $\mu_{max Vc}$ / $\mu_{maxLWBC}$	0.52	0.50	0.47

In addition, competition experiments were performed in the same sterile freshwater samples at 20 °C, 25 °C and 30 °C. As before, equal cell concentrations were inoculated and the final concentrations of *V. cholerae* and total bacterial cells were enumerated in the stationary phase after

48 hours when all substrates had been consumed (Table 2.1). In all experiments *V. cholerae* proliferated from initially 5 x 10^3 cells mL^{-1} during competition with the lake water bacterial community to 1.3 x10^5 cells mL^{-1} (20 °C), 1.76 x 10^5 cells mL^{-1} (25 °C) and 1.97 x 10^5 cells mL^{-1}, respectively. The final fraction of the bacteria was around 10 % of the total bacterial cells at all temperatures tested. All this suggests that within the temperature range tested the fitness of *V. cholerae* was not affected compared to that of the lake water bacterial community. In Table 2.1, the results of the simulation using the same simple growth model used in Figure 2.4 by applying the previously determined μ_{max} of the pure cultures are also listed. They are in good agreement with the experimentally determined values.

Discussion

Although determination of bacterial growth rates in nature - particularly in the marine environment - is an intensively investigated and much discussed research area (Ducklow & Carlson, 1992; Hagström *et al.,* 1979; Hagström *et al.,* 1984; Moriarty, 1986; Robarts & Zohary, 1993; Simon *et al.,* 2002), studies concerning the kinetics of growth of waterborne bacterial pathogens and their competition with indigenous microbial flora for natural organic carbon are rare. This is mainly due to the lack of methods for easily monitoring the growth of microbial cells of a mixed community (most of them are "unculturable") at the low concentrations of substrates and biomass typical for environmental conditions. Here we have used flow cytometry in combination with fluorescent nucleic acid staining of cells to monitor the growth of mixed microbial communities. For the specific detection of *V. cholerae* we have developed a flow cytometric method based on cell surface fluorescent antibodies to enumerate this pathogen in the presence of indigenous freshwater bacterial flora. This combination of techniques allowed us to study the kinetics of growth of the enteric pathogen and its competition for nutrients with a lake water bacterial community in a laboratory system.

Our studies demonstrate for the first time that *V. cholerae* is not only able to survive (Nogueira *et al.,* 2002) but even able to grow in freshwater. In general, *V. cholerae* grew in autoclaved and 0.22 μm-filtered freshwater in batch culture exhibiting first a short lag, then exponential growth with a constant μ_{max} over an extended period of time, which was followed by a decelerating phase of growth and a stationary phase (as commonly seen with e.g., pure cultures of *E. coli* growing on complex medium at much higher substrate and cell concentrations). The specific growth rates achieved by *V. cholerae* during proliferation in sterilized freshwater were roughly 50 % of those observed under optimum salt concentrations (identical freshwater amended with 5 g NaCl L^{-1}). Under such optimum conditions *V. cholerae* reached a surprisingly high μ_{max}, which was similar to that exhibited by the lake water bacterial community on the same freshwater (μ_{max} = 0.84 h^{-1}).

Earlier reports, based on the distribution of *V. cholerae* in estuarine water, had already indicated the preference of this pathogen for moderate salinity with respect to optimum conditions for growth (Huq *et al.,* 2005; Louis *et al.,* 2003; Miller *et al.,* 1982). Also microcosm studies, using artificial seawater and tryptone as a carbon source, had shown before that *V. cholerae* exhibits highest specific growth rates at moderate salinity (Singleton *et al.,* 1982a). However, as far as we are aware, only two studies have been published where growth rates of *V. cholerae* O1 and of natural bacterial

seawater flora were investigated and compared in sterile-filtered natural seawater. Reported specific growth rates (determined by microscopic cell counting) ranged from 0.013 to 0.596 h^{-1} for *V. cholerae* and from 0.004 to 0.404 h^{-1} for seawater microbial flora, respectively, at temperatures between 15 and 28 °C (Mourino-Perez *et al.,* 2003; Worden *et al.,* 2006). Whereas the specific growth rates of *V. cholerae* compare well with our freshwater results, the rates achieved by the lake water bacterial community were considerably higher in our experiments.

It should also be pointed out that the freshwater samples used were first autoclaved (to kill/inactivate autochthonous microbes able to pass 0.22µm membrane filters (Hahn, 2004)) and then 0.22 µm-filtered in order to remove crystalline particles interfering with the flow cytometric analysis. Although several chemical parameters did indicate little change before (pH = 8.09 ± 0.04; DOC = 100 %) and after the treatment (pH = 8.02 ± 0.41; DOC = 104.7 ± 18.3%) the concentration of AOC_{app} increased consistently and was on average 141.5% ± 18.3% after autoclaving. This can be explained by cell breakage and hydrolysis of organic matter during the treatment. Hence, this treatment certainly increased the concentration of AOC_{app} and probably also affected the composition of the organic matter in the different freshwaters.

Several different approaches for the determination of AOC exist (Greenberg *et al.,* 1993; Hammes & Egli, 2005; LeChevallier *et al.,* 1993), and thus care should be taken with comparison of absolute values from different studies. We therefore used the term "apparent AOC" for results obtained with the method developed by Hammes and Egli (Hammes & Egli, 2005). In general, the absolute values obtained with this method are about two to five times higher than those reported with the conventional method (Greenberg *et al.,* 1993). Interestingly, whereas *V. cholerae* was able to grow at AOC_{app} concentrations as low as 100 µg L^{-1}, *S. typhimurium* did not show detectable growth in any of the freshwaters tested (AOC_{app} concentrations up to 800 µg L^{-1}; Figure 2.3 B). This confirms recent reports where growth of *V. cholerae* was observed at low environmental carbon concentrations (Mourino-Perez *et al.,* 2003; Worden *et al.,* 2006), whereas *Salmonella* sp. was reported to grow only under highly eutrophic conditions as found, e.g., in greywater (Ottoson & Stenstrom, 2003) or in compost (Sidhu *et al.,* 2001). Hence, *S. typhimurium* can be considered a strict copiotrophic enterobacterium unable to grow under environmental oligotrophic conditions (Winfield & Groisman, 2003). This is obviously in contrast to the environmental survival and growth ability of *V. cholerae*.

The present results suggest a "threshold" AOC_{app} concentration for growth of *V. cholerae* in the range of 50-100 µg L^{-1}. Interesting in this respect is the report of LeChevallier *et al.* (LeChevallier *et al.,* 1996) who observed a significant negative correlation between the occurrence of coliforms in drinking water systems and AOC concentrations lower than 100 µg L^{-1} (AOC concentration determined according to LeChevallier *et al.,* 1993). Similarly, it was found that *Aeromonas hydrophila* only grew in tap water when starch was added at concentrations higher than 100 µg of C L^{-1} (van der Kooij *et al.,* 1980). Both authors explained the phenomenon by the existence of a substrate threshold concentration for growth in about the same range as observed for *V. cholerae* growing on natural AOC.

For a particular water source the observed cell increase at the expense of AOC for *V. cholerae* was always less than the bacterial AOC-test community. This was the case for all waters tested although the relative numerical cell yields of *V. cholerae* varied strongly between different water samples (0.12 – 0.62). There is certainly not a single reason for this variation. A first explanation is the fact that the spectrum of carbonaceous substrates utilisable for *V. cholerae* is narrower in comparison to that of the bacterial AOC-test community. Therefore, the numerical cell yield must be expected to be strongly dependant on quality of the AOC, i.e. the spectrum of compounds it comprises. A second explanation for varying numerical cell yields of *V. cholerae* could be that the average cell size of *V. cholerae* is changing under different conditions and is different from that of the community used for AOC assessment. Therefore, in spite of a positive correlation between AOC_{app} concentration in different types of freshwater and numerical growth of *V. cholerae* (Figure 2.3 B), it will be very difficult to exactly predict the growth potential of *V. cholerae* in a water sample from the concentration of AOC_{app} only. From an ecological perspective it would be interesting to investigate the relationship between algal growth, AOC_{app} concentration and growth of *V. cholerae*. It is known that algae release considerable amounts of organic carbon compounds during photosynthesis (Münster, 1993) and that the occurrence of *V. cholerae* during algal blooms is elevated in estuary water systems (Epstein, 1993).

In addition to the concentration of utilisable carbon/energy sources, temperature is a further key parameter that governs growth of heterotrophic microbes in aquatic systems (Moriarty & Bell, 1993). Consequently, it has been proposed that an increase in temperature by a few degrees as a result of global warming might lead to a selective advantage for pathogens (see, e.g., Colwell, 1996). It was argued that a shift of the average environmental temperature towards the range optimal for growth for pathogens, which is typically around 37°C, would enable pathogenic bacteria

to compete better for nutrients with the low temperature-adapted indigenous heterotrophic microbial flora. Supporting this hypothesis is that temperature is frequently reported to influence growth and occurrence of *V. cholerae* O1 in natural waters with low salinity (Huq *et al.,* 2005; Louis *et al.,* 2003). Also the faecal indicator bacterium *E. coli* was reported to grow outside its host in tropical waters, but not in the Northern hemisphere where the average annual water temperature is low (Winfield & Groisman, 2003). Our results clearly demonstrate a positive correlation between increasing temperature and the specific growth rate achieved by *V. cholerae* in freshwater within the range of 20 to 30 °C (Table 1.1.). However, it appears from our data that in freshwater the ability of *V. cholerae* to compete with the lake water bacterial community for nutrients was not significantly affected. This result correlates with the observation of Louis and co-workers (Louis *et al.,* 2003) who found, in contrast to an estuarine environment, no year-to-year variability in the occurrence of *V. cholera* O1 in a freshwater system. Certainly, it is difficult to extrapolate from our batch microcosm studies, (where elevated temperatures did not favour *V. cholerae* when competing with the lake water bacterial community for substrates in freshwater), to the much more complex environmental situation where many other biotic and abiotic factors influence competitive microbial interactions. For example, temperature might influence competition in an indirect way by promoting algal growth and excretion of utilizable metabolites, grazing or viral lysis of pathogens and heterotrophic competitors. Competition experiments in the laboratory in AOC-limited continuous culture at different temperatures could provide more insight into the dynamics controlling growth and competition of *V. cholerae* with natural bacterial flora. In such a system competition could be followed over more extended periods of time and nutrient fluxes could be manipulated simulating the varying nutrient supply in nature (Münster, 1993). The presented results clearly demonstrate that growth of *V. cholerae* under oligotrophic conditions is not restricted to estuary or coastal waters but that this pathogen is able to grow in freshwater. Thus, as suggested earlier (Shapiro *et al.,* 1999), freshwater aquifers - particularly when mesotrophic - might provide an environmental reservoir for *V. cholerae* resulting in sporadic epidemic outbreaks originating from these waters. Our results also support the recently made observation (Worden *et al.,* 2006) that being attached to particles or higher organisms is not compulsory for the survival of this pathogen but that it is able to grow in a free-living state. The techniques described here open a way to well-controlled investigations for enhancing our understanding about the behaviour of *V. cholera* in the freshwater environment and to improve risk assessment for the disease of cholera.

Acknowledgements

Spiez Laboratory and the EU project Techneau (018320) are acknowledged for financial support

Supplementary info

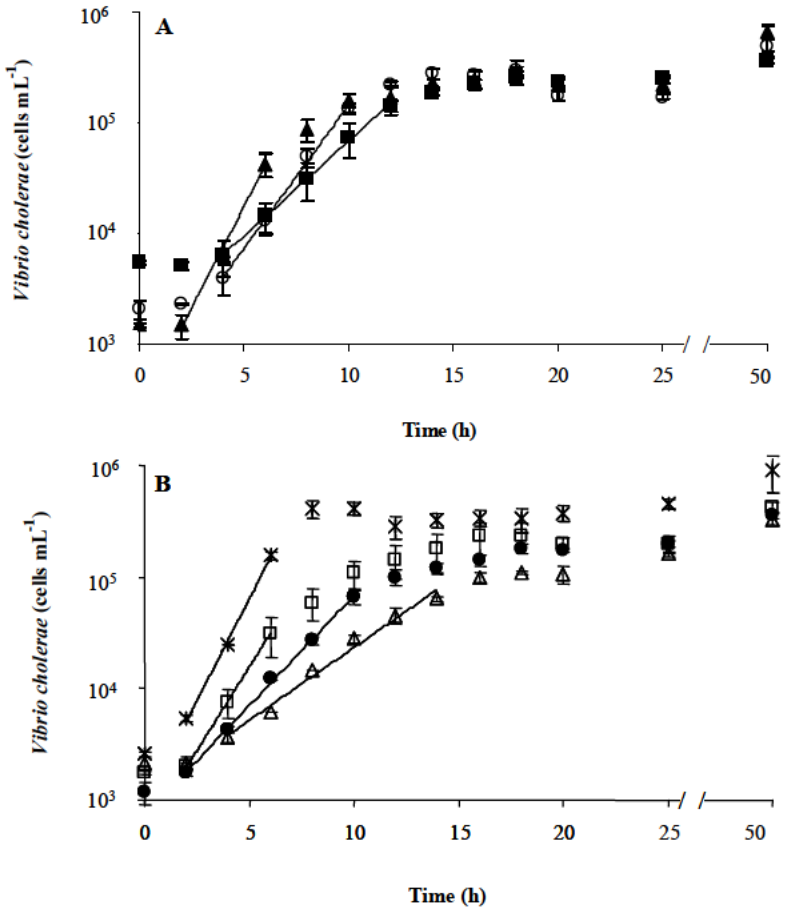

Figure S2.1. Growth of *V. cholerae* in autoclaved and 0.22 µm-filtered river water amended with different concentrations of NaCl (g L^{-1}). Panel A shows the half-log-plot of the growth curves at 0 (■); 1 (○); 5 g NaCl L^{-1} (▲). In panel B, a half-log-plot of growth of *V. cholerae* at 10 (□); 20 (●) and 30 g NaCl L^{-1} (△) is displyed. For comparison, in panel B the growth curve for the lake water bacterial community at 0 g NaCl L^{-1} is shown as well (×). The error bars indicate the standard deviation for triplicate samples.

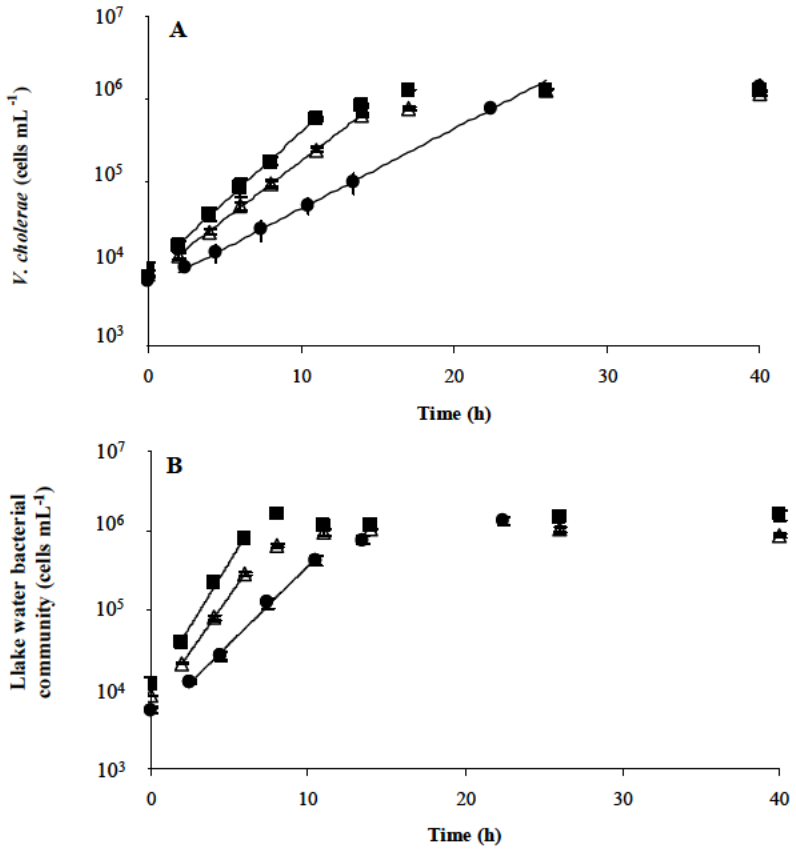

Figure S2.2. Growth of (A) *V. cholerae* and (B) a lake water bacterial community in autoclaved and 0.22 µm-filtered lake water at different temperatures (20 °C (●), 25 °C (△), 30 °C (■)) in batch culture. The corresponding maximum specific growth rates are calculated from the slope of the regression lines and are listed in Table 2.1. Error bars indicate the standard deviation for triplicate samples.

3. Escherichia coli O157 can grow in natural freshwater

Abstract

Whereas much information on the die-off of *E. coli* in the aquatic environment is available, only little data supports its growth under such conditions. We therefore reinvestigated batch growth assays in microcosms containing different types of sterile freshwater. The water samples were inoculated with low starting cell concentrations of *E. coli* O157 (3×10^3 cells mL^{-1}) and growth was followed using nucleic acid-staining combined with flow cytometry. We demonstrated that *E. coli* O157 is able to grow in sterile freshwater at low carbon concentrations, which is against the common view that cell numbers decline over time when added to freshwater samples. A correlation between apparent assimilable organic carbon (AOC_{app}) concentration and the final cell concentration reached by *E. coli* O157 was established ($p << 0.01$). A considerable fraction of the AOC_{app} (34 ± 13 %) was used by *E. coli* O157 but the numerical cell yield was about five-times lower in comparison to the bacterial AOC-test community, which originated from natural freshwater. On average, the maximum specific growth rate (μ_{max}) of *E. coli* O157 growing in sterile freshwater at 30 °C was 0.19 ± 0.07 h^{-1}. Batch growth assays at five different temperatures revealed a positive influence of temperature on μ_{max} of *E. coli* O157. The results give new information on the behaviour of this common pathogen in the aquatic environment and contribute to microbial risk assessment in order to prevent spreading of waterborne diseases.

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Introduction

E. coli can be found in two distinctly different environments: its primary habitat is the large intestine (colon) of warm-blooded animals, while water and soil represent its secondary habitat (Savageau, 1983). The bacterium cycles between these two habitats by faecal-oral transmission. Bacterial concentrations of *E. coli* in the primary habitat vary between 10^5 to 10^8 colony forming units (cfu) per gram faeces, whereas much lower numbers are reported for environments representing the secondary habitat (Bettelheim, 1992; OECD, 2003). Although *E. coli* is part of the normal microbial flora of the gastrointestinal tract of warm blooded animals, certain strains are associated with disease. Of specific interest are the enterohaemorrhagic *E. coli* (EHEC) strains producing cytotoxins, which show similarity to the toxin encoded in *Shigella dysenteriae* (Caprioli *et al.,* 2005). Within this group, the majority of the cases of disease are caused by a strain with the serotype O157 (Nataro & Kaper, 1998). In the period from 1982 to 2002, the Center for Disease Control and Prevention (CDC) reported 350 outbreaks of EHEC O157 in the United States of America, of which 9 % were waterborne (Rangel *et al.*, 2005). Investigations on the growth and survival of *E. coli*, especially pathogenic strains, in natural freshwater could therefore yield important data for microbial risk assessment and hence facilitate prevention of the diseases associated with it.

Proliferation of *E. coli* in the nutrient-rich primary habitat has been studied intensively (Bettelheim, 1992), whereas less information is available on the growth in its secondary habitat where low nutrient concentrations prevail. Most studies in which the behaviour of *E. coli* in natural aquatic environments had been investigated concluded that this enteric bacterium is not able to multiply in these environments (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). As a result, it is the common view that the bacterium can only grow in its primary habitat, and, once disposed into water, *E. coli* 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 continuously supported by laboratory experiments throughout the time (see above), resulted in the implementation of *E. coli* as an index parameter for faecal pollution for the monitoring of drinking water quality (WHO, 2008).

In contrast, only a few studies focused specifically on the growth properties of *E. coli* in synthetic media at low nutrient concentrations (Camper *et al.*, 1991; Wanner & Egli, 1990). *In vitro* growth of coliform bacteria (including *E. coli*) on natural carbon sources, specifically on excreted products of algae, was reported (McFeters *et al.*, 1978). These studies indicated that *E. coli* is able to grow at reduced nutrient levels. However, growth of the bacterium in natural water has not been characterized in detail yet. The bacterium was also isolated from unpolluted tropical rainforest rivers, where annual water temperatures are elevated compared to regions of the Northern hemisphere, leading to speculations that *E. coli* is an active member of the microbial flora in these environments (Carrillo *et al.*, 1985; Rivera *et al.*, 1988; Winfield & Groisman, 2003). Recent studies also indicated that *E. coli* is autochthonous to the soil and sediment environments of Lake Superior, USA (Ishii *et al.,* 2006; Ishii *et al.*, 2007).

Here we investigated the ability of *E. coli* O157 and of two non-pathogenic *E. coli* strains to grow in sterile natural freshwater at low carbon concentrations. Batch growth assays in microcosms were performed. Nucleic acid-staining in combination with flow cytometry was used for bacterial enumeration. We demonstrate that *E. coli* O157 not only survives, but also grows in sterile natural freshwater. A positive correlation between the assimilable organic carbon (AOC) concentrations as well as for dissolved organic carbon (DOC) concentrations and final cell concentrations of *E. coli* O157 was observed.

Material and 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 and *E. coli* WK8 (environmental isolate from surface water (Ihssen *et al.*, 2007)) 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 carbon-free materials

Carbon-free glassware (bottles and vials) was prepared as described in Hammes & Egli (2005). In short: all glassware was first washed with a common detergent, and thereafter rinsed three-times with deionised water. Then it was submerged overnight in 0.2 N HCl and subsequently rinsed with deionised water again and air-dried. Finally, the bottles and vials were heated in a Muffel furnace at 500 °C for at least six hours. Teflon-coated screw caps for the glassware were washed and treated identically with acid (0.2 N HCl). Caps were thereafter soaked in a 10 % sodium persulphate solution (60 °C for 1 h), rinsed three-times with deionised water and finally air-dried.

Preparation of natural freshwater

Different types of freshwater were used: non-chlorinated tap water (DOC = 570 µg L^{-1}; pH = 7.6; conductivity = 476 µS; Dübendorf, Switzerland), water from a shallow stream (DOC = 2546 - 4136 µg L^{-1}; pH = 7.7 - 7.9; conductivity = 402 - 502 µS, Chriesbach stream, Dübendorf, Switzerland), river water (DOC = 3322 - 4048 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}; pH = 7.9 - 8.1; conductivity = 680 - 832 µS, Dübendorf, Switzerland) and wastewater treatment plant (WWTP) effluent (DOC = 6733 - 7198 µg L^{-1}; pH = 7.4 - 7.5; conductivity = 1040 - 1130 µS, Dübendorf, Switzerland). Freshwater was sampled with a 500 mL Duran® flask (Schott, Wertheim/Main, Germany) and pasteurized (30 min at 60 °C) within 30 minutes of sampling. After cooling down to room temperature (minimum 5 h), aliquots of 15 mL were filtered through a 0.1 µm Millex® syringe filter (Millipore, Billerica, MA, USA) into 20 mL carbon-free glass vials (Supelco, Bellefonte, PA, USA) using a 50 mL Luer-Lok™ Syringe (Becton Dickinson, Franklin Lakes, N.J.,

USA). Syringe filters were pre-washed with at least 200 mL of carbon-free water before use to eliminate residual organic carbon. It should be pointed out that pasteurization was included in the sterilization procedure, since it has been shown that 0.1 µm filtration still allows the passage of a fraction of bacteria present in freshwater (Wang *et al.*, 2007). After pasteurization, samples were filtered in order to remove particles, which can interfere with the flow cytometric analysis.

Growth in sterile natural freshwater

E. coli 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) containing pasteurized and 0.1 µm-filtered freshwater (15 mL) originating from different sources (see above) to an initial concentration of 3 x 10^3 cells mL^{-1}. The suspensions were incubated at 30 °C for four days and the final cell concentrations of *E. coli* in the late stationary phase were enumerated with flow cytometry as described below. All batch growth curve experiments were done in sterile river water (Glatt river). 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. An increase in cell concentration and not in biomass was measured. Thus, growth could also be described using the cellular division rate (k), defined as the number of divisions per time unit. Since k is not often used in literature and values differ from that of µ (µ=ln2*k), the term specific growth rate (Equation 1) was used throughout the whole study.

Where growth of *E. coli* O157 in sterile river water (Glatt river) at different temperatures (15, 20, 25, 30, 37 °C) was studied, pre-adaptation of the inoculum was done by cultivating the cells first in minimal medium containing 10 mg glucose L^{-1} into stationary phase at each temperature.

Enumeration of total cell concentration by flow cytometry

Absolute cell-counting was performed flow cytometrically. A volume of 10 µL of SYBR®green (Molecular Probes, Basel, Switzerland), 100-times diluted in dimethylsulfoxid (Fluka Chemie AG,

Buchs, Switzerland), was added to 1 mL of a bacterial suspension and incubated in the dark at room temperature for 15 minutes before analysis. For outer membrane permeabilization EDTA (pH 8) was added (5 mM final concentration) to the sample together with the stain (Berney *et al.*, 2007). All samples were measured on a CyFlow Space flow cytometer (Partec, Münster, Germany) equipped with a 200 mW argon laser emitting at a fixed wavelength of 488 nm and equipped with volumetric counting hardware. For cell enumeration, the trigger was set on the green fluorescence (520 nm) channel and signals were collected on the combined 520 nm / 630 nm (red fluorescence) dot plot. Additionally, sideward scatter (SSC) signals were collected from the combined SSC / 520 nm dot plot. The quantification limit of the instrument was at 1000 cells mL^{-1} with an average standard deviation of less than 5 % (Hammes *et al.*, 2008).

Assimilable organic carbon (AOC) determination

AOC was determined with a batch growth assay as described previously (Hammes & Egli, 2005; Vital *et al.,* 2007). In short: the pasteurized and filtered water samples (15 mL) were inoculated with 10 µL (1 x 10^4 cells mL^{-1} initial concentration) of a bacterial AOC-test community. These suspensions were then incubated at 30 °C for four days (until stationary phase was reached) and the resulting growth was measured with flow cytometry. The AOC inoculum originated from stream water (Chriesbach stream, Dübendorf, CH) and was prepared as described previously (Vital *et al.*, 2007). The same bacterial community was used for all AOC determinations throughout the present study. As standard quality control prior to use, the performance of this inoculum was compared to bacterial AOC-test communities used in previous studies in our group (Hammes *et al.*, 2006; Vital *et al.*, 2007), using different types of natural surface waters as media. A difference of less then 10 % in the average AOC values was deemed acceptable for use. AOC (µg L^{-1}) was estimated from cell concentrations (cells mL^{-1}) using a theoretical conversion factor (Hammes *et al.*, 2006); we will henceforth use the term "apparent AOC" (AOC_{app}) to express the data obtained with this method (Equation 2). All assays were performed in triplicate. The detection limit of the method was 10 µg AOC_{app} L^{-1} and the average standard deviation was ± 10 %.

$$AOC_{app}\ (\mu g\ C\ L^{-1}) = \frac{\text{net grown cells (cells } L^{-1})}{1 \times 10^7 \text{ cells } (\mu g\ C)^{-1}} \qquad \text{(Eq. 2)}$$

AOC_{app} consumption

An indirect measurement was performed to determine the amount of AOC_{app} consumed by *E. coli* during growth. Batch assays in which inoculated *E. coli* cells had reached stationary phase were re-inoculated with the bacterial AOC-test community. Vials were subsequently incubated at 30 °C for four more days in order to allow the complete consumption of the remaining AOC_{app} that had not been used by *E. coli.* Additionally, the total AOC_{app} concentration of the same water sample was determined in a parallel assay as described above. The amount of AOC_{app} consumed by *E. coli* was hence calculated as the difference between the total AOC_{app} and the remaining AOC_{app} in the sample.

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). The detection limit was 10 µg L^{-1} (Meylan *et al.*, 2007).

Adenosine triphosphate (ATP) determination

The BacTiterGlo™ System (Promega, Dübendorf, Switzerland) was used for the determination of total ATP. The BacTiterGlo™-Buffer was mixed with the lyophilized BacTiterGlo™-Substrate and equilibrated at room temperature. The mixture was stored over night at room temperature to ensure that all ATP was hydrolysed ("burned off") and the background signal had decreased. A sample of 100 µL and an equal volume of BacTiterGlo™-reagent (stored on ice) were warmed separately for 2 minutes in a 30 °C water bath. The two liquids were then mixed and the luminescence of the sample was immediately measured with a Luminometer (Model TD-20/20, Turner BioSystems, Sunnyvale, CA, USA). A calibration curve with dilutions of pure ATP (Promega, Dübendorf, Switzerland) was measured for the prepared buffer. The same buffer was used for all experiments. The detection limit of the assay was around 0.003 nM. For all samples, the total concentration of free ATP was also measured. Therefore, water samples were filtered through a sterile 0.45 µm nylon filter (Semadeni, Ostermundigen, Switzerland) to remove all *E. coli* O157 cells. The total ATP of the filtrate was measured (total free ATP) as described above. All samples were analyzed in triplicate. The total cellular ATP was calculated as shown in Equation 4.

Total cellular ATP (nM) = total ATP (nM) - total free ATP (nM) (Eq. 4)

The total amount of ATP-per-cell was calculated as follows (Eq. 5):

$$\text{ATP (ng cell}^{-1}\text{)} = \frac{\text{total cellular ATP (nmol L}^{-1}\text{)} * 507.2 \text{ (g mol}^{-1}\text{)}}{\text{total cell concentration (cells L}^{-1}\text{)}} \quad \text{(Eq. 5)}$$

Plating of *E. coli*

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 ºC 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 ºC before analysis. All measurements were done in triplicate.

Results

Growth of different strains of *E. coli* in sterile freshwater

Three different strains of *E. coli* - a pathogenic strain (O157), a laboratory strain (K12) and an environmental isolate (WK8) - were able to proliferate on three different freshwater samples (Chriesbach stream, Glatt river and a small stagnant pond) at 30 °C. However, the final cell concentrations reached in the stationary phase after four days of incubation were significantly different for the three strains (Table 3.1). In stream and river water samples, most cells were formed by *E. coli* O157 followed by the environmental isolate WK8, whereas final cell concentrations of *E. coli* K12 were clearly lower (Table 3.1). Growth in sterile pond water, which was characterized by the highest AOC_{app} concentration of the three freshwater samples tested, showed a different pattern. Again the final cell concentration of *E. coli* O157 was the highest, but in this case it was followed by *E. coli* K12, and not by *E. coli* WK8.

Table 3.1. Final cell concentration of three different *E. coli* strains (O157, WK8 and K12) grown on three different types sterile freshwater. The error indicates the standard deviation on triplicate samples.

	Final cell concentration (cells mL^{-1} x 10^5)		
	***E. coli* O157**	***E. coli* WK8**	***E. coli* K12**
Chriesbach stream	1.10 ± 0.05	0.98 ± 0.16	0.27 ± 0.06
Glatt river	2.02 ± 0.10	1.49 ± 0.03	0.25 ± 0.04
Stagnant pond	4.07 ± 0.22	2.16 ± 0.15	3.60 ± 0.12

Growth curves in sterile river water followed the typical pattern observed for pure and mixed microbial cultures growing on complex media, consisting of an initial lag phase and an exponential growth phase at a constant specific growth rate (μ), followed by a continuously decreasing μ until stationary phase was reached (Figure 3.1). Approximately 50 % of all cells were formed in the exponential growth phase before μ decelerated. Clear differences in the growth of the different *E. coli* strains were observed. The maximum specific growth rates (μ_{max}) on identical water samples were: O157, 0.20 h^{-1}; WK8, 0.23 h^{-1}; K12, 0.11 h^{-1}. Based on these results and considering the hygienic relevance of *E. coli* O157, all further experiments were carried out using only this strain.

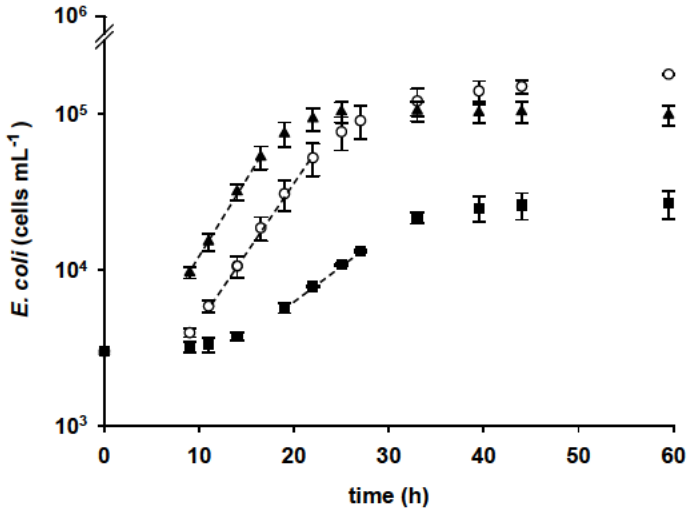

Figure 3.1. Batch growth of three different *E. coli* strains (O157, WK8 and K12) in sterile river water. O157 (○), an environmental isolate - WK8 (▲) and a lab strain - K12 (■) were compared for their growth properties in freshwater. The corresponding final cell concentrations are given in Table 1. The error bars indicate the standard deviation on triplicate samples.

Describing growth with different parameters

Aside from the total cell enumeration with flow cytometry, two additional methods (plating and ATP measurement) were used to verify and describe the growth of *E. coli* O157 in sterile river water (Glatt river). The total cellular ATP concentration showed a very similar pattern as the total cell concentration (Figure 3.2 A). 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). Subsequently, the total cell concentration still increased, whereas the total cellular ATP concentration started to decrease slightly. The ATP content per cell varied between 2.7 - 5.7 x 10^{-7} ng ATP $cell^{-1}$ (Figure 3.2 B). After an initial increase of the ATP content per cell, values stayed approximately stable during the exponential growth phase and finally decreased towards the stationary phase. Throughout all phases of growth, almost all cells measured with flow cytometry also formed colonies on both types of agar used (94.2 ± 4.8 % (PCA) and 90.1 ± 7.9 % (R2A) (Figure 3.3)).

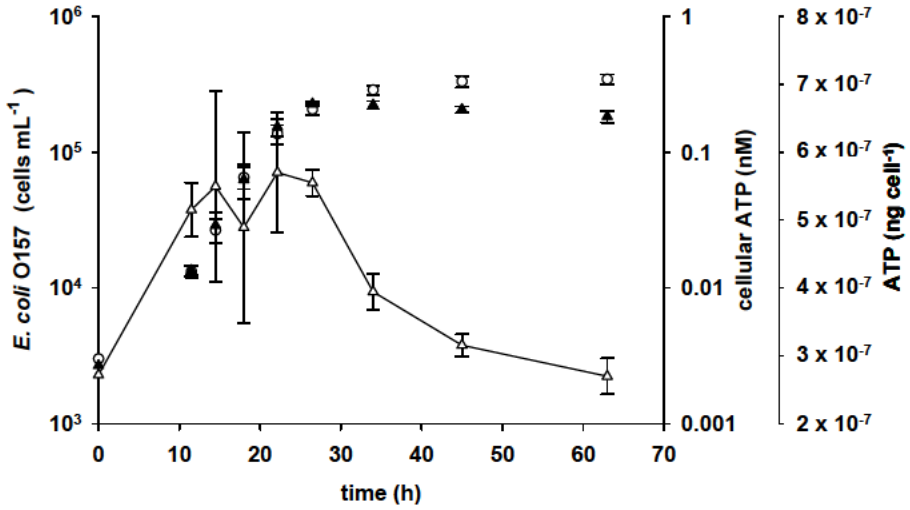

Figure 3.2. Total cellular ATP (▲) and ATP-per-cell (△) during batch growth of *E. coli* O157 in sterile river water. The total cellular ATP increased proportionally to the increase of *E. coli* O157 cell counts (○). Error bars indicate the standard deviation on triplicate samples.

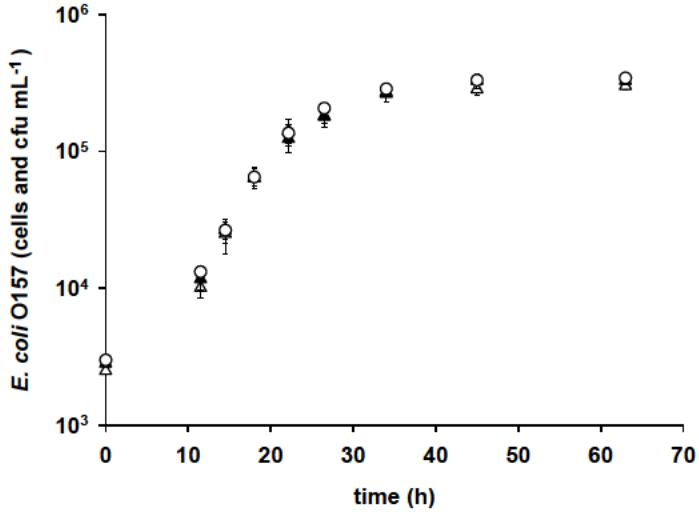

Figure 3.3. Following growth of *E. coli* O157 in sterile river water by plating on PCA, R2A agar and by flow cytometry. During all phases of growth around 90 % in the *E. coli* O157 total cell counts (○) were detected on both PCA (△) and R2A (▲) agar. The error bars indicate the standard deviation on triplicate samples

Correlation between AOC_{app}, DOC and the final cell concentration of *E. coli* O157

A positive correlation ($p << 0.01$; $R^2 = 0.85$; $n = 13$) between AOC_{app} concentrations and final cell concentrations of *E. coli* O157 was observed when cells were grown into stationary phase in different types of sterile freshwater (Figure 3.4 A). A similar correlation ($p << 0.01$; $R^2 = 0.81$; $n = 14$) was detected between DOC concentrations and final *E. coli* O157 cell concentrations (Figure 3.4 B). The final cell concentration of *E. coli* O157 was between 0.82 - 4.07 x 10^5 cells mL^{-1} in all samples tested. AOC_{app} concentrations, where growth of *E. coli* O157 was detected, ranged from

184 - 534 µg L^{-1} (corresponding DOC values of the same samples were between 2322 - 8168 µg L^{-1}). No growth was observed in non-chlorinated tap water characterized by a low AOC_{app} (9.9 ± 5.23 µg L^{-1}, which is at the detection limit of the assay) as well as a low DOC (570 µg L^{-1}) concentration.

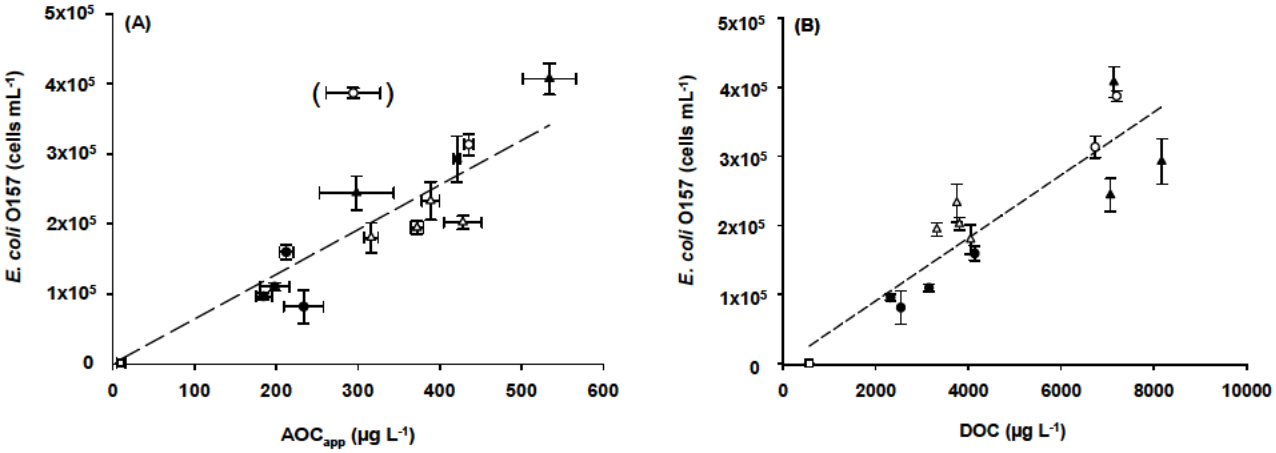

Figure 3.4. The correlation between apparent assimilable organic carbon concentration, AOC_{app} (A; $p << 0.01$; $R^2 = 0.85$; n = 13), dissolved organic carbon concentration DOC (B; $p << 0.01$; $R^2 = 0.81$; n = 14) and the final cell concentration of *E. coli* O157 cultivated in different types of sterile freshwater. Five different types of freshwater (tap water (□), Chriesbach stream (●), Glatt river (△), pond (▲), and effluent of a sewage treatment plant (○)) were inoculated with late stationary phase *E. coli* O157 cells at low concentration ($3x10^3$ cells mL^{-1}) and growth was recorded using flow cytometry. Error bars indicate the standard deviation for triplicate samples. One data point shown in brackets yielded unusually high numbers of cells formed and has therefore not been included in the data set for calculating the correlation.

For all cultivations of *E. coli* O157 in freshwater, the AOC_{app} consumption varied from 20 to 66 % with an average of 34 ± 13 % (n = 13). The average numerical cell yield for growth on AOC_{app} was 1.98 ± 0.73 x 10^6 cells μg^{-1}, which is five-times lower compared to the theoretical value for the bacterial AOC-test community (1 x 10^7 cells (µg AOC_{app})$^{-1}$).

Influence of temperature on growth of *E. coli* O157

The maximum specific growth rate (μ_{max}) of *E. coli* O157 growing in sterile river water (Glatt river) increased with increasing incubation temperature up to 30 °C (Figure 3.5). A slight decrease at 37 °C was observed. The recorded μ_{max} values were: 0.03 h^{-1} (15 °C); 0.04 h^{-1} (20 °C); 0.21 h^{-1} (25 °C); 0.31 h^{-1} (30 °C) and 0.27 h^{-1} (37 °C).

The final cell concentrations of *E. coli* O157 in sterile river water at the different temperatures tested followed a similar pattern to that of μ_{max} (Figure 3.6). The higher the temperature (and the

corresponding μ_{max}), the more cells were formed (1.49 x 10^4 cells mL^{-1} (0.03 h^{-1}); 1.46 x 10^5 cells mL^{-1} (0.04 h^{-1}); 1.73 x 10^5 cells mL^{-1} (0.21 h^{-1}); 2.20 x 10^5 cells mL^{-1} (0.32 h^{-1}) and 1.52 x 10^5 cells mL^{-1} (0.28 h^{-1})). A significantly reduced final cell concentration was observed for growth at 15 °C indicating that *E. coli* O157 struggles to grow in freshwater at lower temperatures.

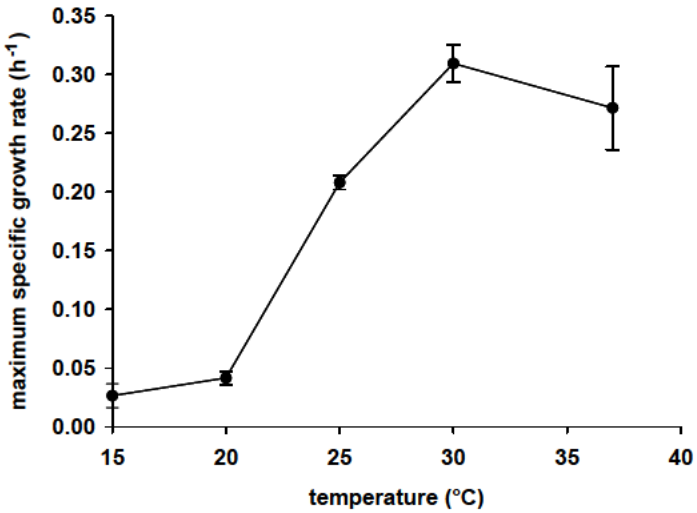

Figure 3.5. Maximum specific growth rate (μ_{max}) of *E. coli* O157 growing in sterile river water (Glatt) as a function of temperature. Error bars indicate the standard deviation on triplicate samples.

The fraction of AOC_{app} utilized for growth was similar at all temperatures analyzed, except at 15 °C, where significantly less AOC_{app} was consumed (15 °C, 17 ± 4 %; 20 °C, 35 ± 5 %; 25 °C, 29 ± 3 %; 30 °C, 35 ± 5 %; 37 °C, 35 ± 6 %; Figure 3.6). The results clearly demonstrate the importance of temperature on growth of *E. coli* O157 in natural freshwater

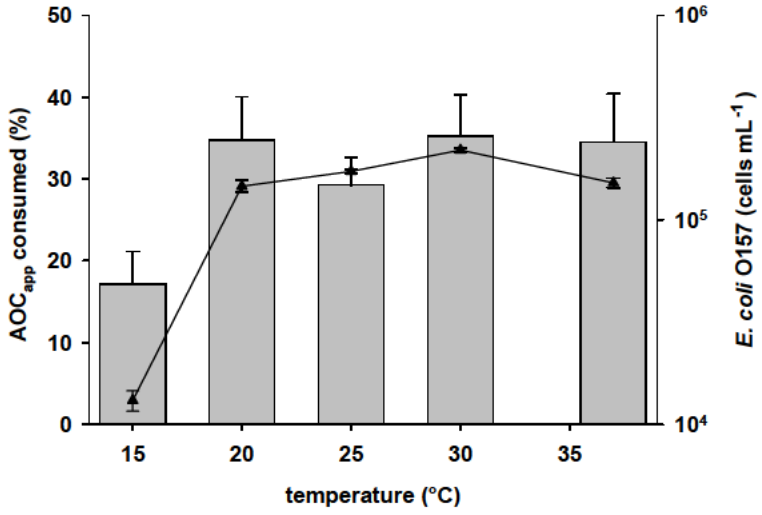

Figure 3.6. Final cell numbers (▲) and AOC_{app} consumption (bars) of *E. coli* O157 growing in sterile river water (Glatt) at five different temperatures. Error bars indicate the standard deviation on triplicate samples

Discussion

This study clearly demonstrates that *E. coli* O157 is able to grow in sterile freshwater with naturally available carbon at the low concentrations encountered in aquatic environments. Furthermore, a positive correlation of utilizable organic carbon and temperature with growth parameters (final cell concentrations and μ_{max}) of the pathogen was established. The data we present here are in contradiction to the common view that enteric pathogens cannot grow in natural freshwater due to the lack of organic nutrients (reviewed by Morita (1997) and Rozen & Belkin (2001)). Flow cytometry-based methods for the determination of total cell concentrations and AOC_{app}-levels allowed us to critically evaluate the experimental set-ups used in many of the earlier studies. The most common pitfall when studying batch growth in natural samples is that the concentration of the bioavailable carbon in a sample has to be known before appropriate inoculation can be done. In most previous studies, water microcosms as well as *in situ* diffusion chambers were loaded with high cell concentrations of *E. coli* (10^6 cells mL^{-1} and higher) (Banning *et al.*, 2002; Bogosian *et al.*, 1996; 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). High starting cell concentrations were often used in order to mimic a "worst case pollution scenario" of freshwater, when investigating the survival of enteric bacteria in these environments. In natural freshwaters, however, bacterial numbers are typically in the range between 10^4 - 10^6 cells mL^{-1} (Berninger *et al.,* 1990; Sanders *et al.,* 1992; Li & Dickie, 2001) because the "carrying capacity" of the ecosystem, determined for heterotrophs mostly by the available carbon concentrations, do not support higher cell densities (Morita, 1997). Thus, the AOC level in a water sample gives an indication on the potential of bacterial growth in that particular aquatic environment. Residual AOC concentrations of 10 µg L^{-1} up to 1000 µg L^{-1} can be observed in natural waters (LeChevallier *et al.*, 1991; Vital *et al.*, 2007). In the present study final cell concentrations of *E. coli* O157 grown in sterile freshwater samples were between 0.82 - 4.07 x 10^5 cells mL^{-1}. Thus, it is obvious that no growth, but rather "die-off" will be observed when inoculum concentrations are high (>10^6 mL^{-1}). Hence, for studying bacterial growth (potential) in freshwater environments inoculum concentrations in batch assays should as a rule not exceed 10^4 cells mL^{-1}.

Additionally, in several previous studies natural waters were treated with 0.22 µm sterile filtration in order to remove all autochthonous bacteria (Chai, 1983; Fujioka & Unutoa, 2006; Henis *et al.*, 1989). However, it has since been shown that this procedure does not remove all bacterial cells from the water. A significant fraction of the natural bacterial flora passes the filter and is able to re-

grow immediately by using the natural bio-available carbon present in the water, leaving no substrates for *E. coli* to grow on (Wang *et al.*, 2007). Such filterable bacteria can usually not be detected with conventional plating techniques but only by culture-independent methods, such as fluorescence microscopy or flow cytometry (Wang *et al.*, 2007). Specifically for this reason we have used a combined pasteurization-filtration procedure for sterilizing our water samples in the present study.

Our data clearly show a positive correlation between (assimilable) organic carbon and the final concentration of *E. coli* O157 reached in batch assays. Such a correlation was also reported for other pathogenic bacteria (Torvinen *et al.*, 2004; Vital *et al.*, 2007). However, only a fraction of the AOC_{app} was consumed by *E. coli* O157 and the numerical cell yield was much lower compared to that of the natural bacterial community used for AOC_{app} determination. The calculated value of 1.98 ± 0.73 x 10^6 cells µg AOC_{app}^{-1} is in the same range as for other pathogenic bacteria growing at low carbon concentrations. van der Kooij *et al.* (1982) report a numerical cell yield (based on cfu's) for *Pseudomonas aeruginosa* growing on a mixture of substrates (n = 45) at a total concentration of 46 µg C L^{-1} of 3.3 ± 0.46 x 10^6 cells µg C^{-1} consumed. For *Aeromonas hydrophila,* growing on 41 single substrates, each at a concentration of 10 µg C^{-1} an average numerical cell yield (based on cfu's) of 5.5 ± 2.7 x 10^6 cells µg C^{-1} is reported (van der Kooij & Hijnen, 1988). The main reason for the different numerical cell yield of *E. coli* O157 compared to that of the natural bacterial community used for AOC_{app} determination is that the average cell size of *E. coli* O157 grown in sterile freshwater to stationary phase is significantly bigger. Mean sideward scatter (SSC) signals, which correspond to cell size (Felip *et al.,* 2007), were higher (139 ± 11 %) for *E. coli* O157 compared to that of the AOC-test community (SSC signal set as 100 %). Thus, more AOC is required to form a new *E. coli* cell, and as a consequence, the numerical cell yield is on average five-times lower than that for the natural bacterial community. Furthermore, the physiological properties of *E. coli* might differ from those of the natural bacterial community, i.e. *E. coli* might respire a bigger part of the carbon for maintenance energy production, and, consequently, less can be used for biomass synthesis (Pirt, 1965).

Nutrient threshold concentrations for enteric bacteria have been reported previously. A minimum concentration of 100 µg AOC_{app} L^{-1} was needed by *V. cholerae* O1 to grow in natural freshwater (Vital *et al.*, 2007). Correspondingly, a significant negative correlation between the occurrence of coliforms in drinking water systems and AOC concentrations lower than 100 µg L^{-1} was reported (LeChevallier *et al.*, 1996). It seems that also for *E. coli* O157 a certain threshold value of AOC_{app}

for growth exists. No growth was detected in tap water, which is characterized by a low AOC_{app} concentration (10 µg L^{-1}), whereas the natural bacterial community grew up to 1 x 10^5 cells mL^{-1} in this sample. Also Camper and coworkers (Camper *et al.,* 1991) showed the ability of *E. coli* to grow on sterile drinking water but only when the DOC concentration was rather high (5 mg L^{-1}).

It is predicted that the global temperature increase due to climate change will affect also biological processes in aquatic environments (IPCC, 2007). It is thought that increasing water temperatures could favour the growth of bacteria, including enteric pathogens that are adapted to higher temperatures (IPCC, 2007; Colwell, 1996; Lipp *et al.,* 2002). Our results support this prediction as much as a positive impact of temperature on the growth of *E. coli* O157 in sterile river water was demonstrated. The μ_{max} reached in batch assays increased with higher temperatures. The average μ_{max} in sterile river water at 30 °C was 0.19 ± 0.07 h^{-1} (n = 7 samples, each in triplicate) and this fits well with μ_{max} values reported (0.08 to 0.26 h^{-1}) for *E. coli* growing at mixed substrate concentrations ranging from 100 to 1000 µg L^{-1} (Camper *et al.,* 1991). A major increase of μ_{max} was observed above 20 °C, whereas only little growth was detected at 15 °C. In contrast to the well documented increase of μ_{max} with temperature up to 39 °C (Herendeen *et al.*, 1979), we observed in our experiments a correlation from 15 to 30 °C only, whereas the μ_{max} at 37 °C was slightly lower (Figure 3.5). This unexpected result might be due to differences in the physiological properties of *E. coli* O157 compared to other *E. coli* strains (e.g., absence of the β-glucuronidase activity (Krishnan *et al.,* 1987)). Indeed Raghubeer & Matches (1990) reported different growth properties of the pathogen at different temperatures in comparison to other *E. coli* strains, suggesting that this EHEC strain can also grow at lower temperatures. However, the optimal growth temperature for *E. coli* O157 and non-O157 strains in nutrient-rich complex media differs only slightly and is between 39 °C and 40 °C (Gonthier *et al.,* 2001). The *E. coli* O157 strain used in this study also exhibited a higher μ_{max} at 37 °C (1.68 ± 0.01 h^{-1}) compared to that at 30 °C (1.21 ± 0.02 h^{-1}) in full strength LB medium (data not shown). This discrepancy may be explained in several ways. For example, low carbon concentrations (below 10 mg C L^{-1}), as typically found in natural freshwaters, might change the temperature optimum of *E. coli* for growth. However, we tested this using 1000 times diluted LB medium (6.5 mg DOC L^{-1}), and found that also under such conditions the μ_{max} of *E. coli* O157 was higher at 37 °C (0.2 ± 0.02 h^{-1}) than at 30 °C (0.16 ± 0.01 h^{-1}) (data not shown). Alternatively, the AOC might be altered during growth experiments at different temperatures. Pre-incubation of sterile water at different temperatures for two days, followed by an AOC determination, however, showed that the concentration of AOC_{app} did not change within this time period (data not shown). Whether the quality of the carbon did change at 37 °C compared to lower temperatures can

presently not be investigated. Since temperatures of natural freshwater systems do not often exceed 30 °C, we did not further consider the issue.

Here we focused specifically on planktonic batch growth of *E. coli* O157 in pure culture in sterile natural freshwaters. The purpose was to investigate the potential of *E. coli* strains to grow with natural nutrients at low concentrations. Growth of the bacterium in natural aquatic environments is definitely more complex and controlled by many additional factors that were not investigated in this study. Further experiments addressing the competitive properties of *E. coli* O157 with the natural bacterial flora for naturally abundant nutrients in freshwater are of great interest in order to gain more information on the behaviour of the pathogen in the natural aquatic environment. The maximum specific growth rates of natural bacterial communities growing in batch in sterile natural freshwater at 30 °C are up to 2 - 4 times higher (0.2 - 0.8 h^{-1}) than the average μ_{max} of *E. coli* O157 (around 0.2 h^{-1}) observed in this study (Vital *et al.*, 2007). Furthermore, although *E. coli* O157 was able to utilize a considerable portion of AOC_{app} (from 20 to 66 %), the resulting cell concentration was low compared to that reached by the natural bacterial community. These observations clearly indicate a limitation for growth of *E. coli* O157 in complex natural environments. Additionally, biofilms are important habitats for bacterial growth in the aquatic environment (Szewzyk *et al.*, 2000) and were not investigated in this study. Another important issue is the infectivity of *E. coli* O157 during growth in natural freshwater. Weather the pathogen is still able to express certain pathogenicity factors during growth at low carbon concentrations remains an open question.

The present study clearly shows, in contrast to many earlier reports, the ability of *E. coli* O157 to grow in sterile natural freshwater. The data give new information on the behaviour of the pathogen in the aquatic environment and contribute to microbial risk assessment. In addition, this study provides a critical assessment on the methodology (and experimental set-up) required for studying the growth of pathogenic bacteria in freshwater at low carbon/nutrient concentrations and low cell numbers.

Acknowledgements

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4. Growth characteristics of three pathogenic bacteria, *Escherichia coli* O157, *Vibrio cholerae* O1, *Pseudomonas aeruginosa,* and a freshwater bacterial community at low nutrient concentrations

Abstract

Many previous studies focused on survival and die-off of pathogenic bacteria outside the host, but only little data is available on their growth properties in the environment at low nutrient concentrations. In this study we compared growth of three distinctly different pathogens (*Escherichia coli* O157, *Vibrio cholerae* O1, *Pseudomonas aeruginosa*) and a freshwater bacterial community in three low nutrient growth media, namely diluted LB medium, diluted wastewater and river water. Flow cytometric bacterial enumeration together with biovolume determination was applied to monitor bacterial growth. Stoichiometric parameters, including nutrient/temperature thresholds for proliferation, consumption of different dissolved organic carbon (DOC) fractions and corresponding yields, as well as kinetic constants, μ_{max} and K_s, were determined. In diluted LB medium, despite the complexity of the substrate mixture, growth followed Monod kinetics and we were able to model it as such. *P. aeruginosa* grew better than *V. cholerae* and *E. coli* O157 under the nutritional conditions applied, whereby kinetic rather than stoichiometric parameters were found to differ considerably between cultures. The freshwater bacterial community performed best in all experiments. The presented results improve our understanding on bacterial growth in the environment and should, furthermore, contribute to risk assessment for the pathogenic bacteria tested.

Introduction

Many pathogenic bacteria cycle between the environment and their host where they can cause disease. In the host, they grow at high nutrient concentrations, low oxygen tensions, and at a constant elevated temperature. Once shed into the environment, their survival and proliferation is severely hampered by stressors such as low temperature, pH variations, oxygen, sunlight, as well as viral lysis and predation (Morita, 1997, Rozen & Belkin, 2001; Bettarel *et al.*, 2003). Furthermore, carbonaceous compounds available for heterotrophic growth are present at low concentrations and vary from the low microgram per litre range in drinking and surface waters, up to a few milligrams per litre in wastewater (LeChevallier *et al.,* 1991; Münster, 1993).

Pathogenic bacteria differ in their ability to grow and survive under environmental conditions. It is commonly accepted that so-called "environmental pathogens", like *Pseudomonas aeruginosa, Aeromonas spp.* or *Legionella spp.* (also *Vibrio cholerae*), have a natural aquatic reservoir where they can proliferate (Szewzyk *et al,* 2000; Steinert *et al.,* 2002; Louis *et al.,* 2003, Torvinen *et al.,* 2004;). The "pathogens from faecal sources", such as the enteric bacteria *Escherichia coli, Campylobacter sp.,* or *Salmonella spp.* are, in contrast, commonly considered to be unable to proliferate in the environment (Morita, 1997; Rozen & Belkin, 2001). They are thought to only survive until finding a host again. However, this crude distinction between environmental and faecal source-derived pathogens is based on assumptions rather than on experimental data. There are several reports indicating that also enteric bacteria may multiply in the environment (Ishii *et al.,* 2006; Walk *et al,* 2007). For example, we have recently demonstrated that *E. coli* O157 is able to grow in river water at low concentrations of natural organic carbon (Vital *et al.,* 2008).

To date, not many reports exist on growth of pathogenic bacteria under conditions similar to those prevailing in the environment (Legnani *et al.,* 1999; Mourino-Perez *et al.*, 2003; Vital *et al.,* 2007; Kirschner *et al.,* 2008) and quantitative data on stoichiometry and kinetics of bacterial growth at low nutrient concentrations are scarce (van der Kooij *et al.,* 1982, 1988; Singleton *et al.,* 1982; Camper *et al.,* 1991). Furthermore, it is difficult to compare data from published studies since they vary distinctly in the methodology applied and the nutritional conditions used. Hence, we are still far away from understanding the basic factors that govern growth of pathogenic bacteria in the environment.

The limited information can partly be explained by technological limitations that hindered investigating growth at low cell and nutrient concentrations. For example, conventional cultivation-based methods face severe drawbacks since only a small fraction of the bacteria present in an environmental sample are able to grow and form a colony on a nutrient agar plate. This was recognized when fluorescent DNA-stains were introduced in microscopy and the phenomenon was referred to as the "great-plate-count-anomaly" (Staley & Konopka, 1985). Although microscopic counting allows investigation of growth under such conditions (Bowden, 1977), the procedure is time consuming. However, technological improvements in flow cytometry over the last two decades made it possible to study growth of bacteria under environmental conditions at a high throughput (Hammes & Egli, 2005; Vital *et al.,* 2007, 2008; Czechowska *et al.,* 2008).

In order to perform an adequate risk assessment we have to better understand growth and survival of pathogenic bacteria in the environment. The aim of the present study was to compare the growth of different pathogenic bacteria at low nutrient concentrations and to determine the stoichiometric and kinetic parameters that govern their growth. Therefore, *Escherichia coli* O157, *Vibrio cholerae* O1, *Pseudomonas aeruginosa* and a freshwater bacterial community were grown in batch culture on three different waters differing distinctly in their organic carbon quality

Materials and Methods

Bacterial strains and precultivation

The verotoxin-negative *E. coli* O157 (Nent 2540-04), *Vibrio cholerae* O1 Ogawa biotype El Tor (Nent 720-95) and *Pseudomonas aeruginosa* (LMG 14073) were used. All strains were kept at -80 °C before use. The cryo-cultures were streaked onto Tryptic soy agar plates (Biorad, Reinach, Switzerland) and incubated for 24 h at 37 °C. Cells from a colony were transferred with a loop into ten-times diluted Lysogeny Broth (LB) and were incubated overnight at 37 °C. Subsequently, cells from the overnight culture were transferred into 10,000-times diluted LB medium (dLB; initial concentration 5 x 10^3 cells mL^{-1}) and incubated for four days at 30 °C prior to use. The freshwater bacterial community (FBC) was isolated from a small river (River Glatt, Dübendorf, Switzerland) as described elsewhere (Vital *et al.,* 2007). All cultures were then pre-grown into stationary phase (for four days at 30 °C; 5 x 10^3 cells mL^{-1} starting concentration) in the three "waters", i.e. dLB, 30-times diluted wastewater (dWW) and sterile river water (RW), in order to acclimate the cells to the different carbon sources. Stationary phase cells from these cultures were then used as an inoculum for our growth experiments.

Waters used for growth

Three different waters with a similar assimilable organic carbon (AOC) concentration were used in the growth experiments: dLB (DOC = 709 µg L^{-1}, AOC = 455 µg L^{-1}, pH = 7.4, conductivity = 540 µS), dWW (DOC = 670 µg L^{-1}; AOC = 339 µg L^{-1}, pH = 7.5; conductivity = 590 µS, Dübendorf, Switzerland), and RW (DOC = 3838 µg L^{-1}; AOC = 334 µg L^{-1}, pH = 8.32; conductivity = 432 µS, River Glatt, Dübendorf, Switzerland). For sterilization, the LB medium was autoclaved, RW was pasteurized (60 °C, 30min) followed by 0.22-µm filtration (Millex®, Millipore, Billerica, MA, USA) and wastewater was 20 kDa filtered (Fresenius Medical Care, Bad Homburg, Germany). For dilution pasteurized (60 °C, 30 min) and 0.22-µm filtered AOC-free bottled mineral water was used. For the temperature threshold experiments, RW from a different day was used, which contained: DOC = 3583 µg L^{-1}, AOC = 530 µg L^{-1}, pH = 8.55, conductivity = 459 µS.

Total dissolved organic carbon (DOC) and fractioning of the NOM by liquid chromatography organic carbon detection (LC-OCD) analysis

Organic carbon was measured 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). The

detection limit was 10 µg L^{-1} (Meylan *et al.*, 2007). The separation of the NOM into different fractions depending on size was obtained using a size exclusion column (Toyopearl TSK HW 50S, Posoh Bioscience, Tokyo, Japan). All samples were pre-filtered prior to analysis using a washed 0.22 µm filter.

Determination of the AOC (cDOC) concentration

AOC determination was based on the method described by Hammes and Egli (2005). As described in the original method, the FBC was grown into late stationary phase (for four days at 30 °C) and growth was measured using flow cytometry. In this study, we did, however, not use a conversion factor to calculate AOC concentration from the produced cells, but measured DOC concentration before and after the assay. The difference in DOC corresponds to the AOC but it was referred to here as "consumable DOC" (cDOC).

Flow cytometry

Absolute cell-counting was performed using nucleic acid staining in combination with flow cytometry as described previously (Vital *et al.,* 2007; Hammes *et al.*, 2008). Additionally, sideward scatter (SSC) signals were collected from the combined SSC / 520 nm dot plots in order to estimate the cellular biovolume, eCV. This was done as described previously following specific calibration of the instrument (Wang *et al.,* 2009; Hammes *et al.,* in press). The estimated total biovolume (eBV) was then calculated by multiplying the eCV with the cell concentration of a sample.

Growth in different types of water

Carbon-free glassware (bottles and vials) was prepared as described in Hammes & Egli (2005). Pre-grown cells of each culture were harvested in stationary phase (see above) and inoculated into carbon-free 40 mL glass vials (Supelco, Bellefonte, PA, USA) containing 30 mL of the three different types of waters (dLB, DWW and RW); the initial cell concentration was 5 x 10^3 cells mL^{-1}. Inoculated samples were incubated at 30 °C for four days and the final cell concentration and eBV in the stationary phase were determined with flow cytometry as described above. Vials containing *P. aeruginosa* were placed for 10 minutes into a sonication-bath prior to sampling to ensure that possible wall-grown cells were released into the liquid (initial tests showed that this was an adequate procedure). DOC was measured in filtered (0.22 µm) samples before and after growth and cDOC was calculated from the difference. The vials containing the stationary phase grown pure cultures were then inoculated with the FBC to an initial concentration of 5 x 10^3 cells mL^{-1}, re-

incubated at 30 °C and analysed four days later for final cell concentration and eBV and remaining DOC concentration (see Vital *et al.,* 2008). For the 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 based on the cell concentration increase (μN) was determined as follows:

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

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. The specific growth rate based on the eBV increase (μB) was determined similarly:

$$\mu B = [\ln (B_t) - \ln (B_0)] / \triangle t \qquad \text{(Eq. 2)}$$

where B_t, B_0 are the eBV at two subsequent time points and $\triangle t$ is the expired time interval between these points.

Preparation of different LB and RW dilutions

For the nutrient threshold experiment as well as to investigate the correlation between μB and cDOC concentration, we prepared a dilution series containing different concentrations of LB medium and RW (only for nutrient threshold) in carbon-free 40 mL glass vials (Supelco, Bellefonte, PA, USA). For dilution, pasteurized (30 min, 60 °C) and filtered (0.22-µm) AOC-free bottled mineral water was used. The cDOC concentration of the different samples was only determined for the highest concentration (10,000-times diluted LB; undiluted RW) and was then calculated for the different dilutions. Samples were inoculated (inoculum preparation see above) to an initial concentration of 1 x 10^3 cells mL^{-1}, incubated at 30 °C and analysed by flow cytometry (final cell concentration and eBV) as described above. Values for μB were determined as described above. For threshold experiments, the samples were incubated for one week in order to assure that the cells growing at very low cDOC concentrations had reached stationary phase. All experiments were performed in triplicate samples.

Determination of the temperature thresholds

Prior to experimentation the bacteria were pre-grown at 30 °C into stationary phase on RW. Bacteria were inoculated to an initial concentration of 1 x 10^3 cells mL^{-1}. The samples were then incubated for up to two weeks until stationary phase was reached at seven different temperatures (4, 7, 10, 12, 15, 17, 20 °C) and analysed by flow cytometry (final cell concentration) as described above. All experiments were performed in triplicate samples.

Determining the K_{cDOC} values

A regression analysis based on Monod kinetics was performed with the obtained results, which returned the best fit for $\mu_{max}B$ and K_s (equation 3). The program "Matlab" was used for this purpose. Since the bacteria did not grow on a single substrate but on a mixture of different organic carbon compounds we will use the term K_{cDOC} instead of K_s in order to express the relationship between $\mu_{max}B$ and the cDOC concentration.

$$\mu B = \mu_{max}B * cDOC / (K_{cDOC} + cDOC) \quad \text{(Eq. 3)}$$

Simulation of the batch growth curves

For the simulation, the three different pathogens and the FBC were grown in batch culture in dLB and the proliferation was followed using flow cytometry as described above. Simulation of the growth curves (using Aquasim) is based on Monod kinetics and uses the parameters $\mu_{max}B$, K_{cDOC} and the biovolumetric yield (equation 4).

$$eBV_t = eBV_{ini} * e^{\mu B * t} \quad \text{(Eq. 4)}$$

where eBV_t is the estimated total biovolume, eBV, at a given time point, eBV_{ini} is the initial eBV inoculated and $\mu_{max}B$ is μ_{max} based on the eBV (equation 3).

Results

Three pathogenic bacteria, *E. coli* O157, *V. cholerae* O1 and *P. aeruginosa*, and a freshwater bacterial community (FBC), were grown in batch culture in three different waters, i.e. diluted Lysogeny Broth (dLB), diluted wastewater (dWW), and river water (RW). The three waters used for cultivation were characterized with respect to their carbon quality using LC-OCD (Figure 1). This method fractionates the complex dissolved organic carbon according to size and charge of the molecules (Huber & Frimmel, 1992). The LC-OCD chromatograms in Figure 4.1 clearly demonstrate the differences in carbon composition for the three waters. For instance, whereas the RW contained a significant DOC fraction between a retention time of 30 to 40 minutes (considered to be polymeric substances), this fraction was missing in the other two waters. Furthermore, although the microbiologically consumable part of organic carbon (referred here as cDOC (see Material and Methods)) was similar in all waters (334 - 455 μg L^{-1}), the cDOC/DOC ratio was distinctly different. In dLB, 64 % of the total DOC was cDOC, while the dWW consisted of 51 % cDOC, and the RW contained only 9 % cDOC.

During batch growth of the three pathogens and the FBC on these waters we determined the following stoichiometric (static, quantitative) parameters: (1) final crop and DOC consumed and the corresponding yields, (2) the carbon fraction utilized for growth, (3) the cDOC threshold concentration, and (4) the minimum growth temperature. Furthermore, we investigated kinetic (time-dependent) aspects, including (5) specific growth rate (μ), and (6) the Monod saturation constant K_s (here K_{cDOC}) which relates nutrient concentration to μ. Finally, we simulated growth based on obtained growth parameters, μ_{max}, K_{cDOC}, and yield.

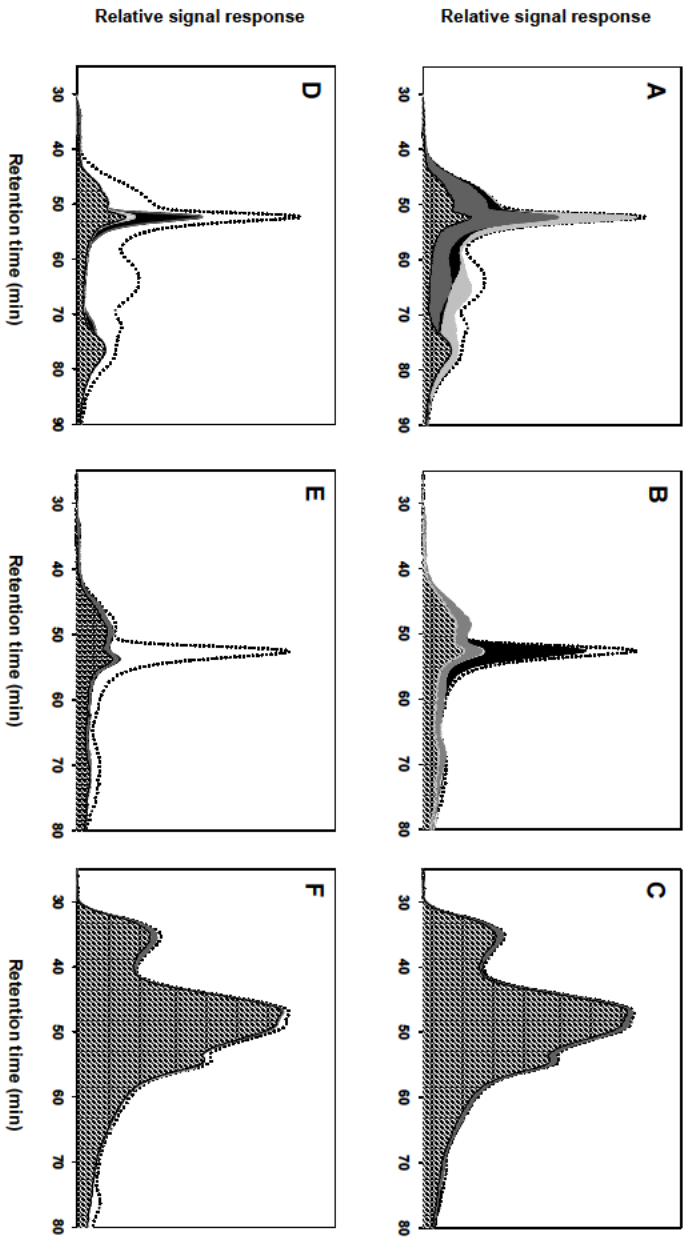


Figure 4.1. LC-OCD choromatograms of different waters after batch growth of different pathogens and the freshwater bacterial community (FBC). Three different waters, containing each a different organic carbon quality (dotted line), are shown, namely, 10,000-times diluted LB medium (A, D), 30-times diluted wastewater (B, E) and river water (C, F). The top row of images (A, B, C) shows the residual carbon fractions after growth of the individual pathogens namely *E. coli* O157 (light grey area), *P. aeruginosa* (dark grey area), *V. cholerae* O1 (black area) and the FBC (hatched area). The bottom row D, E, F) shows the corresponding chromatograms after the growth of the FBC, which was spiked after growth of the pathogens had reached stationary phase.

STOICHIOMETRY

Final crop, DOC consumption, yield, consumption of different fractions of DOC

During growth in all three waters, the FBC consumed most DOC and it utilized the organic carbon from the entire molecular weight spectrum (Figure 4.1 A-C). Significantly less DOC was consumed by the three pathogenic bacteria and the organic carbon fractions utilized by each strain differed distinctly. The results indicate that neither of the pathogens was able to consume organic carbon in the higher molecular weight range (40 to 50 minutes retention time), but primarily grew on the low molecular weight fractions. It must be pointed out that for RW the analysis of the chromatograms was difficult due to a large background of recalcitrant DOC. After the pure cultures had reached

stationary phase, the samples were re-inoculated with the FBC. The bacterial community then further proliferated on the cDOC that was left over by the pathogens, and in all samples the remaining non-biodegradable DOC was finally at a similar level for each individual water (Figure 4.1 D-F). In all three waters, the FBC produced the highest final cell concentration; it was typically three to ten times higher than that of the pathogens (Figure 4.2 A). A general trend concerning the final cell concentration as well as the DOC consumption was not observed for the three different pathogens. Whereas *E. coli* O157 formed least cells on dLB, growth of *V. cholerae* was weaker in dWW (Figure 4.2 A). On RW the final cell concentrations were very similar for all pathogens. An analogous pattern was observed for the consumption of DOC (Figure 4.2 C). Unfortunately, for none of the pathogens it was possible to deduce reliable DOC consumption data for growth on RW because of the large background of recalcitrant carbon (Figure 4.1).

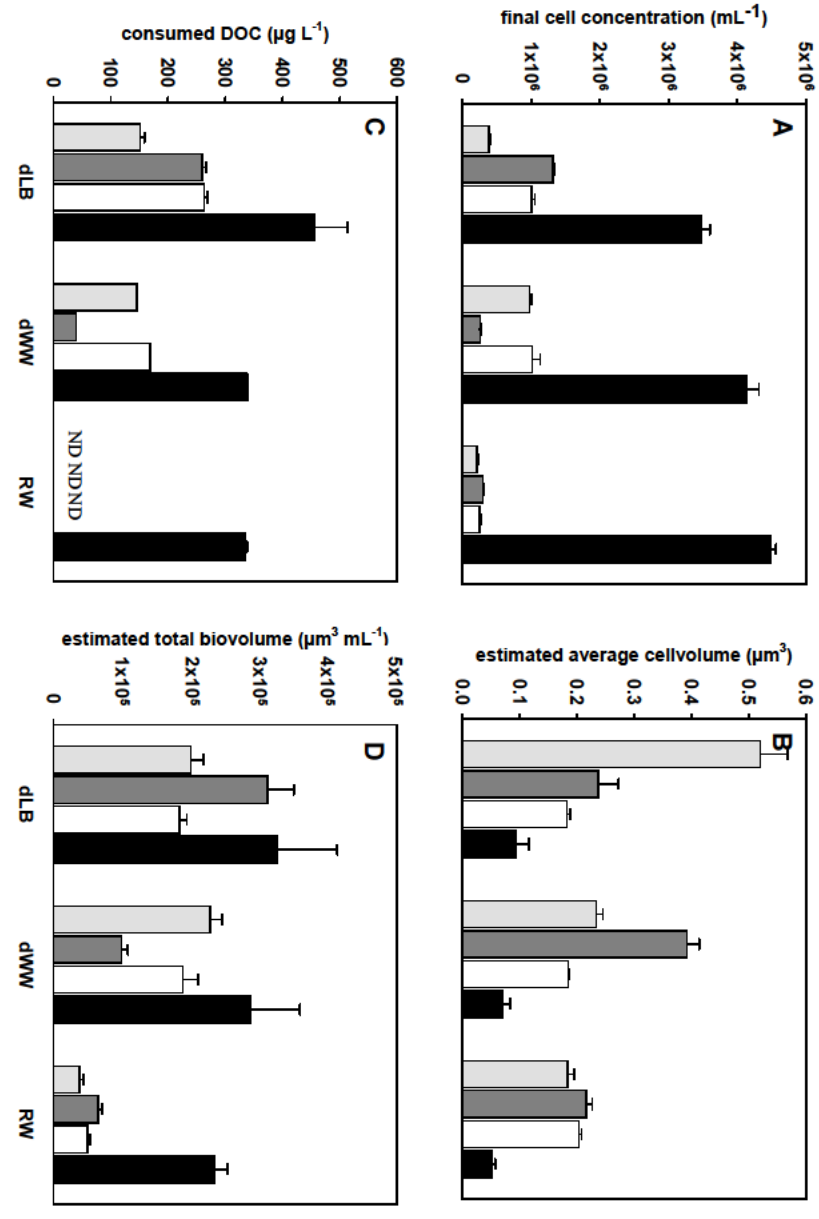


Figure 4.2. Growth of different pathogens and a freshwater bacterial community (FBC) on three different waters. The batch growth of three different pathogens namely *E. coli* O157 (light grey bars), *V. cholerae* O1 (dark grey bars), *P. aeruginosa* (white bars) and the FBC (black bars) in three different waters (10,000-times diluted LB medium (dLB), 30-times diluted wastewater (dWW) and river water (RW)) was investigated. The total final cell concentration (A), the estimated cellular biovolume (B), the consumed DOC (C) and the estimated total biovolume are shown. Error bars indicate the standard deviation on triplicate samples. ND: not detectable

The final estimated average cell volume (eCV) of the individual pathogens was two- to five-times lager in comparison to that of cells of the FBC (Figure 4.2 B). This implies that the three pathogens required more cDOC to form a new cell and, hence, the numerical cell yield, $Y_{\#}$, was considerably lower for the pathogens than for the FBC (Table 4.1). However, when a comparison was done based on the biovolume, eBV, the differences in the final crop between the individual pathogenic bacteria and the FBC were much lower and also corresponding biovolumetric yields, Y_{BV}, were closer for all bacterial cultures (Table 4.1).

Table 4.1. Numerical and estimated biovolumetric yield after batch growth on three different waters**.** The yields for three different pathogens *(E. coli O157, V. cholerae, P. aeruginosa*) and the freshwater bacterial community (FBC) with respect to cell numbers and total estimated biovolume (eBV) formed per µg of consumed DOC are displayed. The presented yields are based on the results shown in Figure 4.2.

	***E. coli* O157**	***V. cholerae***	***P. aeruginosa***	**FBC***
		10,000-times diluted LB		
$\mathbf{Y_{\#}}$	2.54 ± 0.11	5.06 ± 0.20	3.83 ± 0.23	7.69 ± 0.80
$\mathbf{Y_{BV}}$	1.33 ± 0.17	1.20 ± 0.18	0.69 ± 0.05	0.71 ± 0.11
		30-times diluted wastewater		
$\mathbf{Y_{\#}}$	6.69 ± 0.21	2.74 ± 0.1	6.04 ± 0.62	12.2 ± 0.48
$\mathbf{Y_{BV}}$	1.56 ± 0.12	2.47 ± 0.24	1.12 ± 0.12	0.85 ± 0.21
		undiluted river water		
$\mathbf{Y_{\#}}$	ND^{+}	ND^{+}	ND^{+}	13.3 ± 1.2
$\mathbf{Y_{BV}}$	ND^{+}	ND^{+}	ND^{+}	0.96 ± 0.15

$Y_{\#}$: numerical cell yield (cells formed ($x10^6$) µg DOC^{-1} consumed)

Y_{BV}: estimated biovolumetric yield (eBV (μm^3 ($x10^6$)) µg DOC^{-1} consumed)

* FBC: freshwater bacterial community

$^{+}$ ND: not detectable

Nutrient and temperature threshold for growth

As a next step, the existence of a minimum (threshold) substrate concentration for growth was investigated for the three pure cultures. Dilutions of dLB medium and RW were prepared covering a range from 100 % (undiluted) down to 1 %. These waters were then inoculated and the final cell concentrations reached in stationary phase were determined (dLB Figure 4.3; RW data not shown). *E. coli* O157 and *P. aeruginosa* grew at all the different LB concentrations tested. For example, during growth with dLB containing 4 µg cDOC L^{-1} the cell number of *P. aeruginosa* increased from 1 x 10^3 mL^{-1} to 1.04 x 10^4 mL^{-1}, which corresponds to three to four generations and cannot be attributed to reductive division. Also *V. cholerae* proliferated at all cDOC concentrations, however, the $Y_{\#}$ was reduced drastically below cDOC concentrations of ca. 40 µg L^{-1}. Above this concentration a linear correlation between cDOC concentration and final cell concentration was

observed. Also on diluted RW we were unable to detect a distinct nutrient threshold for any of the three pathogens tested (data not shown). All bacteria grew at all dilutions tested down to the most diluted RW, which contained approximately 5 µg of cDOC L^{-1}.

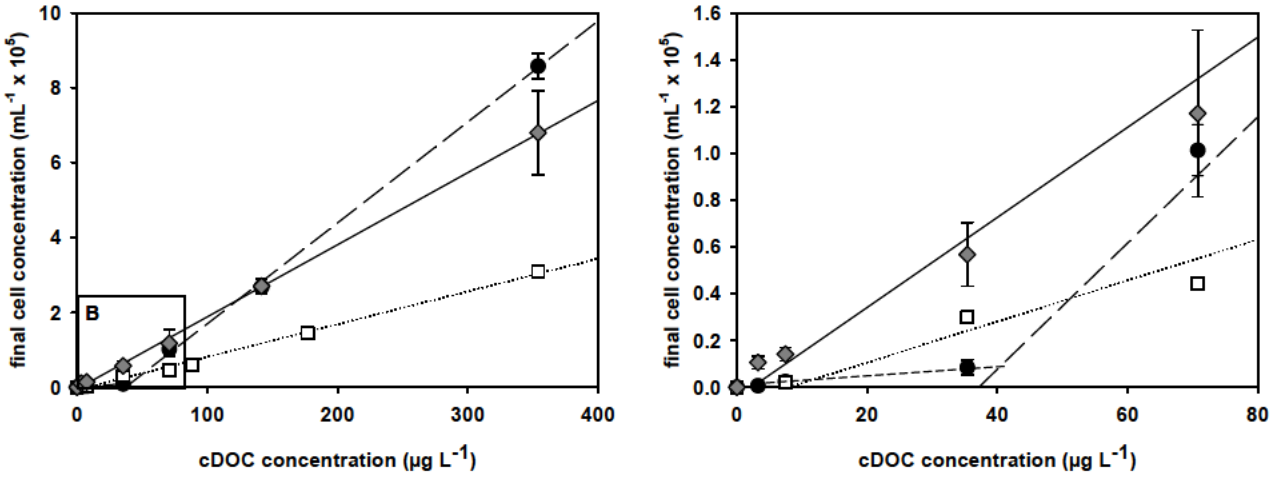


Figure 4.3. Determining the nutrient threshold for growth of three different pathogens. A dilution series of LB medium, containing different concentrations of consumable DOC (cDOC), was established and the final growth of different bacteria namely *E. coli* O157 (□; dotted line), *V. cholerae* O1 (●; dashed line) and *P. aeruginosa* (◆; solid line), grown in batch culture, was determined (Panel A). Panel B specifically illustrate the section of low cDOC concentrations. Error bars indicate the standard deviation on triplicate samples.

The minimum temperatures that were required for proliferation of the individual pathogens on RW are shown in Figure 4.4. *P. aeruginosa* started to grow already at 10 °C, whereas the minimum temperatures for growth of *E. coli* O157 and *V. cholerae* were in the range of 12 and 15 °C, respectively. The numerical cell yields, $Y_{\#}$, achieved by *V. cholerae* and *P. aeruginosa* were similar at all growth-supporting temperatures. In contrast, for *E. coli* O157 the $Y_{\#}$ was, interestingly, significantly reduced below 15 °C. For all bacteria tested, the same temperature thresholds for growth were recorded on dLB as those found with RW (data not shown).

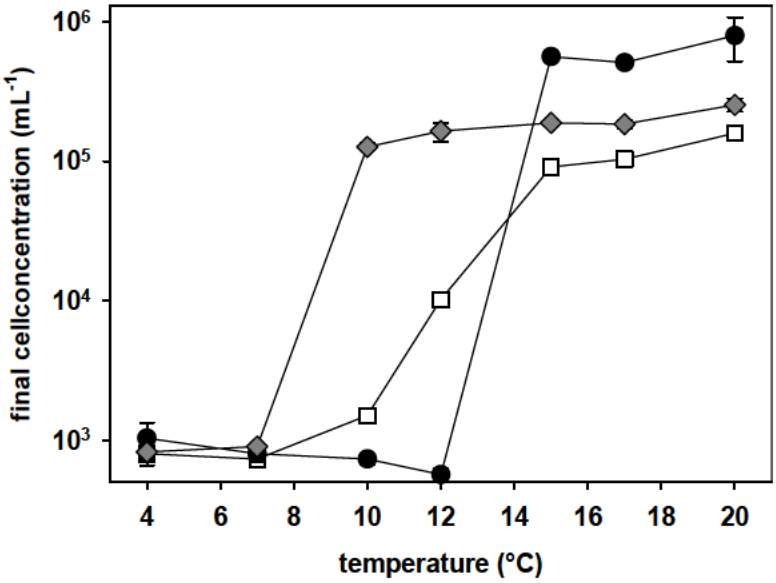


Figure 4.4. Determining the temperature threshold for growth of three different pathogens. The final cell concentration of different pathogens namely *E. coli* O157 (□), *V. cholerae* O1 (●) and *P. aeruginosa* (◆), grown in batch culture at different temperatures on sterile river water, is shown. Error bars indicate the standard deviation on triplicate samples.

KINETICS

The specific growth rate (μ)

Bacterial proliferation was followed based on cell number as well as on estimated total biovolume, eBV (Equations 1 and 2 in Materials and Methods). The two approaches yielded distinctly different growth curve patterns. Initially, the stationary phase cells inoculated increased in their estimated cell volume, eCV, but did not divide yet (Figure 4.5). Thus, when judged solely on an increase in cell number, this initial phase of growth could not have been detected and one would have interpreted this as a lag phase. However, considering the eCV (and corresponding eBV) the cultures showed either no or only a very short lag (Figure 4.5). In all experiments we observed an exponential growth phase characterized by a stable μ. When considering the eBV this phase extended over a larger range than when growth was judged based on cell concentration. The eBV formed at a constant μ was between 40 to 100% of the final eBV (two examples are shown in Figure 4.5). For *E. coli* O157, *V. cholerae* and the FBC, the μ value derived from either cell number or eBV was not significantly different.

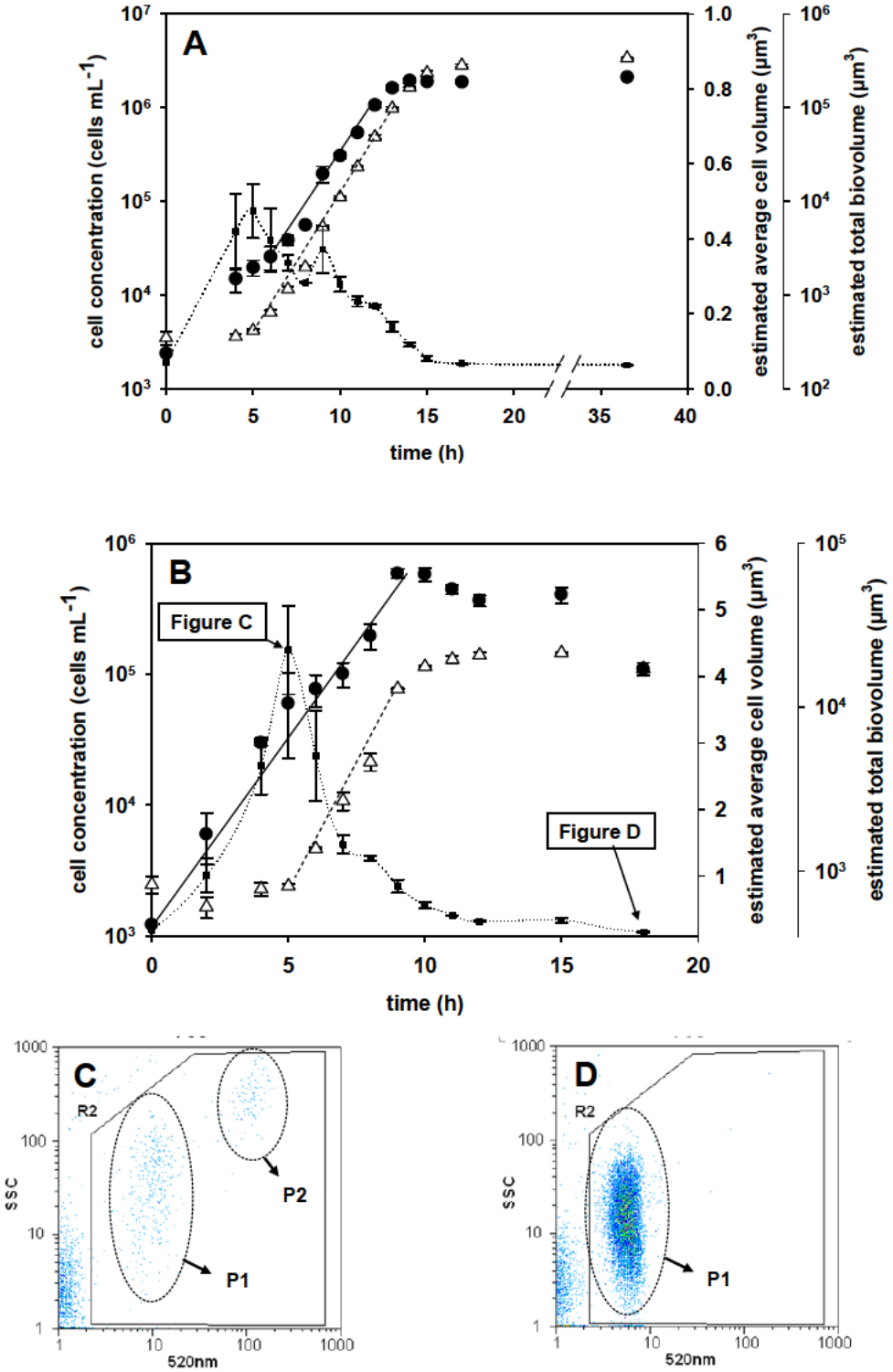


Figure 4.5. Batch growth curves of a freshwater bacterial community (A) and *Pseudomonas aeruginosa* (B) on sterile river water. The plots show the cell concentration (Δ), the estimated total biovolume (eBV; ●) and the estimated average cell volume (■). Trendlines mark the range from which the specific growth rate was calculated (solid line - eBV and dotted line - cell concentration). Plot C and D show corresponding flow cytometric dot plots (sideward scatter-SSC vs green fluorescence-520nm) for two different time points t=5h (C) and t=17h (D) during the batch growth of *P. aeruginosa*. R2 represents the electronic gate used for SSC determinations, whereas the dashed circles (P1 and P2) indicate the different populations formed during growth. Error bars indicate the standard deviation on triplicate samples.

In contrast, *P. aeruginosa* displayed a unique pattern during batch growth. The initial eCV increase was very pronounced and went along with the formation of a second population of very large cells according to the FCM dot plot (P2 in Figure 4.5 C). In this initial phase the eBV-based μ was 0.61 h^{-1} whereas the cell number-based μ was 0 h^{-1}. After 5 hours the cells started to divide. Now the average eCV became smaller and the P2 population gradually disappeared and concomitantly the cell number in P1 increased quickly. Calculating μ for *P. aeruginosa* growing on RW based on eBV (μB) over the whole exponential range resulted in a μB of 0.5 h^{-1}. Based on cell number μ increased during exponential growth to 0.84 h^{-1} (168 %); this value is certainly an overestimation resulting from the fast cell division of large P2 cells in this phase. The same phenomenon was observed for growth of *P. aeruginosa* in the dLB and dWW. The resulting μ values based on cell concentration were increased by 166 % (dLB) and 154 % (dWW), respectively, in comparison to those derived from eBV.

Based on the eBV, μB of the individual bacterial cultures exhibited a clear trend in all the three waters, despite of the different quality of cDOC available for growth. The FBC exhibited the highest μB, followed by *P. aeruginosa* and *V. cholerae*, whereas *E. coli* O157 grew slowest (Figure 6). Only on dWW μB of *V. cholerae* was similar to that of *E. coli* O157, but on RW *V. cholerae* achieved a very high μB, comparable to that of *P. aeruginosa*.

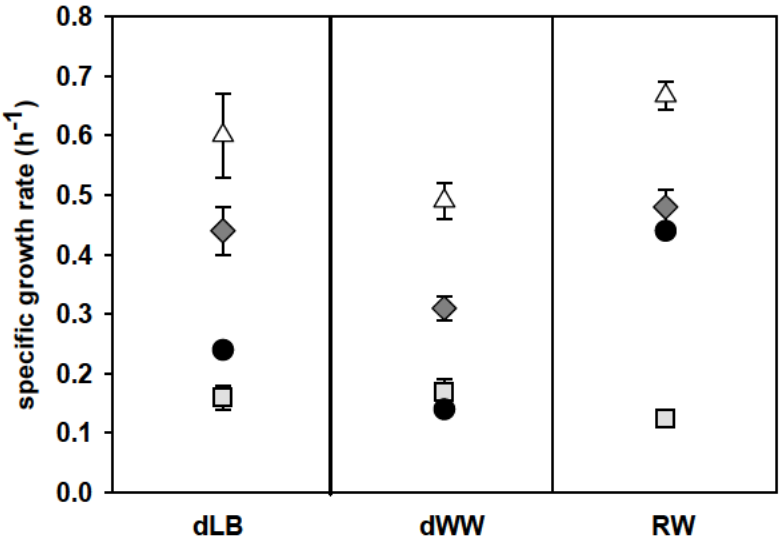


Figure 4.6. Specific growth rates (μ) of different pathogens and a freshwater bacterial community (FBC) on three different waters. The batch growth of three different pathogens namely *E. coli* O157 (□), *V. cholerae* O1 (●), *P. aeruginosa* (◆) and the FBC (Δ) was investigated on three different waters (10,000-times diluted LB medium (dLB), 30-times diluted wastewater (dWW) and sterile river water (RW). The μ_{max} values are based on total biovolume estimations. Error bars indicate the standard deviation on triplicate samples.

Correlation between µB and nutrient concentration (K_{cDOC} values) and simulation of batch growth

The specific growth rate of a microbial culture is usually correlated to the concentration of a growth-limiting substrate in a saturation-type relationship. Most frequently the Monod equation is used to relate µ with the concentration of a single substrate (s) via the two constants μ_{max} and K_s (Monod, 1949; Kovarova-Kovar and Egli, 1998). To test whether a similar simple relationship exists for the much more complex situation during growth with diluted LB medium, where cells grow simultaneously with a multitude of different carbon/energy sources, we exposed the three pathogens and the FBC to different concentrations of cDOC in batch culture and recorded the resulting µ*B* values.

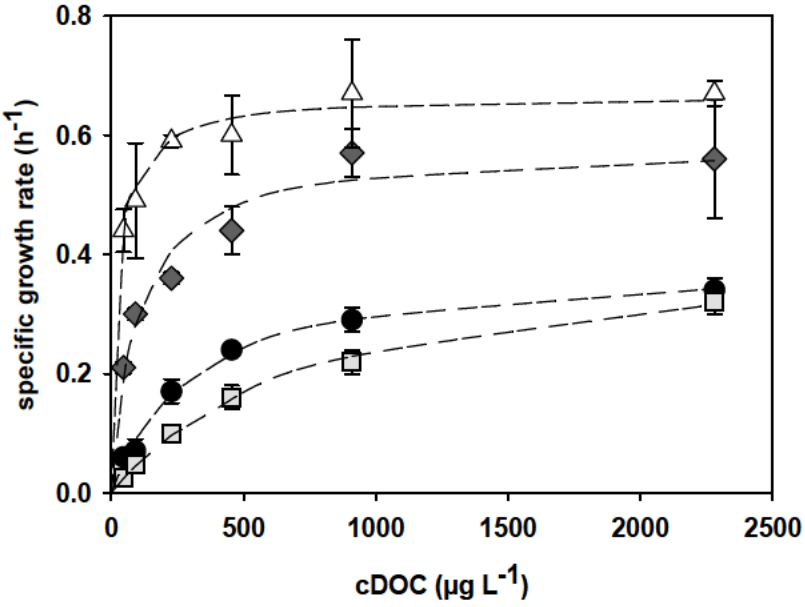


Figure 4.7. A correlation between the specific growth rate (µ) and the cDOC concentration on different dilutions of LB medium was established for three different pathogens namely *E. coli* O157 (□), *V. cholerae* O1 (●), *P. aeruginosa* (◆), and the freshwater bacterial community (Δ). The µ values were determined using total biovolume estimations. The dashed lines illustrate the best fit with the variables $\mu_{max}B$ and K_{cDOC} based on Monod kinetics (see Materials and Methods). Error bars indicate the standard deviation on triplicate samples.

For all cultures the resulting µ*B* vs. cDOC patterns exhibited a clear saturation-type-relationship with striking similarity to the Monod model (Figure 4.7). Therefore, we fitted the data using Monod kinetics to extract the two model constants, defined here as $\mu_{max}B$ and K_{cDOC} (see eq. 3). K_{cDOC} values in Table 4.2 represent the cDOC concentration, where the µ*B* reaches 50 % of $\mu_{max}B$, based on the simulation. The three pure cultures could only use a fraction of the total cDOC present in the medium (see above) and we therefore adjusted the K_{cDOC} values for each of the pathogens (Table 4.2).

The kinetic constants obtained demonstrate clear differences between the different bacterial cultures. Whereas the curves for *P. aeruginosa* and the FBC reached saturation within the cDOC concentration range used in the experiment, those for *V. cholerae* and *E. coli* O157 did not. Whereas *E. coli* and *V. cholerae* reached similar $\mu_{max}B$ around 0.4 h^{-1}, the simulated value of $\mu_{max}B$ for *P. aeruginosa* was higher and close to that of the bacterial community. Obvious were the large differences in the obtained affinity constants, K_{cDOC}. Based on available cDOC, the FBC and *P. aeruginosa* exhibited a considerably lower K_{cDOC} compared to *E. coli* O157 and *V. cholerae*.

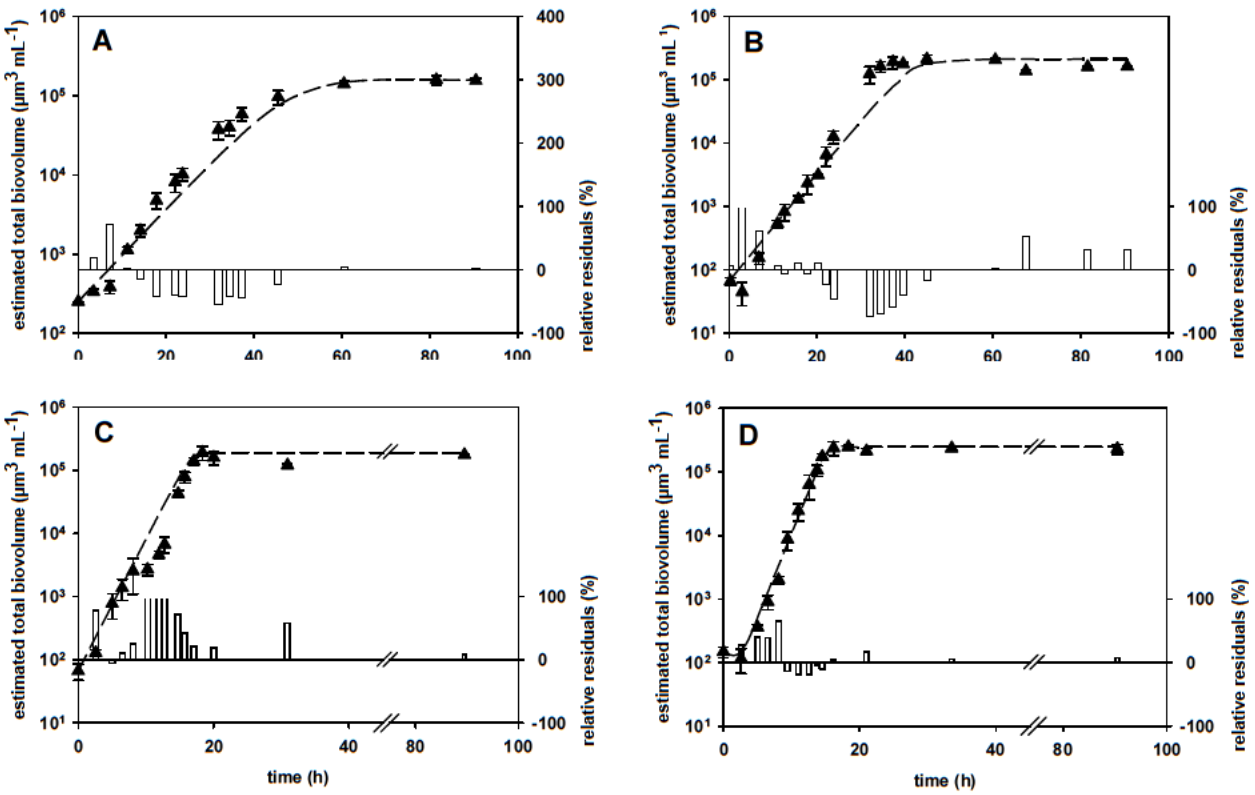
Table 4.2. Correlation between specific growth rate (μ) and the consumable DOC (cDOC) concentration**.** The simulated K_{cDOC} values for three different pathogens, *E. coli O157* (K_{cDOC}E); *V. cholerae* (K_{cDOC}V); *P. aeruginosa* (K_{cDOC}P), and the freshwater bacterial community (K_{cDOC}FBC) based on Monod kinetics are displayed. The corresponding measured data are presented in Figure 4.7.

	cDOC (μg L^{-1})	**cDOC available (μg L^{-1})***	**$\mu_{max}B$ (h^{-1})$^{+}$**
K_{cDOC}E	768 ± 52	252 ± 17	0.42 ± 0.01
K_{cDOC}V	305 ± 33	174 ± 19	0.39 ± 0.01
K_{cDOC}P	98 ± 22	57 ± 12	0.58 ± 0.03
K_{cDOC}FBC	27 ± 4	27 ± 4	0.66 ± 0.01

***** This row displays the K_{cDOC} values considering the proportion of DOC available for growth for the individual bacteria (Figure 2).

$^{+}$ Simulated $\mu_{max}B$ values based on Monod kinetics.

The kinetic parameters listed in Table 4.2, together with the individual biovolume yields (Y_{BV}), were used to simulate batch growth in dLB (Figure 4.8). For all cultures, the simulation adequately described the time-course of the estimated biovolume (eBV) data derived from flow cytometry. The influence of different substrate affinities, K_{cDOC}, became nicely visible when we compared the cultures of *E. coli* and the microbial community. For the former a clear deceleration phase was observed when the culture approached stationary phase, whereas the FCB maintained a constant μB virtually throughout the whole growth cycle and entered the stationary phase abruptly.



Figur 4.8. Simulated batch growth curves of different pathogenic bacteria and a freshwater bacterial community (FBC) on diluted LB medium. The batch growth of three different pathogens namely *E. coli* O157 (A), *V. cholerae* O1 (B), *P. aeruginosa* (C) and the FBC (D) on 10,000-times diluted LB medium is displayed. Each plot depicts the measured values (▲), the mathematical prediction (dashed line) and the corresponding relative residuals (white bars). The simulation is based on the $\mu_{max}B$ and the K_{cDOC} values presented in Table 4.1 and 4.2. Error bars indicate the standard deviation on triplicate samples.

Discussion

Who is eating what?

In the environment, the variety of organic carbon molecules is very divers and they are present at very low concentrations (Münster, 1993). This is particularly relevant for heterotrophic microbes; each species has its own spectrum of carbon compounds that it can utilize for growth and this characterization is a pillar of traditional taxonomy. Thus, in an ecosystem the carbon pool present influences growth of individual heterophic strains and in the end also governs the composition of the resulting microbial**.** The chromatograms obtained with LC-OCD analysis shown in Figure 4.1 nicely demonstrate the differences in utilized carbon by the individual cultures. Each of the three pathogens displayed a specific utilization pattern and the freshwater community clearly consumed the most DOC throughout all fractions. Since this community consists of many different bacterial strains, its range of consumable substrates is logically broader than that of a pure culture. This is clearly demonstrated in the "spiking experiment", where the freshwater bacterial community polished away all cDOC that was left over by the individual pathogens. To obtain preliminary information of the influence of carbon quality on the composition of the developing bacterial freshwater community in the different waters we analyzed the resulting stationary phase communities with denaturing gradient gel electrophoresis (DGGE). On all three waters the grown-up bacterial community consisted indeed of several bacteria varying in their 16S sequence and the band pattern for each water varied distinctly confirming the influence of carbon quality on the selection for specific bacterial species (data not shown).

The LC-OCD method employed here is accepted to be one of the best and most sensitive methods presently available for the fractionation of natural organic matter; from the chromatograms one is able to differentiate between four to six organic carbon fractions containing unknown compounds of different molecular size (Huber & Frimmel, 1992). Hence, unfortunately this method does not allow identifying individual substrates utilized during the growth of our cultures. Nevertheless, the data obtained provide a first glimpse at the quality and quantity of carbonaceous substrates consumed during growth of microbial cultures under environmental conditions. New technologies, which enable sensitive measurements on a single substrate level in a complex mixture of organics are needed. Presently, MS/MS-techniques are being developed that should allow a better separation and identification of compounds in NOM (Zhang *et al.,* 2004; Frazier *et al.,* 2005) and may contribute to a better understanding in this area.

Cell concentration versus biovolume and corresponding yields

Two different types of methods are applied to determine growth in dilute (oligotrophic) systems: those based on single cell (number) measurements (e.g., plating, microscopic or flow cytometric counting), and methods based on bulk parameters that yield information on the entire population (e.g., sensitive turbidity measurement, ATP, radioactive substrate incorporation). Bacteria can, however, distinctly differ in their cell size and the actual biomass per cell is varying (Stephenson, 1949, Wang *et al.,* 2009). Hence, measurements based on either cell number or a bulk parameter solely, yield only limited information on growth. An ideal method would allow to determine both, cell concentration and biomass of single cells at a high throughput. We approached this using flow cytometry, which allowed us to estimate the bio-volume of individual cells from their light scattering signal (Felip *et al.* 2007; Hammes *et al., in press*). The results in Figure 4.2 demonstrate that stationary phase cells of the pathogens tested were always bigger than the average cell of the freshwater bacterial community; this explains the observed discrepancy of the numerical cell yield ($Y_{\#}$) and the yield based on biovolume estimation (Y_{BV}) in Table 4.1. The FBC achieved a much higher $Y_{\#}$ than the pathogens, whereas Y_{BV} was lower for the community. The lower Y_{BV} of the FBC may result from the fact that they consumed not only the "easy" cDOC used as well by the pathogens but were able to utilize also some of the more complex cDOC, which probably supported lower growth yields.

Kinetics

Bacterial growth kinetics had its heydays in the 1950-70ies and a number of reviews were published over the years (e.g., Stephenson, 1949; Monod, 1949; Powell, 1958; Kovarova-Kovar & Egli, 1998). It was for a long time considered to be a closed book and the original concepts were applied in biotechnology and microbial ecology. However, the kinetic models used were based on the concept that a single compound only is controlling the rate of growth (see discussion in Kovarova-Kovar & Egli, 1998). Although this is valid for many laboratory investigations, the conditions experienced by microbial cells growing in Nature are usually drastically different. Probably the best example is that of heterotrophic microbes that grow in the environment typically under carbon/energy-limited conditions (Morita, 1988, 1997). Here, not a single substrate but myriads of carbonaceous compounds, usually present in the low microgram to nanogram per litre range, are available for growth. Under such conditions microbial cells take up and metabolize different carbonaceous substrates simultaneously instead of utilizing them individually. It has become clear that in Nature this so-called "mixed substrate growth" (Harder and Dijkhuizen, 1976) is the rule rather than the exception and confers a number of advantages to a microbial cell, such as improved

kinetics and competitive ability, better metabolic flexibility, and it probably also helps to minimize threshold concentrations for growth (see Egli, 1995).

Kinetics and modelling

Assuming Monod kinetics and that a cell is specializing on growth with one single growth-limiting carbon source only, the predicted specific substrate consumption rate (and, hence, μ) would be extremely low (equation 5). However, under mixed substrate growth conditions the cell is able to combine the small rates from all simultaneously consumed substrates to a decent total carbon flux, which can support a much higher specific growth rate (equation 6) (see Egli, 1995). However, for growth with an undefined substrate mixture such as diluted complex media or natural waters, it will be impossible to quantify the contribution of each individual carbon sources in equation 6 (for a discussion see Kovarova-Kovar & Egli, 1998). Therefore, we tested whether it is possible to use the bulk parameter cDOC as the variable controlling the specific growth rate of a bacterial culture (equation 7). The data shown in Figure 4.7 convincingly demonstrate that the relationship between cDOC and specific growth rate can be indeed described well with the Monod model, taking cDOC as the limiting carbon source. Considering that each substrate used probably varies in its kinetic constants μ_{max} and K_s (equation 6), the quality of the fit of the data was indeed surprising. Particularly, if one takes into account that during the batch growth kinetic experiments performed here, not only the cDOC concentration but furthermore the quality of carbon/energy substrates used by a cell was probably continuously changing.

$$\mu = \mu_{max}s * s / (K_s + s) \quad \text{Eq. 5}$$

$$\mu = \mu_{max}s_1 * s_1 / (K_{s1} + s_1) + ... + \mu_{max}s_n * s_n / (K_{sn} + s_n) \quad \text{Eq. 6}$$

$$\mu = \mu_{max}cDOC * cDOC / (K_{cDOC} + cDOC) \quad \text{Eq. 7}$$

We would like to mention that for modelling, the fact that not the total DOC but only a part of the organic carbon present in an eco-system is readily available for microbes (i.e. the AOC referred here as cDOC) has to be considered. Furthermore, for modelling growth of individual strains the parameters used have to be adapted to the individual cDOC availability.

In this respect, the main differences for growth of the used bacterial cultures in low nutrient environments was primarily in their affinity to cDOC, with the natural community and *P. aeruginosa* exhibiting significantly better affinities than the two enteric pathogens *E. coli* O157 and *V. cholerae* (Table 4.2; Figures 4.6, 4.7). Differences in the stoichiometric properties appeared to be

less important. This may be the reason for the better performance and survival of *P. aeruginosa* in aquatic ecosystems compared to enteric pathogens. Although in river water *V. cholerae* was astonishingly fit and achieved a higher specific growth rate than the predicted μ_{max} with dLB (Figure 4.6), which indicates that next to carbon concentration also carbon quality can be important for the kinetic performance of individual strains.

A nice example for the advantage of the method used for assessing growth on the information obtained was seen for the batch cultivation of *P. aeruginosa*. In the initial phase of the batch culture the cell number for this bacterium stayed constant, despite the fact that cellular biomass increased exponentially (Figure 4.5). In this phase two distinct cell populations became visible in the FCM dot plots. This demonstrates the power of flow cytometry to assess both cell number and size simultaneously, which yields additional insights into the growth behaviour of certain strains.

Threshold

On one hand, many reports exist in literature that suggest the existence of a nutrient threshold concentration for microbial growth, frequently in the range of 1 - 100 µg L^{-1} (see e.g., Jannasch, 1967; 1969; Jannasch & Egli, 1993; Alexander, 1994; Kovarova *et al.* 1996; Vital *et al.,* 2007). On the other hand, there is also much evidence that microbes still grow in the presence of minute concentrations of individual carbon substrates (van der Kooij, 1980, 1982; Egli, 1995). Based on this it has been proposed so far that enteric pathogens are microorganisms adapted to live at high nutrient concentrations in their primary habitat and, hence, are unable to grow at the low nutrient concentrations found in most environments (e.g. Morita, 1997; Winfield & Groisman, 2003), whereas the adapted natural microbial flora is still able to grow at very low substrate concentrations. Our results (Figure 3) clearly demonstrate that all the three pathogens are able to multiply at all cDOC concentrations tested (down to a few µg L^{-1}). The only exceptional growth pattern was that of *V. cholerae* on dLB with a biphasic growth, where the numerical cell yield was significantly reduced at cDOC concentrations below 40 µg L^{-1}. It should be pointed out that experimental determination of threshold concentrations is fraught with difficulties; the presence of background AOC (of natural origin or introduced during sample handling) can affect the result and we therefore used a negative control, i.e. the water used for dilution (cDOC-free bottled mineral water) in our experiments.

The results demonstrate that growth of pathogenic bacteria can already occur at minute substrate concentrations of a few µg C L^{-1}, which supports the early reports of van der Kooij & Hijnen (1988)

who showed growth of the opportunistic pathogen *Aeromonas hydrophilia* already at a concentration of 0.1 µg C L^{-1}. This is of particular importance for drinking water treatment and distribution where the production of a so called "biostable water" - i.e. drinking water without any nutrients available for bacterial growth - should be a central focus in order to prevent the re-growth of pathogenic bacteria in the system (Juhna *et al.,* 2007; Hammes *et al.,* 2010).

Next to the available nutrients, temperature can be a limiting factor governing microbial growth. Especially in the northern hemisphere, where average daily temperatures below 20 °C prevail, the ability of a bacterium to multiply at cold temperatures is important for growth in the environment. Whereas many studies focus on the optimal growth temperature of a bacterium, data on the minimum temperature required for growth are scarce. For different strains of *E. coli*, the reported results vary significantly and range from 13.5 to 19 °C (Herendeen *et al.,* 1979; Raghuber & Matches, 1990), whereas in this study we observed growth of *E. coli* O157 starting already at 12 °C. The observed minimum temperatures required for proliferation of *V. cholerae* and *P. aeruginosa* were similar to those reported by others (Rajkowski & Rice, 2001; Rajkowski *et al.*, 1996).

Conclusions

- *P. aeruginosa* grew better than *V. cholerae* and *E. coli* O157, whereby kinetic rather than stoichiometric parameters were found to differ considerably between cultures
- The freshwater bacterial community performed best in all aspects investigated
- Not only cell concentration but also the individual cell volume should be considered for growth investigations
- With the determined parameters it was possible to model growth based on Monod kinetics

5. Competition of *Escherichia coli* O157 with a drinking water bacterial community at low nutrient concentrations

Abstract

In the environment, heterotrophic bacteria grow with a multitude of different organic carbon compounds, all present at minute concentrations. Investigations on bacterial competition under such nutritional conditions are rare and the principles involved are unexplored. However, knowledge on this topic is crucial in order to understand the behaviour of specific bacterial species such as pathogens in the environment and, furthermore, to comprehend the mechanisms shaping the structure of microbial communities in general. Therefore, the pathogen *E. coli* O157 was grown in competition with a drinking water bacterial community on natural assimilable organic carbon (AOC), namely diluted waste water, in both batch and continuous culture. Growth was monitored by flow cytometry enabling enumeration of total cell concentration and specific *E. coli* O157 detection using fluorescently labelled antibodies. The influence of AOC concentration, temperature and dilution rate was investigated, demonstrating that all these parameters are positively influencing competitive fitness of *E. coli* O157. The obtained results could be explained by the determined growth properties of competitors in pure culture and it was even possible to model many of the underlying dynamics based on Monod kinetics. However, during competition in continuous culture, additional mechanisms not explainable by Monod kinetics were observed. The study provides first insights into the principles governing bacterial competition under mixed substrate conditions at low AOC concentrations. It contributes to microbial risk assessment by improving the understanding on the behaviour of *E. coli* O157 in the environment.

Introduction

Given the enormous bacterial diversity in aquatic ecosystems, any particular heterotrophic strain is usually competing with many other microbes for nutrients and it is acknowledged that competition processes are, thus, a key element shaping microbial communities (Gottschal, 1993; Torsvik *et al.,* 2002; Konopka, 2009). Furthermore, understanding competitive interactions between bacteria are important for microbial risk assessment as well because they partly determine the growth behaviour of pathogenic bacteria in ecosystems closely related to human health such as drinking water. However, controlled *in vitro* experiments on this topic are scarce and, hence, the basic understanding of the principles governing competitive growth of heterotrophs in the environment is poor. Microbiologists still rely on concepts established in the period from 1950 - 1970, where the focus was on pure and simple competition, i.e. a single growth-limiting substrate (usually an organic carbon compound) is available for all competitors and competition for this substrate is the only interaction between the competitors, under totally defined conditions (see Powell, 1958; Fredrickson & Stephanopoulos, 1981). Results of such experiments were explainable by Monod kinetics (Janasch 1967; Harder & Veldkamp, 1971; Veldkamp & Jannasch, 1972; Hansen & Hubbell, 1980), and much of the subsequent research on competition of heterotrophic bacteria was done *in silico*, where different scenarios were theoretically investigated (e.g., Hsu, 1980; Hale & Somolinos, 1983; Gottschal 1993; Dukan *et al.,* 1996).

Also in the environment, growth of heterotrophic bacteria is usually carbon-limited (Morita, 1997). However, the organic carbon pool that is readily available for growth, referred to as assimilable organic carbon (AOC), is not composed of a single substrate but is a mixture of many different individual compounds, all present at very low concentrations (Münster, 1993). Hence, growth is simultaneously limited by many homologous nutrients, i.e. all satisfy the same physiological function (Harder & Dijkhuizen, 1976). In other words, bacterial cells that are growing in the environment use many AOC molecules simultaneously as the carbon and energy source, referred to as “mixed substrate growth” (Egli, 1995). This phenomenon should not be mixed up the simultaneous limitation of multiple non-homologous nutrients such as nitrogen, phosphorous and carbon, each satisfying a different physiological function (e.g., Egli, 1991; Huisman & Weissing, 2001; Grover, 2004). There are reports available, which focus on growth of bacteria on natural AOC in pure culture, including pathogens (e. g., van der Kooij & Hijnen, 1982; Pomeroy & Wiebe, 2001; Vital *et al.,* 2008; Kirschner *et al.,* 2008), but our understanding of their competitive behaviour in the environment is still very limited. To our knowledge, studies specifically addressing

the principles governing competition of heterotrophic bacteria under "mixed substrate growth" conditions on natural AOC *in vitro* do not exist.

This lack of data is at least partly due to previous methodological limitations. In the environment, bacteria are present at low concentrations (White *et al.,* 1991) and only a minute fraction of them can be cultured on conventional media (see e.g., Staley & Konopka, 1985). Hence, methods relying on high cell concentrations such as optical density measurements or those based on cultivation are not suitable for investigating bacterial growth and competition under environmental conditions *in vitro*. Fluorescence-staining in combination with microscopy circumvents these problems but is very time consuming (Bowden, 1977). Furthermore, this approach often neglects a big part of bacteria present in freshwater (Wang *et al.,* 2009). In contrast, flow cytometry-based methods are well suited for this purpose as they allow multiparametric data acquisition of all cells at low concentrations at a high throughput (Vital *et al.,* 2007; Czechowska *et al.,* 2008; Hammes *et al,* 2008).

The aim of this work was to improve the knowledge of the principles governing bacterial competition in the environment with special focus on the behaviour of *E. coli* O157. For this purpose, the pathogen was grown in competition with a bacterial community derived from drinking water on natural AOC in both batch and continuous culture. This approach represents a classical "opportunist" versus "gleaner" relationship, where *E. coli* O157 is the "opportunist", specialised for growth at high nutrient concentrations, and the bacterial community is the "gleaner", which is adapted to nutrient-poor environments (Veldkamp & Jannasch, 1972, Grover, 1990). The influence of the nutrient concentration, the temperature and the dilution rate on the outcome of the competition was studied. Growth was monitored using flow cytometry-based methods. Furthermore, it was investigated whether it is possible to model the obtained data using Monod kinetics.

Materials and Methods

Bacterial strains and pre-cultivation

The verotoxin-negative *E. coli* O157 (Nent 2540-04) was stored at -80 °C. The cryo-culture was streaked onto a Tryptic soy agar plate (Biorad, Reinach, Switzerland) and incubated for 24 h at 37 °C. Cells from a single colony were transferred with a loop into ten-times diluted Luria-Bertani (LB) broth and were incubated overnight at 37 °C. Subsequently, cells from this overnight culture were transferred into 10,000-times diluted LB medium (starting concentration 5 x 10^3 cells mL^{-1}) and incubated for four days at 30 °C. The cells were then inoculated in sterile 100-times diluted wastewater (starting concentration: 5 x 10^3 cells mL^{-1}) and grown for four days at 30 °C before being used as an inoculum. To prepare the drinking water bacterial community (BC), indigenous bacterial communities from both bottled mineral water and unchlorinated tap water (Dübendorf, Switzerland; initial concentration ~ 3 x 10^3 cells mL^{-1} from each water) were grown together in 100-times diluted wastewater for four days at 30 °C. This BC was kept at room temperature and served as a stock culture for all experiments.

Preparation of carbon-free materials

Carbon-free glassware (bottles and vials) was prepared as described in Hammes & Egli (2005). In short: all glassware was first washed with a common detergent and thereafter rinsed three-times with deionised water. Then it was submerged overnight in 0.2 N HCl and subsequently rinsed with deionised water again and air-dried. Finally, the bottles and vials were heated in a Muffel furnace at 500 °C for at least six hours. Teflon-coated screw caps for the glassware were washed and treated identically with acid (0.2 N HCl). Caps were thereafter soaked in a 10 % sodium persulphate solution (60 °C, 1 h), rinsed three-times with deionised water and finally air-dried.

Flow cytometry

For total cell counts, an aliquot of 10 µL of SYBR®green (Molecular Probes, Basel, Switzerland), 100-times diluted in dimethylsulfoxid (Fluka Chemie AG, Buchs, Switzerland), was added to 1 mL of a bacterial suspension and incubated for 15 min at room temperature in the dark before analysis. EDTA (pH 8) was added (5 mM final concentration) to the sample together with the stain for outer membrane permeabilization (Berney *et al.,* 2007). If a sample contained more than 10^6 cells mL^{-1}, it was appropriately diluted before staining. Direct detection of *E. coli* O157 was achieved using fluorescent antibodies (0.5 mg mL^{-1}, KPL, MD, USA) in combination with flow cytometry. 1 µL of

a ten-times diluted solution was added to 1 mL of sample. Before analysis, the suspension was incubated for 20 min at room temperature in the dark. All samples were measured on a CyFlow Space flow cytometer (Partec, Münster, Germany) equipped with a 200 mW argon laser emitting at a fixed wavelength of 488 nm and equipped with volumetric counting hardware. The trigger was set on the green fluorescence (520 nm) channel and signals for total cell counting were collected on the combined 520 nm / 630 nm (red fluorescence) dot plot. For specific *E. coli* O157 detection, the signals were collected on the combined 520 nm / sideward scatter (SSC) dot plot. The quantification limit of the instrument was about 1000 cells mL^{-1} with an average standard deviation of less than 5 % (Hammes *et al.*, 2008).

Determination of the assimilable organic carbon (AOC) concentration

AOC determinations were based on the method described by Hammes and Egli (2005). As described in the original method, the BC was grown into late stationary phase (four days at 30 °C) and final growth was measured using flow cytometry. However, in this study not a conversion factor was used to calculate AOC concentration from the produced cells, but the DOC concentration before and after growth of the BC was measured. The difference in DOC corresponds to the AOC but it was here referred to "consumable DOC" (cDOC). This procedure was only applied on 40-fold diluted wastewater; cDOC concentrations of other dilutions were calculated from the dilution factor. Identical measurements were done for *E. coli* O157. The consumed DOC of *E. coli* O157 was considered as a fraction of the total cDOC determined with the BC.

Preparation of diluted wastewater

Undiluted wastewater (Dübendorf, Switzerland; DOC= 51.7 mg L^{-1}; cDOC/AOC = 36.4 mg L^{-1}) was sterilized by 20 kDa filtration (Fresenius Medical Care, Bad Homburg, Germany), kept at 4 °C and served as a stock for all performed experiments. To prepare the different dilutions, the stored wastewater was diluted with pasteurized (60 °C for 30 min) mineral water containing no cDOC. The obtained solutions were then directly filtered (0.22 µm) into sterile 40 mL carbon-free glass vials. From the stock solution (cDOC = 36.4 mg L^{-1}), the following diluted wastewater media were prepared (calculated cDOC concentrations in µg L^{-1} are included in parentheses): 10-fold (3644), 40-fold (911), 100-fold (364), 200-fold (182), 500-fold (73) and 1000-fold (36 µg L^{-1} cDOC). All continuous culture experiments were performed with a dilution containing 820 µg L^{-1} cDOC. In order to check whether the wastewater was indeed carbon-limited, an experiment was performed where inorganic nutrients (Ihssen & Egli, 2004) were added to 40-times diluted sterile wastewater in triplicate samples and the solutions were inoculated either with *E. coli* O157 or the BC. The final

cell concentrations reached in batch cultivation in both cultures were not significantly different from control cultures with no additional nutrients (data not shown).

Non-competitive growth in diluted wastewater

Batch culture: Cells used for inoculation were harvested from stationary phase cultures (see above) and inoculated into carbon-free 40 mL glass vials containing different dilutions of sterile wastewater (starting concentration 3 x 10^3 cells mL^{-1}). The cultures were incubated at 30 °C and the final cell concentration was enumerated with flow cytometry as described above. All results are given as netto growth, where the inoculum concentration was subtracted from the final result. For the 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. The specific growth rate (µ) was calculated from the initial phase of growth as described earlier (Vital *et al.,* 2007). For investigating growth at 12, 15, 20, 25 and 30 °C the inocula were pre-cultivated at the corresponding temperatures. All temperature experiments were performed with sterile diluted wastewater with a cDOC concentration of 364 µg L^{-1}.

Continuous culture: For growth experiments in continuous culture, three reactors (200 mL Schott flasks) containing 100 mL of diluted wastewater were simultaneously fed from the same medium reservoir (5 L Schott flask). The reactors were operated at 30 °C for 40 - 110 h. Experiments with three different dilution rates (D = 0.1 h^{-1}, D = 0.2 h^{-1} and D = 0.3 h^{-1}) were performed. The reactors were inoculated with stationary-phase grown cells (starting concentration 3 x 10^3 cells mL^{-1}), run in the batch-mode until the beginning of the stationary growth phase was reached and then switched to the continuous culture mode. The steady-state, defined by a stable cell concentration over time, was achieved after two to four volume changes. No active aeration was necessary and the medium exchange in and out of the reactors was achieved by a peristaltic pumping system. No substantial wall growth was observed.

Competition in batch culture

Bacteria from the two competitors were separately pre-grown in different dilutions of sterile wastewater and different temperatures, respectively, into exponential growth phase. The harvested cells were then re-inoculated into a new pre-warmed medium at a ratio of 1 : 1 (3 x 10^3 cells mL^{-1} of each competition partner). The increase in total cell concentration and of *E. coli* O157 was then determined directly after inoculation and further monitored over the whole competition period using flow cytometry (see above).

Competition in continuous culture

To investigate the competition during continuous culture growth, *E. coli* O157 and the BC were separately pre-grown in continuous culture at the same dilution rate into steady-state. Then 50 mL of each culture was combined with the corresponding competition partner and the culture was monitored as a function of time using flow cytometry (see above). Specific growth rates (μ) were calculated from three adjacent points as follows:

$$\mu = D - (\ln (N_t) - \ln (N_0)) / \triangle t \quad (1)$$

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

Simulations

The equations used for simulations are shown in Box 5.1. All simulations are based on single substrate saturation kinetics established by Monod (1949). However, here the single growth limiting substrate (s) was replaced by the amount of DOC available for a bacterial culture, namely for *E. coli* O157 ($cDOC_E$) and for the BC ($cDOC_{BC}$), where it was assumed that all substrates available for *E. coli* O157 were also accessed by the BC. Furthermore, the Monod saturation constants (K_s), referred to as K_{BC} and K_E, respectively, were adjusted to the amount of DOC available for each culture. No experiments were performed on the influence of the temperature on K_s values of the two competitors because previous reports suggest constant K_s values with changing temperatures (Kovarova *et al.,* 1996; Hall *et al.,* 2008). Hence, the values measured at 30 °C were applied for simulations at all temperatures. For simulations of the competition in continuous culture in the second phase during experiments where a stable wash-out of *E. coli* O157 was observed (Figure 5.6 and 5.7), residual cDOC concentrations, referred to "cDOC steady state", i.e. $_{ststst}$cDOC, were not derived from Monod kinetics. At each dilution rate a parameter estimation analysis for $_{ststst}$cDOC was performed yielding the best fit (based on the Monod equation) with the experimentally determined washout of *E. coli* O157, where the amount of cDOC available for *E. coli* O157 was set as 53 % of $_{ststst}$cDOC. The estimated values were subjected to regression analysis from which the $_{ststst}$cDOC was subsequently used for simulations. Thus, if cDOC concentrations in the simulations equaled $_{ststst}$cDOC the μ of the BC was set as the dilution rate, whereas that of *E. coli* O157 was still modeled according to the Monod equation.

Box 5.1. Equations used for the simulations

Batch growth & competition

$\mu_{BC} = \mu_{maxBC} * cDOC_{BC} / (cDOC_{BC} + K_{BC})$ (2)

$\mu_{E} = \mu_{maxE} * cDOC_{E} / (cDOC_{E} + K_{E})$ (3)

$\Delta cDOC_{BC} / \Delta t = cDOC_{ini} - ((BC_{\#} - BC_{\#ini}) * Y_{BC} + (E_{\#} - E_{\#ini}) * Y_{E})$ (4)

$\Delta cDOC_{E} / \Delta t = 0.53 * cDOC_{ini} - (0.53*((BC_{\#} - BC_{\#ini}) * Y_{BC}) + (E_{\#} - E_{\#ini}) * Y_{E})$ (5)

$\Delta BC_{\#} / \Delta t = BC_{\#} + (BC_{\#} * e^{\mu BC * \Delta t} - BC_{\#})$ (6)

$\Delta E_{\#} / \Delta t = E_{\#} + (E_{\#} * e^{\mu E * \Delta t} - E_{\#})$ (7)

The measured parameters used for simulations are displayed in Table 1.

Effect of temperature

$Y_{BC} = 21000 * (°C) + 9 * 10^{6}$ (8)

$Y_{E} = - 13000 * (°C)^{2} + 738000 * (°C) - 5 * 10^{6}$ (9)

$\mu_{maxBC} = 0.0134 * (°C) - 0.085$ (10)

$\mu_{maxE} = 0.0426 * (°C) - 0.439$ (11)

Corresponding values are displayed in Figure 2

Continuous culture growth & competition

$\Delta BC_{\#} / \Delta t = \mu_{BC} * BC_{\#} - D * BC_{\#}$ (12)

$\Delta E_{\#} / \Delta t = \mu_{E} * E_{\#} – D * E_{\#}$ (13)

$\Delta cDOC_{BC} / \Delta t = D * 820 - \mu_{E} * E_{\#} / Y_{E} - \mu_{BC} * BC_{\#} / Y_{BC} - D * cDOC_{BC}$ (14)

$\Delta cDOC_{E} / \Delta t = D * 820 - \mu_{E} * E_{\#} / Y_{E} – 0.53 * \mu_{BC} * BC_{\#} / Y_{BC} - D * cDOC_{E}$ (15)

$_{ststst}cDOC = 8300 * D^{2} - 1580 * D + 115$ (16)

The parameter $_{ststst}cDOC$ was not derived from conventional Monod kinetics, but obtained from parameter estimation analysis. It was set that 53 % of $_{ststst}cDOC$ was available for *E. coli* O157.

μ_{BC}: specific growth rate for the BC; μ_{E}: specific growth rate for *E. coli* O157; μ_{maxBC}: maximum specific growth rate for the BC; μ_{maxE}: maximum specific growth rate for *E. coli* O157; K_{BC}: Monod saturation constant for the BC; K_{E}: Monod saturation constant for *E. coli* O157; Y_{BC}: Yield for the BC; Y_{E}: Yield for *E. coli* O157; $BC_{\#}$: cell concentration of the BC; $BC_{\#ini}$: initial cell concentration of the BC; $E_{\#}$: cell concentration of *E. coli* O157; $E_{\#ini}$: initial cell concentration of *E. coli* O157; $cDOC_{BC}$: cDOC for the BC; $cDOC_{E}$: cDOC for *E. coli* O157; $cDOC_{ini}$: initial concentration of cDOC; $_{ststst}cDOC$: estimated residual cDOC concentration in second phase during competition; D: dilution rate.

Results

Determination of stoichiometric and kinetic parameters

In order to determine several growth parameters of *E. coli* O157 and the drinking water bacterial community (BC), respectively, the two competitors were separately grown at different dilutions of wastewater at 30 °C in batch culture (Table 5.1). The BC was able to utilize 70 % of the total DOC, which represents the assimilable organic carbon (AOC), but was referred here to "consumable DOC" (cDOC; see Material and Methods). Only 53 % of that cDOC was also available for *E. coli* O157. The numerical cell yield, i.e. cells formed per consumed µg of DOC, of *E. coli* O157 was only half of that of the BC (Table 5.1). This is consistent with previous data, where it was shown that *E. coli* O157 cells were bigger than those of a freshwater bacterial community and, hence, needed more carbon than the community in order to produce a new cell (Vital *et al.,* 2008). The modelled kinetic Monod constants, i.e. the maximum specific growth rate (μ_{max}) and the saturation constant (K_s), where the growth limiting substrate, s, was replaced by cDOC (see Material and Methods), showed a clear "opportunist" versus "gleaner" relationship (Figure 5.1). Since only a fraction of cDOC was available for *E. coli* O157 its "K_s" value was adjusted accordingly and the values of the two cultures are, thus, referred to as K_{BC} and K_E, respectively (Table 5.1). The opportunist *E. coli* O157 displayed a high μ_{max} together with a high K_E, and the BC, adapted to nutrient-poor conditions, exhibited both, a lower μ_{max} and a lower K_{BC}. The results presented in Figure 5.1 suggest a cross-over of the two curves at a cDOC concentration of approximately 590 µg L^{-1}.

Table 5.1. Basic stoichiometric and modelled kinetic parameters of the two cultures at 30 °C.

Parameter	*E. coli* O157	BC
DOC consumed (% of total DOC)*	37.7 ± 1.5	70.5 ± 0.4
DOC consumed (µg L^{-1})*	486.5 ± 19.8	911 ± 4.9
Cells formed (x 10^6 mL^{-1})*	2.78 ± 0.22	10 ± 0.7
Yield (cells formed x 10^6 (µg consumed DOC^{-1}))*	5.72 ± 0.68	11 ± 0.52
μ_{max} $(h^{-1})^+$	0.873	0.329
K_E and K_{BC} (µg L^{-1})$^+$	489	7.4

* values obtained from 40-fold diluted wastewater (DOC = 1.29 mg L-1).
\+ parameters based on simulations using Monod kinetics.
BC: Drinking water bacterial community
µmax: maximum specific growth rate
KE and KBC: saturation constants for E. coli O157 (KE) and the BC (KBC); the values were modelled considering that only a fraction of DOC was available for growth.

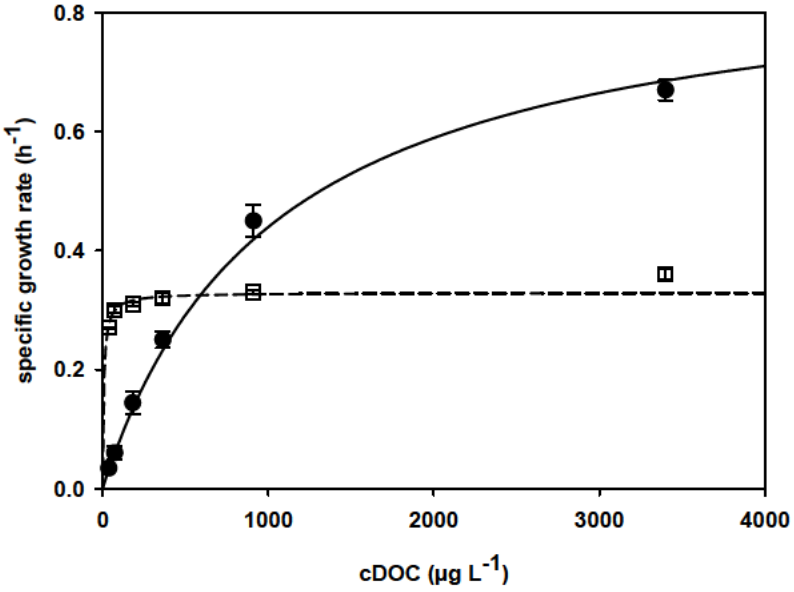


Figure 5.1. The opportunist versus the gleaner relationship of the two cultures at 30 °C. The specific growth rates of *E. coli* O157 (●) and the drinking water bacterial community (□) in correlation with the consumable DOC (cDOC) are displayed. The lines represent the simulated correlation based on Monod kinetics. Corresponding modelled maximum specific growth rates (μ_{max}) and saturation constants (K_s) are presented in Table 5.1. The error bars represent the standard deviation on triplicate samples.

As a next step, the effect of temperature on the growth parameters of the two individual cultures was investigated in 100-times diluted wastewater containing 364 µg cDOC L^{-1} (Figure 5.2). Whereas *E. coli* O157 produced considerable fewer cells per µg of consumed DOC with decreasing temperatures, the yield of the BC was hardly affected by temperature (Figure 5.2 A). Both competitors showed a similar increase of their specific growth rate (µ) with increasing temperature (Figure 5.2 B). This suggests that the temperature has no influence on the competition via the specific growth rate. However, the experiment was performed at a cDOC concentration where the determined specific growth rate of the BC at 30 ºC equalled its μ_{max}, whereas that of *E. coli* O157 was even below $\mu_{max}/2$ (compare Figure 5.1). Hence, the recorded influence of temperature on µ (slope in the experiment) was also the actual influence on μ_{max} of the BC, whereas it had to be modelled for the μ_{max} of *E. coli* O157 (Figure 5.2 C). This revealed that *E. coli* O157 had an increasing competition advantage with rising temperatures based on its μ_{max}.

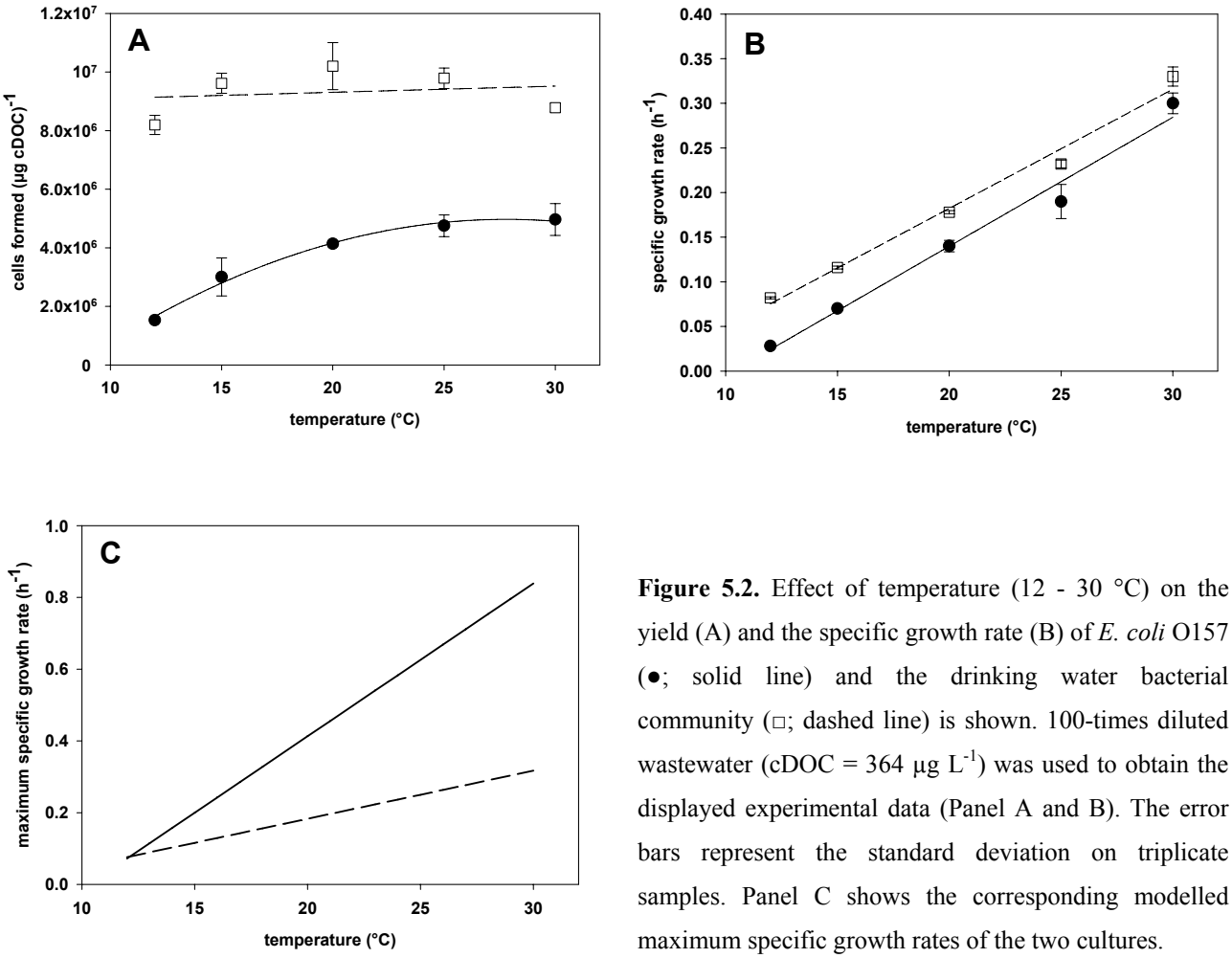


Figure 5.2. Effect of temperature (12 - 30 °C) on the yield (A) and the specific growth rate (B) of *E. coli* O157 (●; solid line) and the drinking water bacterial community (□; dashed line) is shown. 100-times diluted wastewater (cDOC = 364 µg L^{-1}) was used to obtain the displayed experimental data (Panel A and B). The error bars represent the standard deviation on triplicate samples. Panel C shows the corresponding modelled maximum specific growth rates of the two cultures.

Competition in batch culture - influence of the nutrient concentration

Elevated nutrient concentrations favoured the competition of *E. coli* O157 with the BC in batch culture (Figure 5.3). The higher the concentration of cDOC, the higher was the percentage of *E. coli* O157 of the total counts at the end of competition experiments. Although the starting cell ratio of the two competitors was always around 1:1, *E. coli* O157 never represented more than 30 % of the total cell concentration in stationary phase at all cDOC concentrations tested. According to Figure 5.1, one would expect that the pathogen was growing faster than the BC above a cDOC concentration of 590 µg L^{-1} and, hence, competition experiments above this value should have favoured growth of *E. coli* O157. Indeed, at a concentration of 911 µg cDOC L^{-1}, *E. coli* O157 exhibited a higher µ than the BC and after 12 h the bacterium consequently accounted for 85 % of the total cell concentration (Figure 5.4). However, since only 53 % of the cDOC was available for the pathogen, the BC continued to grow when growth of *E. coli* O157 had already stopped. Thus, the proportion of *E. coli* O157 of the total cell concentration was finally reduced to around 30 %.

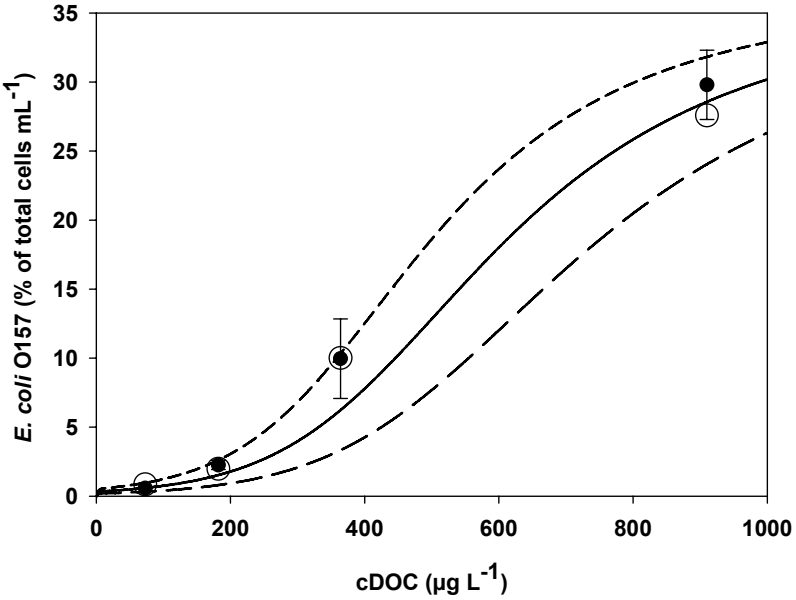


Figure 5.3. Final results of batch culture competition between *E. coli* O157 and a drinking water bacterial community (BC) at different concentrations of cDOC at 30 °C are displayed. The measured cell concentrations of *E. coli* O157 as a percentage of the total cell concentration (●) are shown. The error bars represent the standard deviation on triplicate samples. The lines represent *in silico* predictions on competition outcome at different starting ratios of *E. coli* O157 and the BC (ratios between competitors in parenthesis); solid line (1:1 – original model); short-dashed line (7:3) and long-dashed line (3:7). The open circles (O) illustrate the modelled results of individual competition experiments taking the measured starting ratios of the two competitors at the beginning of experiments into account.

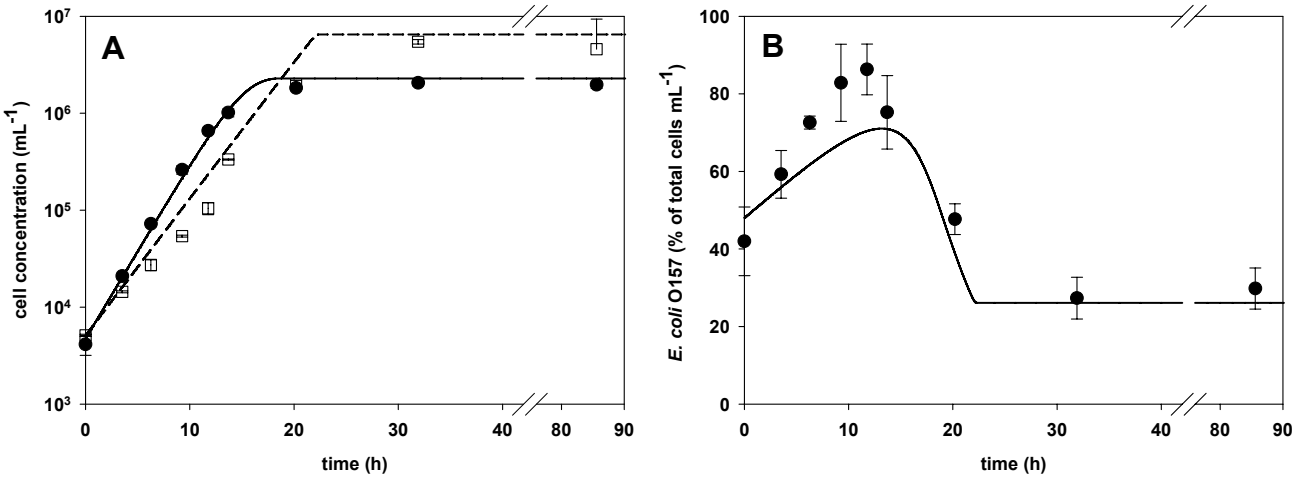


Figure 5.4. Batch culture competition between *E. coli* O157 (●) and the drinking water bacterial community (□) at a cDOC concentration of 911 µg L^{-1} and a temperature of 30 °C (A) is displayed. Panel B shows the corresponding percentage of *E. coli* O157 (●) of the total cell concentration. The lines represent the modelled results based on Monod kinetics. Error bars indicate the standard deviation on triplicate samples.

Based on the measured parameters of the competitors in pure culture (Table 5.1), it was possible to predict the influence of cDOC concentration on competition outcome; the model could explain the experimentally derived data (solid line in Figure 5.3). Simulations on the influence of different starting ratios of the two individual bacterial cultures at the beginning of the batch competition suggest a considerable influence of this parameter on the outcome of the competition (dashed lines in Figure 5.3). Since, in the experiments performed the starting ratios indeed varied and were not always exactly 1:1, additional simulations for individual competition experiment were performed, which took the measured ratios of competitors at the beginning of the experiments into account. Considering this parameter significantly improved *in silico* predictions (open circles in Figure 5.3).

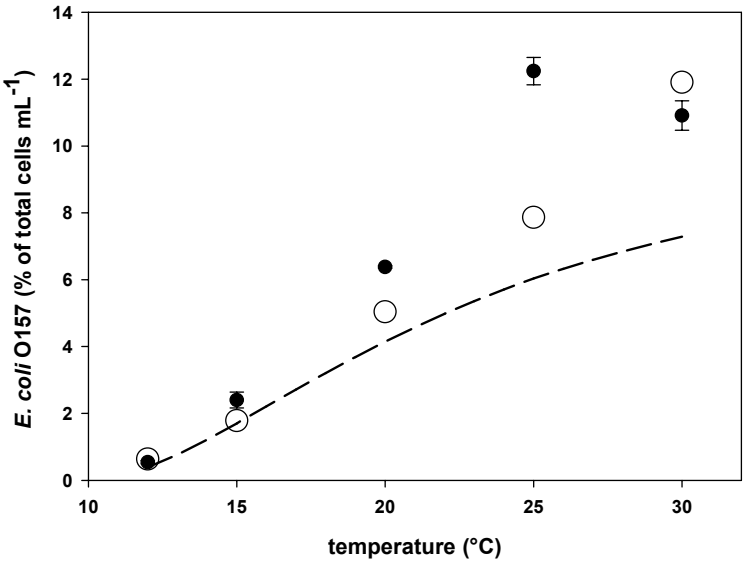


Figure 5.5. The influence of temperature on batch culture competition between *E. coli* O157 and a drinking water bacterial community (BC) is displayed. The measured *E. coli* O157 cell concentrations as a percentage of the total cell concentration (●) are shown. The dashed line indicate the prediction of the model, whereas the open circles represent the simulated result considering the measured starting ratios of the two competitors at the beginning of each individual competition experiment (see Figure 5.2). 100-times diluted wastewater (cDOC = 364 µg L^{-1}) was used for the experiment. The error bars represent the standard deviation on triplicate samples.

Competition in batch culture - influence of temperature

Increasing temperatures had a positive influence on competitive growth of *E. coli* O157 in batch culture (Figure 5.5). Except for 25 °C, the model could explain the experimental data. It should be pointed out that competition experiments were performed at a cDOC concentration of 364 µg L^{-1}. However, based on the measured growth properties of competitors one would expect that the influence of temperature on the outcome of competition is not constant but depending on the cDOC concentration and this aspect was consequently investigated *in silico* (Figure S5.1). According to

the model, temperature was affecting competition the most at cDOC concentrations around 500 to 800 µg L^{-1} with a maximum at 565 µg cDOC L^{-1}. At this concentration the percentage of *E. coli* O157 cells in relation to the total cells was predicted to be around 30-times higher at 30°C than at 12°C. Below and above this maximum, the influence of temperature was not as strong.

Growth in continuous culture

In steady-state of a continuous culture, the bacterial cell concentration should be constant over time. Figure 5.6 displays steady-state cell concentrations of the two competitors growing separately at different dilution rates. *E. coli* O157 established at lower cell concentration than the BC due to both its lower yield and the ability to consume only 53 % of the cDOC (see above).

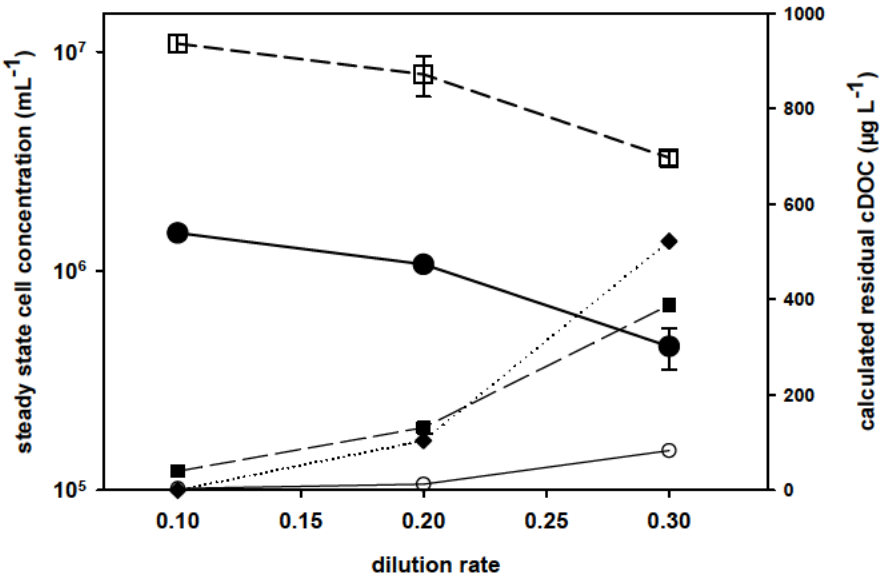


Figure 5.6. Steady-state cell concentrations of *E. coli* O157 (●) and the drinking water bacterial community (BC; □) at different dilution rates. Predicted steady-state cDOC concentrations for the BC ($_{stst}$cDOC) based on Monod kinetics (○; Figure 5.1), $_{stst}$cDOC concentrations based on the measured BC cell concentrations in steady-state and the yield (Table 5.1) (◆), as well as, the estimated $_{stst}$cDOC concentrations used for the following competition simulations (■) are displayed. The error bars represent the standard deviation on triplicate samples.

Steady-state cell concentrations of both cultures correlated negatively with the dilution rate (D). This observation can partly be explained by Monod kinetics shown in Figure 5.1 and partly by the substrate utilization pattern during growth in complex media. With respect to the former aspect, Monod kinetics imply that the residual concentration of cDOC in steady-state is increasing with increasing D. Consequently, less carbon is available for biomass production at a high D than at lower growth rates. This effect should be particularly pronounced in the culture of *E. coli* O157

because K_E was considerable higher than K_{BC}. The latter aspect can be attributed to the complex composition of the organic carbon pool in wastewater where an increasing number of carbon sources becomes available at reduced dilution rates. In other words, slowly growing cells have access to a broader cDOC spectrum in comparison to fast growing ones (Sepers, 1984; Egli, 1986). Hence, the availability of substrates for growth in steady-state was dependent on the dilution rate and, thus, determined the measured cell concentrations.

Unfortunately, it was not possible to measure the residual concentrations in the reactors in steady-state and the corresponding values had to be estimated for simulations. In Figure 5.6, three different scenarios for steady-state cDOC for the BC ($_{ststst}$cDOC) concentrations are presented. The lowest concentration was predicted based on Monod kinetics (scenario 1), which did not take the heterogeneity of the substrate pool into account (as explained above). Calculations using the measured cell concentration of the BC in steady-state together with the determined yield from Table 1 resulted in higher concentrations of $_{ststst}$cDOC (scenario 2). However, for the following simulations on competition in continuous culture the $_{ststst}$cDOC concentrations were fitted using parameter estimation analysis (see Materials and Methods). The obtained values are shown as squares in Figure 5.6 (scenario 3).

Competition in continuous culture

During competition experiments in continuous culture the BC was expected to control the residual cDOC concentration in the reactor because of its kinetic properties (Figure 5.1). Figure 5.7 presents the outcome of the competition at D = 0.2 h^{-1}. The competitors were grown separately to steady-state and then mixed at a ratio of 50 mL : 50 mL. It should be pointed out that the cell concentration of *E. coli* O157 was lower than that of the BC at the beginning of the competition, because the bacterium produced a lower cell concentration in steady-state than the BC (Figure 5.6). Two distinctly different phases were recognized. At the beginning of the competition, the cell concentration of *E. coli* O157 was only slightly affected and this was followed by a phase where stable washout of *E. coli* O157 occurred (Figure 5.7). This behaviour of *E. coli* O157 can be explained in the following way: at the start of the experiments half of the liquid in the reactor stemmed from the *E. coli* O157 culture, which contained an elevated residual cDOC concentration; the residual cDOC concentration was, thus higher than that required by the BC to grow at this D (Figure 5.6). In addition, the cell concentration of the BC was reduced to 50 % of its original steady-state cell concentration. This low initial BC cell concentration together with the high residual cDOC concentration allowed stable growth of *E. coli* O157 with a μ almost equal to D at the

beginning of the competition experiments. The following increase of the BC cell concentration resulted in a decrease of the residual cDOC concentration approaching steady-state concentration of the BC, which consequently promoted washout of *E. coli* O157 at a constant rate. Hence, an increased amount of cDOC became available for the BC resulting in a slow further increase in their cell concentration (Figure 5.7). However, the washout-rate of *E. coli* O157 was lower than the theoretically predicted total washout ($\mu = 0\ h^{-1}$) suggesting that the pathogen was still able to multiply, also when competing with the BC.

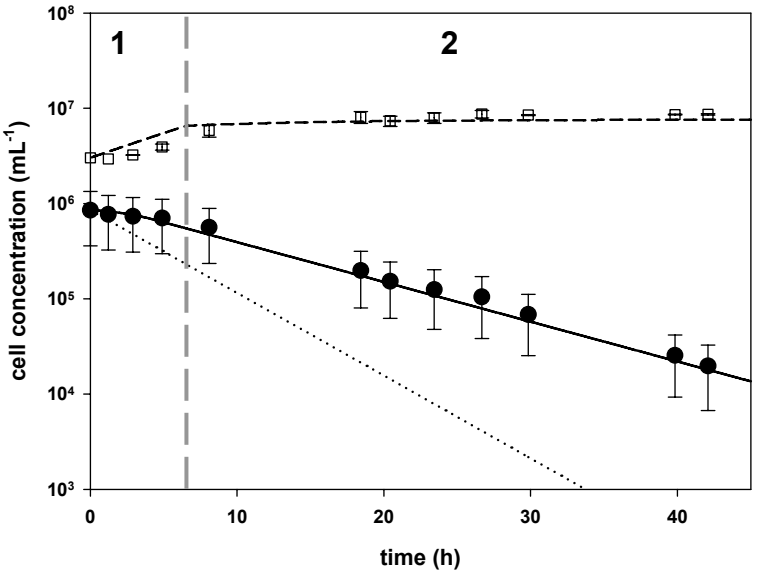


Figure **5.7.** Competition of *E. coli* O157 (●) and the drinking water bacterial community (BC, □) in diluted wastewater in continuous culture at a dilution rate of $D = 0.2\ h^{-1}$. The lines represent the modelled results (solid line: *E. coli* O157; dashed line: the BC and dotted line: theoretical total washout ($\mu = 0\ h^{-1}$)). The Figure is separated into two parts indicating the biphasic behaviour of *E. coli* O157 during competition with the BC in continuous culture (see text). The error bars represent the range on duplicate samples.

The effect of the dilution rate on the competition between *E. coli* O157 and the BC is shown in Figure 5.8. At all dilution rates investigated ($D = 0.1\ h^{-1}$, $D = 0.2\ h^{-1}$ and $D = 0.3\ h^{-1}$), the biphasic competition dynamics was observed (discussed above). Furthermore, a correlation between dilution rate and the competitive fitness of *E. coli* O157 with the BC was established. The higher the D, the better *E. coli* O157 was able to compete. The predictions from the model were only partly able to explain the observed results. At $D = 0.2\ h^{-1}$, the model was only slightly deviating from the experimental results (also shown in Figure 5.7). The simulation at $D = 0.1\ h^{-1}$ predicted the dynamics correctly, but underestimated the slow washout of *E. coli* O157 in the first phase. At $D = 0.3\ h^{-1}$ the model failed to predict the experimental data. Over the whole period of ten volume changes the simulation predicted a slow increase in the *E. coli* O157 cell concentration (μ slightly

higher than D), which was in contradiction to the observed washout of the pathogen. As the set dilution rate of 0.3 h^{-1} is approaching μ_{max} of the BC of 0.329 h^{-1} one would predict the initial increase in cell concentration to be very slow, which should result in a high residual cDOC concentration over a long time, promoting considerable growth of *E. coli* O157. However, the observed initial increase in cell concentration of the BC from the experimental data was faster (μ = 0.38 h^{-1}; data not shown), which might explain the discrepancy between the prediction of the simulation and the experimental results. In this respect it should be pointed out that the BC is not rigid but has to be regarded as a changing entity, which is continuously adapting to prevailing growth conditions. Bacterial communities, cultivated under similar conditions as in this study, were displaying considerable higher specific growth rates (μ) than the μ_{max} established here (Vital *et al.,* 2007; Wang *et al.,* 2009). Hence, the fact that the BC displayed a higher μ at D = 0.3 h^{-1} than the predicted μ_{max}, can be ascribed to adaptation processes during cultivation at a high growth rate

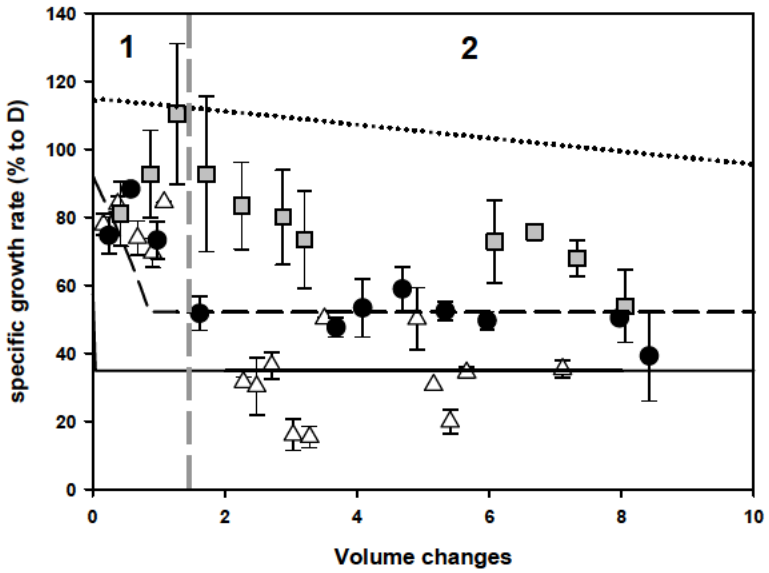


Figure 5.8. Specific growth rates (μ) relative to the dilution rate of *E. coli* O157 growing in competition with the drinking water bacterial community in a continuous culture system (calculation of μ see Materials and Methods). The results are based on competition experiments at three different dilution rates (D = 0.3 h^{-1}: □; D = 0.2 h^{-1}: ● and D = 0.1 h^{-1}: Δ); an example (D = 0.2 h^{-1}) is shown in Figure 7. The lines represent the modelled results (dotted line: D = 0.3; dashed line: D = 0.2 and solid line: D = 0.1). The Figure is separated into two parts indicating the biphasic competition dynamics of *E. coli* O157 and the BC (see text). The error bars represent the range on duplicate samples

Discussion

This study sheds new light on the principles of competitive growth between enteric pathogens and autochthonous bacterial communities under environmental conditions, i.e. bacteria are growing on a mixture of substrates at low concentrations. It could be demonstrated for the first time that competition dynamics under such nutritional conditions can be described by the measured growth properties of the individual competitors in pure culture, namely (1) availability of DOC in batch and continuous culture, respectively, (2) yield and (3) kinetics (maximum specific growth rate (μ_{max}) and Monod saturation constant, K_s).

The results show that *E. coli* O157 is not only able to proliferate at low substrate concentrations in pure culture, as reported earlier (Vital *et al.,* 2008), but can also grow in competition with bacteria originating from nutrient-poor environments. However, growth of *E. coli* O157 was drastically restricted in the presence of competing bacteria. Also many other studies reported that autochthonous bacteria considerably limit growth of enteric bacteria in open environments (e.g., Camper *et al.,* 1985; van Elsas *et al.,* 2007), but in these studies the factors involved, specifically the growth properties of competitors, were only vaguely considered leaving the underlying competition dynamics in the dark.

According to theory, the possibility of invasion correlates with ecosystem stability (Tilman, 1999). Hence, if waters contain well adapted microbial communities acting as "placeholders", i.e. they occupy all nutritional niches, growth of enteric pathogens should be severely hampered or even not occur. However, disturbing events such as changing substrate concentration/composition or a reduction/structural alteration of the "placeholder community" could generate a risk for invasion by opening a niche for enteric pathogens to grow. This is supported by the results of the competition experiments in continuous culture presented here. Whereas a steady wash-out of *E. coli* O157 occurred in the second phase of the experiments (representing a stable ecosystem), no or only a slight wash-out of the pathogen was observed in the first unstable phase, which was characterized by both elevated nutrient concentrations and a reduction of the drinking water bacterial community (BC; Figure 5.6). These principles were also important during batch competition, where simulations predicted that the initial cell concentration of the drinking water bacterial community (BC) strongly influences growth of *E. coli* O157; according to the model, the higher the initial cell concentration of the BC, the less growth of the pathogen would occur (Figure S5.1). These observations are specifically relevant for drinking water treatment and distribution. Often, a philosophy prevails to

get rid-off all bacteria present in treated drinking water through a final disinfection step. However, by killing all "placeholders", uncontrolled re-growth occurs that creates the possibility of "unwanted bacteria" such as pathogens to multiply. For example, growth of *E. coli* was reported in a drinking water distribution system where all autochthonous bacteria were eliminated by chlorination, and which was characterized by high nutrient concentrations together with a lack of disinfectant residuals during distribution (Juhna *et al.,* 2007). The production of so called "biologically stable" water, where low nutrient concentrations are combined with viable autochthonous bacteria acting as "placeholders" (van der Kooij, 1992; Hammes *et al.,* 2010), is thus a suitable way to close the "niche for growth" of pathogenic bacteria.

This study demonstrates that the main drawback of *E. coli* O157 during growth in competition at low substrate concentrations was due to its weaker kinetic properties in comparison to the BC (Figure 5.1). The established kinetic relationship between the competitors, also known as the "opportunist" versus the "gleaner" relationship, was often proposed for autochthonous microorganisms and enteric bacteria, but has up to now never been verified in the laboratory yet (e.g., Jannasch, 1968; Grover, 1990). This relationship implies that competition fitness of *E. coli* O157 is correlated with both increasing nutrient concentrations and increasing dilution rates, which was indeed observed in our experiments (Figure 5.3 and 5.8). Also several *in situ* observations indicate a positive effect of elevated nutrient concentrations on growth of *Enterobacteriaceae* in water. For example, both LeChevallier and colleagues (1996) and Camper and co-workers (2000) reported a correlation between the assimilable organic carbon (AOC) concentration and the occurrence of coliform bacteria in different drinking water systems during broad surveys across North America. Hence, these observations together with the results presented here suggest that competition behaviour and, thus, occurrence of enteric pathogens is indeed directly linked to their kinetic properties.

Next to nutrients, temperature is a key factor governing heterotrophic growth and competition in the environment. It is a well known fact that growth of *E. coli* is positively influenced by increased temperatures (Raghubeer & Matches, 1990) and it is suggested that the bacterium is part of the indigenous microbial flora in the tropics due to elevated annual water temperatures (Winfield & Groismann, 2003). However, with this study it could be shown for the first time that *E. coli* O157 performs better at elevated temperatures not only in pure culture, but that temperature is also directly controlling the competition between the pathogen and indigenous aquatic bacteria through both the yield and μ_{max} of competitors (Figure 5.4). The results strengthen the suggestions that

ongoing global climate changes enhance growth of enteric pathogens in aquatic ecosystems due to rising annual water temperatures (IPCC, 2007). However, it should be pointed out that the BC used here originated from moderately tempered environments (< 20 °C; Wang *et al.,* 2008; Hammes *et al.,* 2010). Although it was adapted to the individual temperatures (one batch cultivation; ~ 10 generations), long term adaptation processes, as they occur in the environment, might increase its competitive fitness at high temperatures.

In the present study it was possible to mathematically describe many of the obtained results using conventional single substrate kinetics based on Monod (Monod, 1949). This is astonishing, because Monod based his concepts on experiments performed with pure cultures growing with a single limiting substrate. In contrast, the nutrient pool used in this study was composed of many different carbon substrates, all simultaneously limiting growth (Egli, 1995). Furthermore, with respect to the BC, the culture did not consist of one, but many different bacterial species; denaturing gradient gel electrophoresis (DGGE) of stationary phase grown cells revealed several bands on the gel (data not shown). However, ignoring all these additional complexities by simply replacing the single growth limiting substrate (s) within Monod kinetics with the consumable DOC (cDOC), namely $cDOC_{BC}$ and $cDOC_{E}$, respectively, it was possible to simulate many of the obtained results. In this respect it should be pointed out that also the saturation constants, K_{BC} and K_{E}, were adjusted accordingly. Hence, mathematical modelling provides an opportunity to postulate different scenarios not experimentally investigated. Examples are given in Figures S5.1 and S5.2. Another interesting aspect to be explored using simulations would be the effect of varying nutrient supply during competition in continuous culture (Hsu, 1980; Hale *et al.,* 1983). The experiments in this study were performed by continuously feeding the reactors with an identical concentration of nutrients. However, in the environment a discontinuous supply of nutrients is the rule rather than the exception, which is considerable altering the competition dynamics (Grover, 1990; Gottschal, 1993).

Furthermore, it would be interesting to investigate how adaptation of *E. coli* O157 during growth under oligotrophic conditions would influence competition dynamics. For *E. coli* K12 it was shown that the K_s during growth on glucose in a continuous culture system was reduced from 400 – 500 µg glucose L^{-1} (interestingly the K_E presented here is very similar, namely 489 µg $cDOC_E$ L^{-1}) down to as low as 30 µg glucose L^{-1} (Wick & Egli, 2002). Implementation of this reported value in the presented model would shift the crossing point of the two competitors from 590 µg L^{-1} (Figure 5.1) down to 21 µg L^{-1} (data not shown). As a result, the outcome of the competition experiments would

be dramatically altered in favour of *E. coli* O157. However, it is unclear whether *E. coli* has the same adaptation potential during growth in the environment, where a multitude of substrates are limiting growth simultaneously and, thus, the selection pressure of an individual compound is considerably reduced. Moreover, this study indicate that during growth on complex substrate mixtures additional mechanisms, not explainable by single substrate kinetics, are influencing growth and competition of bacteria such as a growth rate dependent access to the substrate pool. Hence, the presented results suggest that application of Monod kinetics for theoretical investigations on bacterial competition in the environment can give first indications on the dynamics involved, but obtained results should not be regarded as facts, but rather as a stimulus to explore the proposed scenarios in the laboratory.

Supplementary info

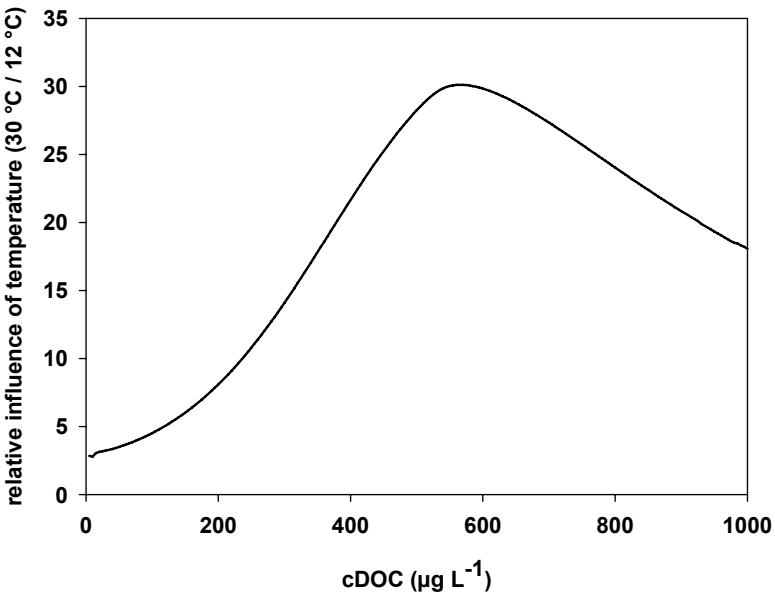


Figure S5.1. The relative influence of temperature on the outcome of batch competition between *E. coli* O157 and a drinking water bacterial community at different cDOC concentrations is shown. The relative influence was defined as the modelled % of *E. coli* O157 of the total counts in stationary phase at 30 °C divided by the modelled % *E. coli* O157of the total counts in stationary phase at 12 °C.

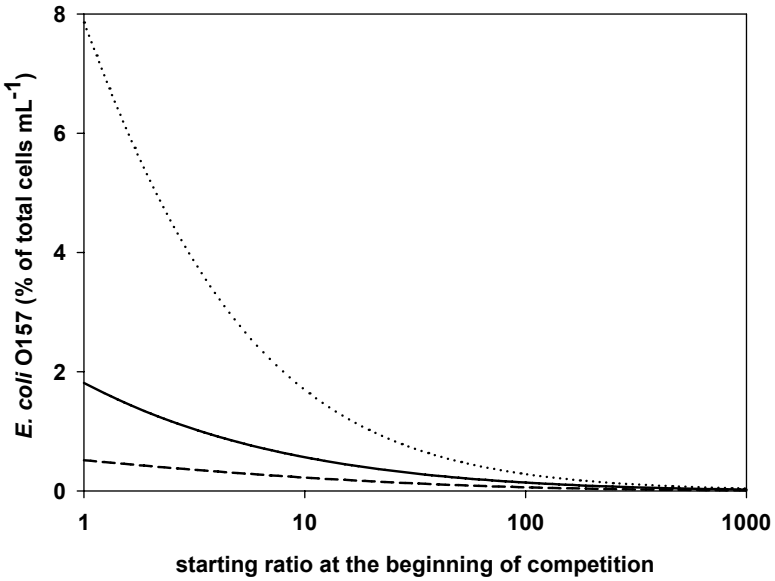


Figure S5.2. Modelled influence of different starting ratios of *E. coli* O157 and a drinking water bacterial community (BC) on the outcome of competition at 30 °C in batch culture. The starting concentration of *E. coli* O157 (3 x 10^3 cells mL^{-1}) was kept constant, whereas the concentration of the BC was continuously increased from 3 x 10^3 cells mL^{-1} to 3 x 10^6 cells mL^{-1}. Three different cDOC concentrations were investigated (dotted line 400 µg L^{-1}, solid line 200 µg L^{-1}; dashed line 50 µg L^{-1}).

A considerable decline of the percentage of *E. coli* O157 in stationary phase with increasing starting concentrations of the BC was obtained and the effect was more pronounced at higher cDOC concentrations. The fraction of *E. coli* O157 then gradually decreased towards the point where almost no growth of the pathogen was predicted anymore. Since, no threshold concentration for growth could be detected for this pathogen (Vital, unpublished data) the point of zero growth of *E. coli* O157 can, theoretically, never be reached even in the presence of a vast excess of BC bacteria. This is also reflected in the simulations where curves asymptotically approach the x-axis

6. Evaluating the growth potential of pathogenic bacteria in water

Abstract

The degree to which a water sample can potentially support growth of human pathogens was evaluated. For this purpose, a pathogen growth potential (PGP) bioassay was developed based on the principles of conventional assimilable organic carbon (AOC) determination, but using pure cultures of selected pathogenic bacteria (*Escherichia coli* O157, *Vibrio cholerae*, or *Pseudomonas aeruginosa)* as the inoculum. We evaluated 22 water samples collected after different treatment steps from two drinking water production plants, a wastewater treatment plant, river water and migration water from plastic materials used for in-house water installation systems. Each pathogen was batch-grown into stationary phase in sterile water samples and the concentration of produced cells was measured using flow cytometry. In addition, the fraction of AOC consumed by each pathogen was estimated. Pathogen growth did not correlate with dissolved organic carbon (DOC) concentration and correlated only weakly with the concentration of AOC. Furthermore, the three pathogens never grew to the same concentration in any water and the relative ratio of the cultures to each other was unique in each sample. These results suggest that the extent of pathogen growth is affected not only by the concentration but also the composition of AOC. Through this bioassay, PGP can be included as a parameter in water treatment system design, control and operation. Additionally, a multi-level concept is discussed that integrates the results from the bioassay into the bigger framework of pathogen growth in water. The proposed approach provides a first step for including pathogen growth into microbial risk assessment.

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Introduction

Pathogenic bacteria can survive and also grow in low nutrient aquatic environments such as surface waters or man-made water treatment systems (Camper *et al.,* 1991; LeChevallier *et al.,* 1991; van der Kooij *et al.,* 2005). Studies on pathogen survival and/or die-off (including disinfection) in water are common, but little is known about the fundamental factors governing their growth in the environment (see Vital *et al.,* 2007, 2008). Understanding the growth of pathogenic bacteria in aquatic ecosystems is essential for a holistic approach to microbial risk assessment as well as for improving drinking water treatment design and operation.

A key factor governing growth of all organisms is nutrient availability. All human pathogens are heterotrophs, utilizing organic compounds as their carbon and energy source. Natural organic matter in water comprises a broad spectrum of many different compounds; it is usually determined as a bulk parameter such as dissolved organic carbon (DOC). Only a fraction (0.1 - 44 %) of this DOC pool is readily available for bacterial growth (Kaplan *et al.,* 1993; van der Kooij, 2002). This bio-available fraction is quantified using bioassays such as the biodegradable dissolved organic carbon (BDOC) assay (Servais *et al.,* 1987), or the assimilable organic carbon (AOC) assay (van der Kooij *et al.,* 1982). Results from both of these assays are commonly used as indicators for bacterial growth potential and have been previously linked to re-growth and biofilm formation in drinking water distribution systems (LeChevallier *et al.,* 1991; van der Kooij, 1992; Escobar *et al.,* 2001).

Previous studies have pointed towards an apparent correlation between the concentration of AOC and the presence of enteric bacteria. For example, during two large surveys of drinking water treatment systems across North America, the occurrence of coliform bacteria was found to be elevated above an AOC concentration of 100 µg L^{-1} (LeChevallier *et al.,* 1996; Camper *et al.,* 2000). Other studies also found that AOC concentrations were directly correlated to growth of pathogenic bacteria (Torvinen *et al.,* 2004; Vital *et al.,* 2007, 2008). However, AOC is a bulk parameter, which includes many different substrates (e.g., amino acids, sugars or fatty acids) readily available for heterotrophic growth. Hence, its composition can differ distinctly and it is assumed that every aquatic environment carries a complex and unique “fingerprint” of utilizable organic carbon compounds (Münster, 1993). However, the spectrum of growth-supporting substrates of individual bacterial strains is specific - a fact used for the classification of bacteria for taxonomic purposes. This principle has been also integrated into conventional AOC assays, where the specific substrate spectrum of different pure cultures can be used to quantify different types of compounds

present in water (van der Kooij, 2002; Sack *et al.,* 2009). Thus, we have hypothesized that the total concentration of AOC alone is not a sufficient parameter for describing the growth potential of pathogenic bacteria; the quality of the available carbon compounds has to be considered aswell.

Presently, no method exists that is capable of fractionating organic carbon in a way that allows for the quantification of individual carbonaceous compounds that support growth of specific pathogens. In this study, we have developed a pathogen growth potential (PGP) assay by combining the conventional AOC assay (van der Koij *et al.,* 1982) with flow cytometric quantification of bacterial growth (Hammes & Egli, 2005) and using pathogens as inocula. The PGP assay yields two main results, namely (i) the extent of pathogen growth, and (ii) the relative fraction of AOC consumed by a pathogen. With this approach, we investigated the growth potential of three model pathogens, namely *Escherichia coli* O157, *Vibrio cholerae* O1 and *Pseudomonas aeruginosa*, in a broad range of water samples, differing considerably in their origin and quality.

Materials and Methods

Bacterial strains used and precultivation

The verotoxin-negative *Escherichia coli* O157 (Nent 2540-04), *Vibrio cholerae* O1 Ogawa biotype El Tor (Nent 720-95) and *Pseudomonas aeruginosa* (LMG 14073) were kept at -80 °C before use. The cryo-cultures were streaked onto Tryptic Soy Agar plates (Biorad, Reinach, Switzerland) and incubated for 24 h at 37 °C. One colony was transferred with a loop into ten-times diluted Luria-Bertani (LB) broth and the culture was incubated overnight at 37 °C. Cells from this overnight culture were transferred into 10,000-times diluted LB medium (initial concentration 5 x 10^3 cells mL^{-1}), incubated for four days at 30 °C into stationary phase and subsequently used as inoculum. Fresh inocula were prepared for each experiment.

Preparation of carbon-free materials

Carbon-free glassware (bottles and vials) was prepared as described in Hammes & Egli (2005). In short: all glassware was first washed with a common detergent, and thereafter rinsed three-times with deionised water. Then it was submerged overnight in 0.2 N HCl and subsequently rinsed with deionised water again and air-dried. Finally, the bottles and vials were heated in a Muffel furnace at 500 °C for at least six hours. Teflon-coated screw caps for the glassware were washed and treated identically with acid (0.2 N HCl). Caps were thereafter soaked in a 10 % sodium persulphate solution (60 °C, 1 h), rinsed three-times with deionised water and finally air-dried.

Water samples

A list of the 22 water samples used in the present study is given in Table 6.1. Two different full-scale drinking water treatment plants (A & B), one wastewater treatment plant, and river water (river Glatt, Dübendorf, Switzerland) were included in this study. The river water was treated with ozone (5 mg L^{-1}) for different time periods (resulting in ozone exposures, i.e. c * t values, of 0, 1, 5 and 10 mg min L^{-1}) and subsequently quenched with nitrite before use. Furthermore, migration products of three commonly available plastic materials that are used in drinking water in-house installations were tested. The migration procedure was performed according to the European norm (DIN EN 12873 - 1). In short: The materials were incubated at 60 °C for 24 h in AOC-free mineral water at a surface-to-volume ratio of 2 (migration 1). The water was discarded, replaced by fresh AOC-free mineral water and the samples were again incubated at 60 °C for 24 h (migration 2). This

procedure was repeated until migration 4 and this water, containing migration products of the plastic materials, was then used for testing.

Table 6.1. Parameters of the different water samples analysed. The samples were always taken immediately after the treatment process.

	DOC (mg L^{-1})	AOC (µg L^{-1})	pH	salinity (µS)
DWTP A				
RW	1.21	56 ± 4	7.79	296
1. Oz	1.17	129 ± 4	7.8	290
RSF	0.97	66 ± 4	8.05	301
2. Oz	0.94	65 ± 6	8.06	299
GAC+SSF	0.73	37 ± 2	7.9	297
DWTP B				
RW	20.9	395 ± 46	7.82	514
1. Oz	21.4	520 ± 20	7.6	293
RSF	9.40	67 ± 15	6.78	344
2. Oz	7.25	177 ± 2	6.57	337
GAC	6.45	66 ± 5	6.51	341
Cl	7.04	75 ± 22	6.71	341
WWTP				
RW	33.6	5497 ± 730	7.16	649
Bio	3.56	349 ± 23	7.17	657
Oz	3.38	753 ± 38	7.14	623
SSF	3.15	424 ± 18	7.86	633
Ozonated river water				
RW	3.70	331 ± 61	8.22	415
ct 1	3.70	439 ± 32	8.13	468
ct 5	3.66	515 ± 15	8.16	438
ct 10	3.71	525 ± 39	8.14	434
Materials				
pipe material 1	0.17	121 ± 12	7.24	585
pipe material 2	0.62	237 ± 145	7.29	583
sealing material	0.83	295 ± 100	7.20	590

DOC: dissolved organic carbon; DWTP A, B: drinking water treatment plant A, B; WWTP: waste water treatment plant; RW: untreated (raw) water; Oz: ozonation; RSF: rapid sand filtration; GAC: granular activated carbon filtration; SSF: slow sand filtration; Cl: chlorination; Bio: biological wastewater treatment; ct 1-10 : degree of oxidation (mg * min / L). The standard deviation for DOC measurements was always < 5 %.

Sampling & sterilization

Water (500 mL) was sampled into a carbon-free Schott flask and closed with a carbon-free teflon cap. To quench the remaining oxidant in waters treated with ozone or chlorine, nitrite (double molar to the oxidant) was added to the flask before sampling. The samples were then prepared as described in Vital *et al.* (2008), i.e. pasteurized (60 °C for 30 min), filtered (0.2 µm Millex® syringe filter (Millipore, Billerica, MA, USA) and aliquoted (15 mL) into 30 mL carbon-free glass vials

(Supelco, Bellefonte, PA, USA). Pasteurization was specifically included in the procedure, since it was shown that 0.2 µm filtration still allows the passage of a considerable fraction of bacteria present in freshwater (Wang *et al.,* 2007). After pasteurization, samples were filtered in order to remove particles, which can interfere with the flow cytometric analysis. Syringe filters were pre-washed with at least 200 mL of carbon-free water before use in order to eliminate residual organic carbon.

Pathogen growth potential (PGP) assay

Each pathogen was pre-cultivated as described above and separately inoculated into three vials, containing 15 mL of the sterile sample (starting concentration of 1 x 10^3 bacteria mL^{-1}). Three extra vials served as a sterile control. Furthermore, AOC-free bottled mineral water was treated in the same way and inoculated with the individual pathogens serving as a negative control to assess for possible AOC contamination. The inoculated water samples were then incubated at 30 °C in the dark until stationary phase was reached (up to seven days). A schematic presentation of the PGP assay is shown in Figure 6.1. All vials were put into a sonication bath (10 min) before analysis in order to remove possible surface attached bacteria into the liquid.

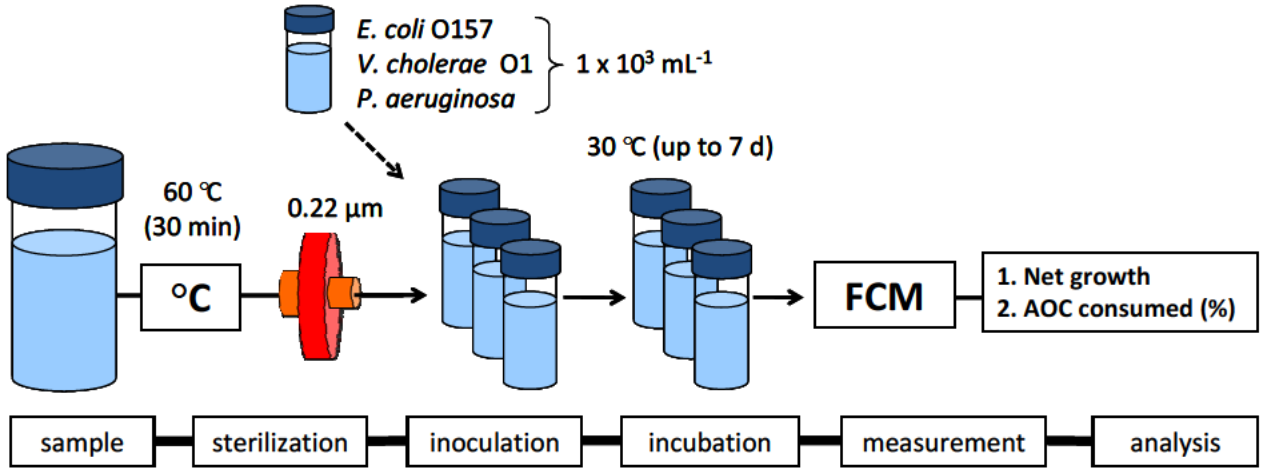

Figure 6.1. Schematic overview of the assay developed investigating the growth potential of pathogenic bacteria in different water samples.

Flow cytometric measurements

Flow cytometry was used to determine the final cell concentration and average biovolume after growth. A 10 µL aliquot of SYBR®green stain (Molecular Probes, Basel, Switzerland), 100-times diluted in dimethylsulfoxid (Fluka Chemie AG, Buchs, Switzerland), was added to 1 mL of a bacterial suspension and incubated for 15 min at room temperature in the dark before analysis. For outer membrane permeabilization, EDTA (pH 8) was added (5 mM final concentration) to the sample together with the stain (1). If a sample contained more than 10^6 cells mL^{-1}, a dilution prior to

the staining procedure was done. All samples were measured on a CyFlow Space flow cytometer (Partec, Münster, Germany) equipped with a 200 mW argon laser emitting at a fixed wavelength of 488 nm and equipped with volumetric counting hardware. The trigger was set on the green fluorescence (520 nm) channel and signals for total cell-counting were collected on the combined 520 nm / 630 nm (red fluorescence) dot plot. For cellular biovolume estimations, additional signals were collected on the combined 520 nm / sideward scatter (SSC) dot plot. An experimentally derived correlation factor was then used to determine the cellular biovolume (Hammes *et al.,* 2010). The quantification limit of the instrument was below 1000 cells mL^{-1} with an average standard deviation of less than 5 % (Hammes *et al.,* 2008).

AOC determination

AOC was determined as described previously (Hammes & Egli, 2005; Vital *et al.,* 2007). This bioassay is similar to the PGP assay presented here; a water sample is filtered, inoculated with a natural bacterial community and the final cell concentrations are measured after 3 days using flow cytometry (Hammes & Egli, 2005). In this study pasteurization of the water samples was included as well (as described above). The bacterial community used for AOC determination consisted of bacteria derived from river water (Glatt, Dübendorf, Switzerland) and unchlorinated tap water (Dübendorf, Switzerland). The same community was used for all experiments.

DOC determination

The total organic carbon (TOC) was measured based on the combustion catalytic oxidation/NDIR method (TOC-V CPH, Shimadzu, Duisburg, Germany). Before analysis, samples were 0.22 µm filtered and the TOC was, hence, referred as DOC.

Data analysis and presentation

"Growth potential": net growth of pathogens

The growth potential in a sample was measured as the final cell concentration in stationary phase. A minimum increase of 1×10^3 cells mL^{-1} was required for a result to be judged as positive. The presented results are expressed as net growth where the inoculum cell concentration was subtracted from the final cell concentrations of individual samples.

"AOC quality": fraction of consumed AOC by the individual pathogens

AOC assays normally estimate the amount of AOC from the concentrations of cells produced. However, cells of the pathogenic bacteria used are considerably bigger than those of a bacterial community (Vital *et al.,* 2008). Hence, relating the net growth of the different pathogens to that of the bacterial community used for AOC determination (see below) would underestimate the growth of the pathogens (thus also AOC consumption) relative to the bacterial community. Multiplying the average cell volume (μm^3 $cell^{-1}$) derived from flow cytometric analysis (see above) with the final cell concentration yielded the estimated total biovolume (μm^3) for individual cultures. We then calculated the percentage of AOC available for the pathogens by dividing the estimated total biovolume of each pathogen with the estimated total biovolume of the bacterial community used for AOC determination. Where the obtained value exceeded 100 % (four out of 66 samples) we display this point as 100% in the figures.

Statistics

Correlations were derived from linear regression analysis and are expressed as R^2 values. All correlations shown in Table 6.1 are also displayed as figures in the appendix (Figure S6.1 - S6.4).

Results

We modified an existing AOC assay (Hammes & Egli, 2005) to assess the growth potential of selected pathogenic bacteria in any water sample (Figure 6.1). In this bioassay, a water sample is tested with respect to (i) the net growth of a pathogenic bacterium, measured as an increase in cell concentration, representing the "growth potential" and (ii) the fraction of AOC consumed by a pathogen, representing the "AOC quality". With respect to the latter, "AOC quality" is not a compositional analysis of individual AOC compounds, but rather an expression of the accessibility of AOC for a specific organism.

Table 6.2. Correlation (R2) between the growth potential of the individual pathogens and the DOC and AOC concentration (n = 22). The corresponding Figures are presented as supplementary material (Figure S6.2 – S6.4).

	P. aeruginosa	***V. cholerae* O1**	***E. coli* O157**
DOC	0.00	0.03	0.28
AOC	0.18	0.51	0.56
***E. coli* O157**	0.11	0.2	-
***V. cholerae* O1**	0.46	-	-
P. aeruginosa	-	-	-

DOC: dissolved organic carbon; AOC: assimilable organic carbon. Due to the very high concentration of AOC and DOC, the results from the raw wastewater were not included into correlations

General overview of all data

We analysed 22 water samples from different locations. DOC and AOC concentrations of the different samples varied considerably, ranging between 0.17 – 33.6 mg L^{-1} of DOC and 37 – 5497 µg L^{-1} of AOC, respectively (Table 6.1), with AOC accounting for 0.7 % up to 22 % of the DOC. For the migration products of the plastic materials, between 36 – 71 % of the DOC consisted of AOC. Overall, no correlation (R^2 = 0.08; Figure S6.1) between AOC and DOC was observed, which reaffirms that mere DOC analysis is insufficient evidence of the general growth potential of a water sample. Looking at all results in general, the growth potential of *E. coli* O157 ranged from 0 – 2.87 x 10^6 cells mL^{-1} and for *V. cholerae* from 0 – 1.35 x 10^7 cells mL^{-1}, whereas *P. aeruginosa* grew in all samples tested and its growth potential varied from 4 x 10^3 – 7.5 x 10^6 cells mL^{-1} (Figures 6.2 to 6.6). There was no correlation between DOC concentration and net growth of the pure cultures (Table 6.2; Figure S6.2), whereas the concentration of AOC, reflecting the fraction of nutrients readily available for heterotrophic growth, correlated to some extent with the growth of the different pathogens (Table 6.2; Figure S6.3). The correlations between the growth potential and AOC ranged from R^2 = 0.18 for *P. aeruginosa* up to R^2 = 0.56 for *E. coli* O157 (Table 6.2; Figure

S6.3). From this, one can conclude that in general, elevated concentrations of AOC also support enhanced pathogen growth.

However, it was only partly possible to predict the growth potential of the individual pathogens using the concentration of AOC. The data-scattering along the regression lines was considerable, with notable outliers (Figure S6.3). Furthermore, correlations between the pure cultures with each other were poor, ranging from only $R^2 = 0.11$ (*E. coli* O157 vs *P. aeruginosa*) to $R^2 = 0.46$ (*V. cholerae* vs *P. aeruginosa*) (Table 6.2; Figure S6.4) and the pathogen displaying the highest growth potential was different between samples. The three pathogens never grew to the same concentration in any water and the relative ratio of the cultures to each other was unique in each sample (Figures 6.2 – 6.6). Additionally, we also investigated the "quality" of AOC in the different water samples by estimating the fraction of AOC consumed by a pathogen. For *E. coli* O157 and *V. cholerae*, the obtained values ranged from 1 % up to 100 %, whereas *P. aeruginosa* consumed between 7 % and 89 % (Figure S6.5). This further illustrates the differing accessibility of AOC for pathogen growth. All these results imply that the pathogens responded dissimilar in the different waters tested and that not only the concentration, but also the "quality" of AOC is a key factor controlling their growth.

PGP in drinking water treatment plant A

The treatment train of plant A is composed of two sequential ozonation-biofiltration steps (Figure 6.2). Overall, the PGP in water samples from this plant was very low, as were AOC concentrations. *V. cholerae* was only detected in the raw water samples and no other water sample allowed proliferation of this pathogen, whereas *E. coli* O157 grew only in the water sample collected after the first ozonation treatment (Figure 6.2 A). In contrast, *P. aeruginosa* multiplied in all samples. The two ozonation steps resulted in increased growth of *P. aeruginosa*, whereas the growth potential of this pathogen was markedly decreased after slow sand filtration and very low (net growth of only 4×10^3 cells mL^{-1}) in the finished water. We observed a similar pattern for the AOC concentration (Table 6.1). The estimated fraction of AOC consumed by *P. aeruginosa* was, however, always below 20 % and, hence, always only a small fraction of the AOC was available for the pathogen (Figure 6.2 B).

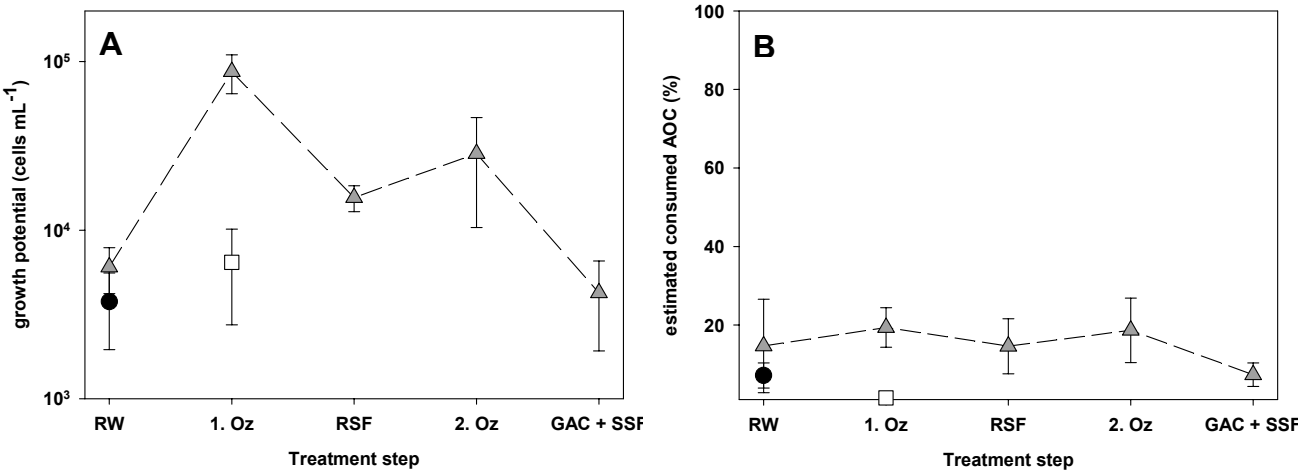

Figure 6.2. Growth of the three pathogens, *E. coli* O157 (□), *V. cholerae* (●) and *P. aeruginosa* (△), in samples taken after the individual treatment steps from drinking water treatment plant A. The growth potential, i.e. net grown cells (A), as well as the fraction of estimated consumed assimilable organic carbon (AOC) for each pathogen (B) are shown. RW: untreated (raw) water; Oz: ozonation; RSF: rapid sand filtration; GAC: granular activated carbon filtration; SSF: slow sand filtration.

PGP in drinking water treatment plant B

The treatment train of the second drinking water treatment plant is similar to plant A and includes two sequential ozonation-biofiltration steps as well (Figure 6.3). However, it includes no slow sand filtration step and the finished water is chlorinated at the end of the treatment as a final disinfection step. The water quality was, however, distinctly different in comparison to plant A: DOC as well as AOC concentrations were considerably higher, as was the growth for all three pathogens (Table 6.1, Figure 6.3 A). The growth potential of *E. coli* O157 and of *P. aeruginosa* displayed the same trend, i.e. more growth in the samples after ozonation followed by a decline in the growth potential after biofiltration. Interestingly, although the total AOC concentration decreased considerably through the treatment process, the growth potential of *E. coli O157* and of *P. aeruginosa* in samples of the finished water (2.27 x 10^5 cells mL^{-1} and 1.39 x 10^5 cells mL^{-1}, respectively) were similar to those observed in the raw water (2.81 x 10^5 cells mL^{-1} and 1.21 x 10^5 cells mL^{-1}, respectively). This observation is also illustrated by Figure 6.3 B, where the accessed fraction of AOC for the pathogens increased during the water treatment steps and displayed very high values, i.e. 94 % for *E. coli* O157 and 71 % for *P. aeruginosa*, in the finished water. *V. cholerae* displayed a distinctly different pattern. After a slight increase in growth after the first ozonation step, its growth potential sharply decreased after rapid sand filtration and second ozonation. Finally, only a little growth potential of *V. cholerae* was detected in the finished water (Figure 6.3 A).

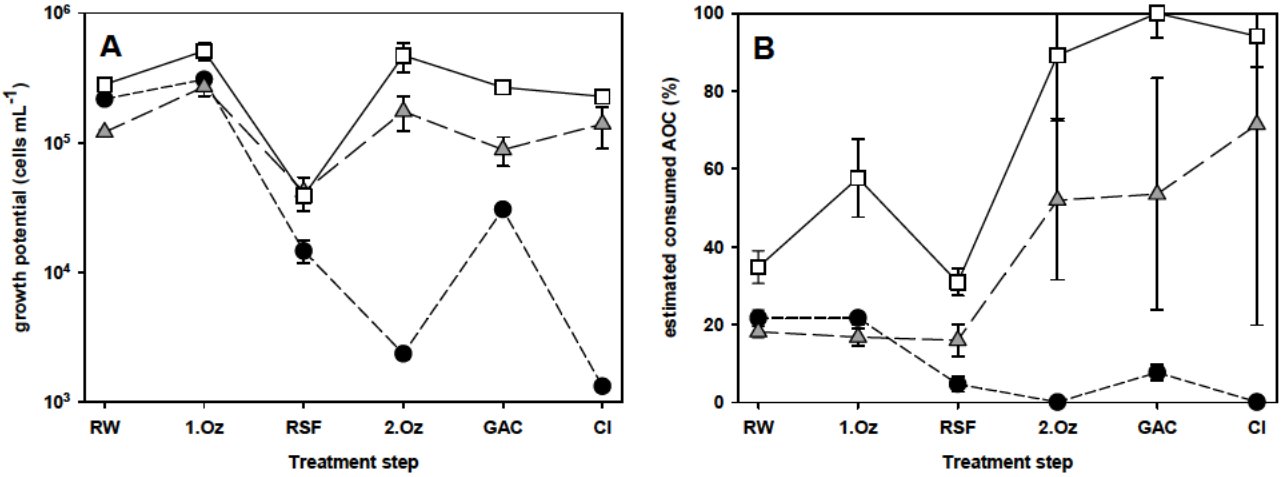

Figure 6.3. Growth of the three pathogens, *E. coli* O157 (□), *V. cholerae* (●) and *P. aeruginosa* (▲), in samples taken after the individual treatment steps from drinking water treatment plant B. The growth potential, i.e. net grown cells (A), as well as the fraction of estimated consumed assimilable organic carbon (AOC) for each pathogen (B) are shown. RW: untreated (raw) water; Oz: ozonation; RSF: rapid sand filtration; GAC: granular activated carbon filtration; Cl: chlorination.

PGP in wastewater treatment plant

In the wastewater treatment plant, the effluent of the conventional biological treatment is subjected to post-treatment using a combined ozonation-biofiltration step before it is finally fed into a river. For all three pathogens, the raw wastewater, which contained a high AOC concentration (5.5 mg L^{-1}), promoted the highest growth of all waters tested in this study (Figure 6.4 A). In samples after biological treatment, the growth potential was reduced by 93 % for *E. coli* O157 and 98 % for *V. cholerae* and *P. aeruginosa*, respectively. The following ozonation-biofiltration step resulted in a similar pattern as observed in the two drinking water treatment plants, i.e. ozonation generated AOC and led to an increased growth potential of *E. coli* O157 and *P. aeruginosa*; followed by reduced growth after biofiltration (Figure 6.4 A). In this case, the growth potential of *V. cholerae* displayed a similar pattern to that of the two other pathogens during treatment. The estimated AOC consumption differed distinctly between the individual pure cultures; the data suggest that *V. cholerae* consumed the most AOC followed by *E. coli* O157 and *P. aeruginosa* (Figure 6.4 B).

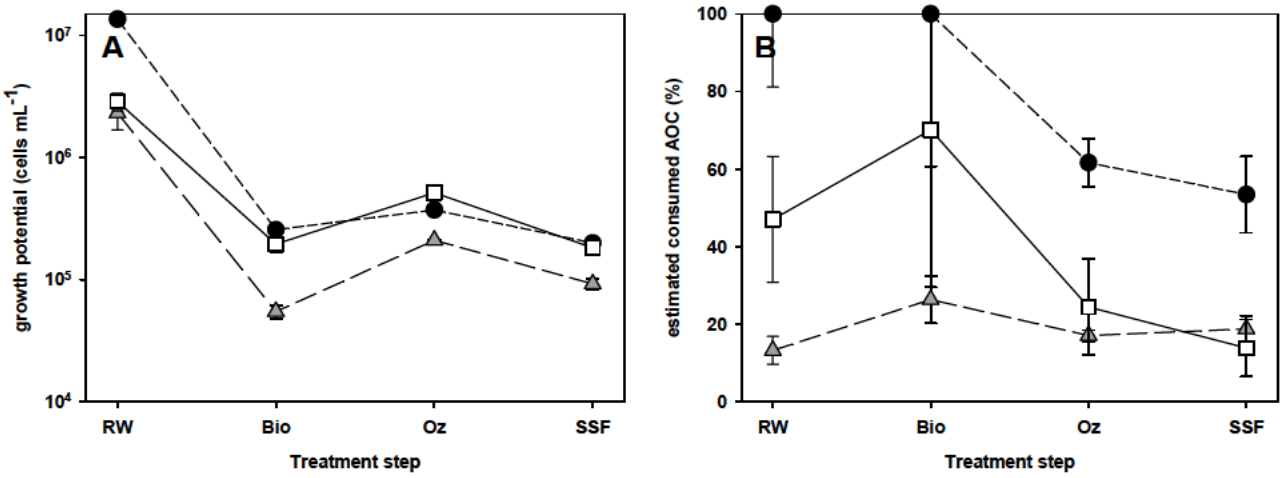

Figure 6.4. Growth of the three pathogens, *E. coli* O157 (□), *V. cholerae* (●) and *P. aeruginosa* (▲), in samples taken after the individual treatment steps from a wastewaterwater treatment plant. The growth potential, i.e. net grown cells (A), as well as the fraction of estimated consumed assimilable organic carbon (AOC) for each pathogen (B) are shown. RW: untreated (raw) water; Bio: biological treatment; Oz: ozonation; SSF: slow sand filtration

PGP in ozonated river water

Ozonation is a key step in the water treatment plants studied above. Since it is an emerging method to treat water of different types, we investigated the effect of ozonation on the growth of the three pathogens in detail. As observed before (Figure 6.2 A, 6.3 A, 6.4 A), the AOC concentration increased with increasing ozone exposure and also the growth potential of *E. coli* O157 and of *P. aeruginosa* was positively affected by ozonation; both pathogens produced around three times more cells in the most oxidized water (ct = 10) compared to untreated river water (Figure 6.5 A). However, the estimated AOC consumption of the two pathogens showed a decrease with increasing oxidation suggesting that the nutrient availability for the pathogens was considerably altered during the treatment (Figure 6.5 B). The data presented in Figure 6.3 and 6.4 indicate that *V. cholerae* followed a different pattern to the other pathogens when growing in oxidized water. Also in this experiment, the growth potential of this pathogen was hardly affected by the oxidation treatment (Figure 6.5 A).

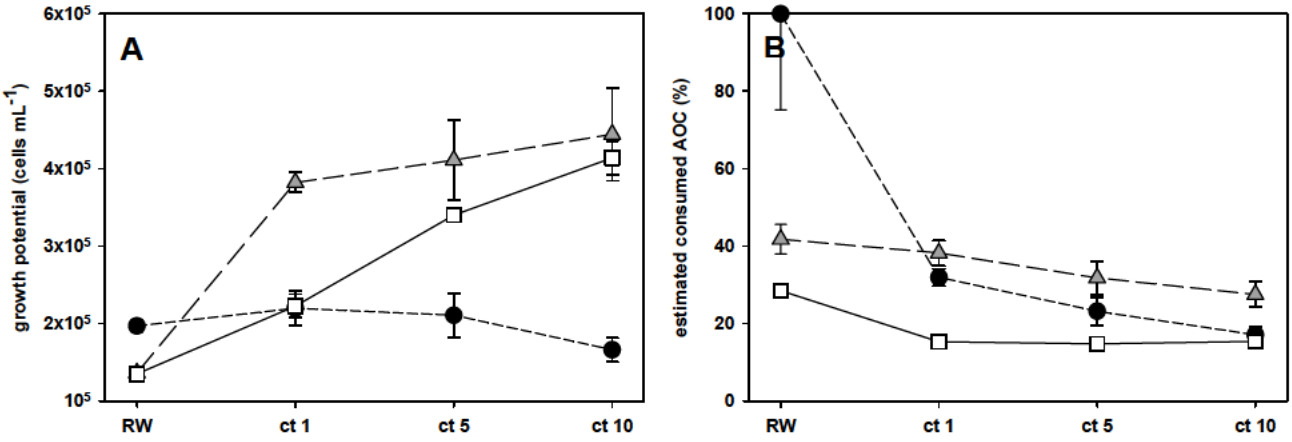

Figure 6.5. Growth of the three pathogens, *E. coli* O157 (□), *V. cholerae* (●) and *P. aeruginosa* (▲), in river water exposed to ozone. The growth potential, i.e. net grown cells (A), as well as the fraction of estimated consumed assimilable organic carbon (AOC) for each pathogen (B) are shown. RW: untreated (raw) river water; ct 1-10: degree of oxidation (mg * min / L).

PGP in migration products from plastic materials

Finally, we investigated the pathogen growth potential for compounds migrating from three plastic materials commonly used for drinking water installations. All three pathogens were able to multiply on the migrating compounds of all three materials tested (Figure 6.6 A). *P. aeruginosa* displayed higher growth potential than *E. coli O157* and *V. cholerae*, respectively. Furthermore, on all three materials tested the estimated fraction of AOC consumed for growth by this pathogen was high (above 30 %; Figure 6.6 B).

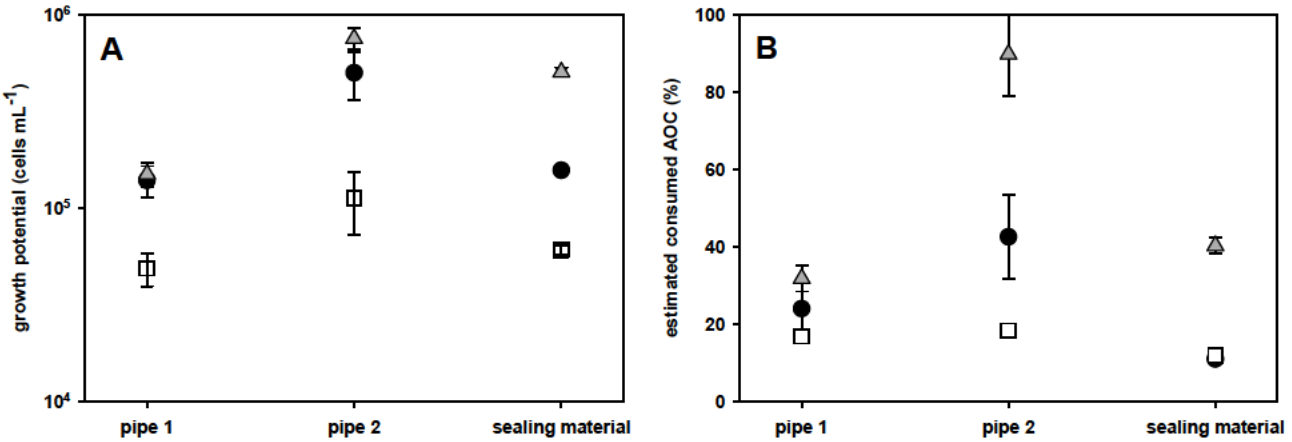

Figure 6.6 Growth of the three pathogens, *E. coli* O157 (□), *V. cholerae* (●) and *P. aeruginosa* (▲), on compounds migrating from plastic materials commonly used for in-house drinking water distribution systems. The growth potential, i.e. net grown cells (A), as well as the fraction of estimated consumed assimilable organic carbon (AOC) for each pathogen (B) are shown.

Discussion

Despite the holistic approaches of modern day risk assessment, growth of pathogenic bacteria is only vaguely considered (OECD/WHO, 2003). This risk component is usually restricted to tables grouping pathogens into those that may grow in water and those that do not (WHO, 2008). The neglect of pathogen growth in risk assessment can partly be explained by the limited knowledge of the factors governing their growth (Vital *et al.,* 2008). It has, however, been shown previously that pathogenic bacteria, including enteric pathogens, can grow at low nutrient concentrations in waters used for drinking or personal hygiene (Camper *et al.,* 1991; Szewzyk *et al.,* 2000; Torvinen *et al.,* 2004; Juhna *et al.,* 2007; Vital *et al.,* 2007, 2008). This warrants further investigation on this topic and we have, therefore, developed a bioassay specifically assessing the pathogen growth potential (PGP) in water.

Nearly all treatment processes affect the water´s carbon pool with respect to both, concentration and composition. An analysis of the PGP in water provides researchers and water utilities with an additional decision making tool for optimum design and operation of water treatment systems in order to minimize the risk of pathogen growth. For example, ozonation is used in (drinking) water treatment for the oxidation of micro-pollutants and for disinfection (Escobar *et al*, 2001; von Gunten, 2003a, b). It is commonly known that oxidation processes generate AOC and BDOC, thus increasing the overall bacterial growth potential of a water (Hammes *et al.,* 2006, 2007; von Guten, 2003a; Table 6.1). This increase in AOC also enhanced the growth potential of *E. coli* O157 and *P. aeruginosa* after ozonation (Figure 6.2, 6.3, 6.4, 6.5), albeit to a different extent in different waters. Ozonation is, however, an effective disinfection step against pathogenic bacteria (Juhna *et al.,* 2007; von Guten, 2003b) and our work should not be understood as a suggestion to omit this process to improve treatment efficiency and lower the risk of pathogen growth. Rather, the presented PGP assay can be used to characterize and optimise the ozonation or subsequent processes in terms of the trade-off between maximum disinfection and minimum formation of AOC available for pathogens. Moreover, to reduce the risk of pathogen growth, our results suggest that a biofiltration step should always directly follow this oxidation step, as done in the treatment trains analysed here and also described previously (Siddiqui, 1997; Chen *et al.,* 2007). All biofiltration steps analysed in this study (Bio, RSF, GAC, SSF) reduced AOC concentrations as well as the growth potential of *E. coli* O157 and *P. aeruginosa*, respectively. The PGP assay can, therefore, be used to evaluate, optimize and compare different biofilter systems. Interestingly, *V. cholerae* often responded uniquely in samples collected after oxidation steps. It appears that during oxidation the quality of carbonaceous

compounds in the water is altered in such a way that the potential risk for growth of this pathogen is in fact reduced, although the concentration of AOC increases (Figure 6.2, 6.3, and 6.5). This illustrates the necessity to test the growth potential for different pathogen separately. Another good example demonstrating the advantage of individual testing is drinking water treatment plant B (Figure 6.3). Although a low concentration of AOC (75 µg L^{-1}) was recorded in the finished water, the growth potential of *E. coli* O157 and of *P. aeruginosa* was disproportionally high. The supporting data presented in Figure 6.3 B suggest that the quality of AOC was favouring growth of these two pathogens. Interestingly, a previous study demonstrated that *E. coli* can indeed grow in biofilms of this particular drinking water treatment system, contributing up to 0.1 % of the total bacterial population (Juhna *et al.,* 2007).

Another application of the presented PGP assay is the evaluation of (plastic) materials in contact with water. Most plastic materials, especially when new, slowly release organic carbon, part of which can be biodegradable (van der Kooij & Veenendaal, 2001). Although the analysis of total organic carbon (TOC) leaching from such materials is compulsory in European states (DIN EN 12873 - 1), this validation normally does not include AOC or similar parameters. In our research group we developed an assay for the testing of such migration products leaching from materials used in drinking water installations that include testing for AOC (unpublished data). Our data clearly indicate that DOC analysis is inadequate for knowing the general bacterial growth potential (expressed as AOC) or the specific PGP (Figure S6.1 and S6.2). The relevance of this information is underpinned by studies indicating that hygienic relevant bacteria such as *Legionella pneumophilia* can grow on plastic materials (Rogers *et al.,* 1994; van der Kooij *et al.,* 2005). The migrating compounds originating from all of the plastic materials tested in the present study supported the growth of the three pathogenic bacteria used here. In particular, the opportunistic pathogen *P. aeruginosa* showed a high potential of growth on migrating compounds from the three plastics analysed. The high AOC/DOC ratios recorded suggest that the substrates favoured bacterial growth in particular. Choosing the correct materials for water distribution, especially in environments where older and/or immuno-comprised people are exposed such as hospitals or retirement homes, could thus be critical in order to limit pathogen growth and associated problems.

In this study we used the three pathogenic bacteria tested previously on their growth properties at low nutrient concentration (Vital *et al.,* 2007, 2008). However, the PGP assay is not restricted to these organisms, but is essentially applicable to any bacterium of interest. For example, Rice *et al.* (1990) used a similar approach to analyse coliform growth in water samples. However, for some

heterotrophic bacterial species there are other factors than the availability of AOC that affect their growth potential. One example is *Legionella pneumophilia* which requires cystein and iron for proliferation (Devos *et al.,* 2005); such specific physiological properties should thus be considered when designing a PGP assay and interpreting the results.

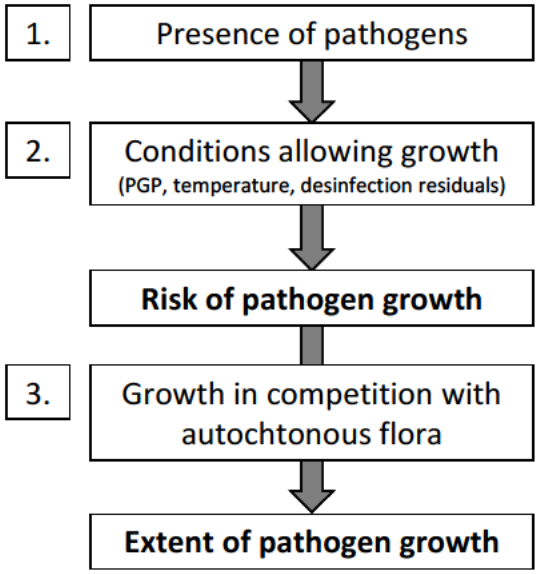

Figure 6.7. A schematic overview of the three different levels, which are important to assess the risk of pathogen growth in water. For explanation see text. PGP: pathogen growth potential..

Several other factors also determine the growth of pathogenic bacteria in water, along with nutrient availability. Integration of the developed PGP assay into a bigger framework of pathogen growth in water is outlined in Figure 6.7. In this flow-scheme we identified three main levels to be considered, namely: (i) the presence of pathogens, (ii) an environment supporting their growth, and (iii) competition with the indigenous microbial flora for AOC. First, the starting concentration of present (or contaminating) pathogens has to be known, since growth cannot occur in their absence. The second level pertains to whether the physicochemical environment allows growth. This information is obtained directly from the results of the PGP assay. For example, in the finished water of drinking water treatment plant A (Figure 6.2), no growth of *E. coli* O157 and *V. cholerae* was detected and, hence, their growth potential can be considered zero. Moreover, the PGP assay does not consider the possible presence of disinfection residuals and *in situ* temperatures of the water. It is well-documented that pathogens require a certain "minimum temperature" for growth that is normally in the range between 10 and 20 °C, depending on the bacterial species (Raghubeer & Matches, 1990). Therefore, if the PGP assay yields a positive result but the actual water temperature is below the "minimum temperature" or disinfection residuals are present at concentrations inhibiting bacterial proliferation, the risk of growth is zero as well. If all the three parameters (PGP

assay, temperature and disinfection residuals) allow for growth, a risk of pathogen multiplication in the system is automatically given. In other words, if all requirements for level two are fulfilled then there is a risk of growth (Figure 6.7). At the third level, the extent of pathogen growth in a given water sample is of interest. The results of the PGP assay can already serve as a first indication; the higher the growth potential the more growth can be expected. Since pathogens, however, normally grow together with the indigenous bacterial flora, and not in pure culture, the extent of their growth is strongly influenced by competition for AOC (level three in Figure 6.7). Competition is a complex interplay controlled by several factors such as the concentrations of competing species, their stoichiometric and kinetic properties, several physicochemical parameters as well as the nutrient supply dynamics (Frederickson & Stephanopoulos. 1981; Gottschal, 1993). Unfortunately, the current state of knowledge on the mechanisms governing competition of bacteria in natural waters is very limited. However, new methods measuring bacteria at low cell concentrations at a high throughput are rapidly providing new information that are shifting existing paradigms regarding pathogen growth in water (Kirschner *et al.,* 2008; Vital *et al.,* 2007, 2008). Such data combined with mathematical modelling could be used in future to estimate the actual degree of pathogen growth in a given water sample and to investigate different scenarios of interest *in silico*.

Conclusions

- A novel bioassay was developed to investigate the potential of any water sample to support growth of pathogenic microorganisms.
- The data showed unique growth behaviour of the different pathogens in each water sample, relative to each sample and to each other. Furthermore, AOC correlated only to some extent with their net growth, indicating that the quality/composition of AOC is critical for pathogen growth.
- The developed pathogen growth potential (PGP) assay can be used as a screening tool complimentary to conventional AOC assays in order to compare different water samples or treatment steps.
- The results of the PGP assay can also be incorporated into a multi-level concept assessing the risk of pathogen growth in water.

Acknowledgements

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Supplementary info

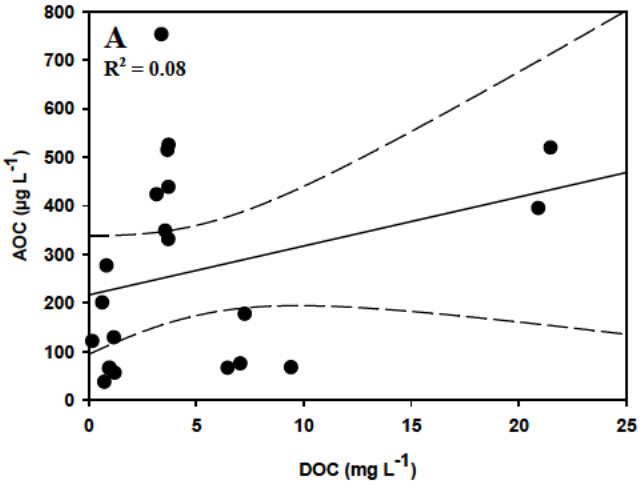

Figure S6.1. Correlation (R^2) between DOC and AOC. Due to the high DOC and AOC concentration the values from the raw wastewater were not included. The solid line shows the regression line, whereas the dashed lines represent the confidence intervals for 95 %.

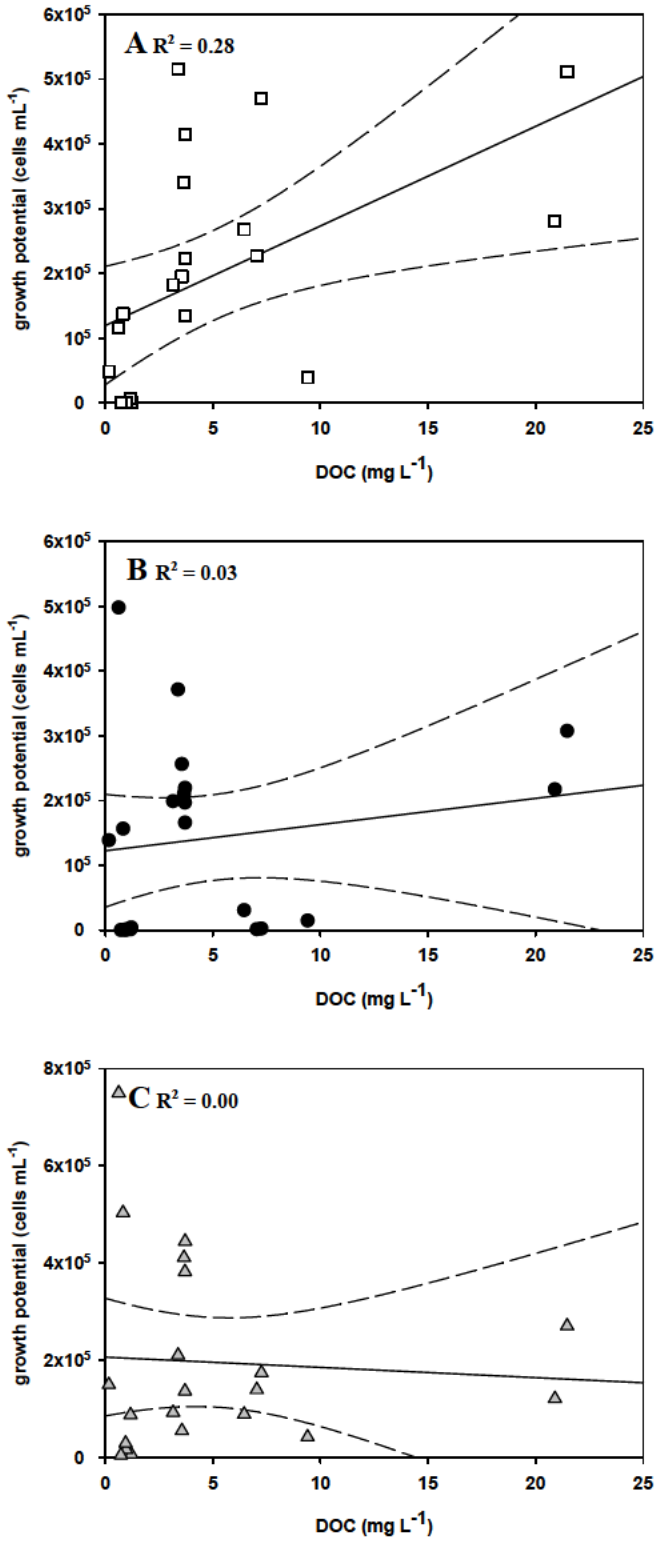

Figure S6.2. Correlation (R^2) between DOC and growth potential (net growth) of the three pathogens, *E. coli* O157 (A), *V. cholerae* (B) and *P. aeruginosa* (C). Due to the high DOC concentration the values from the raw wastewater were not included. The solid line shows the regression line, whereas the dashed lines represent the confidence intervals for 95 %.

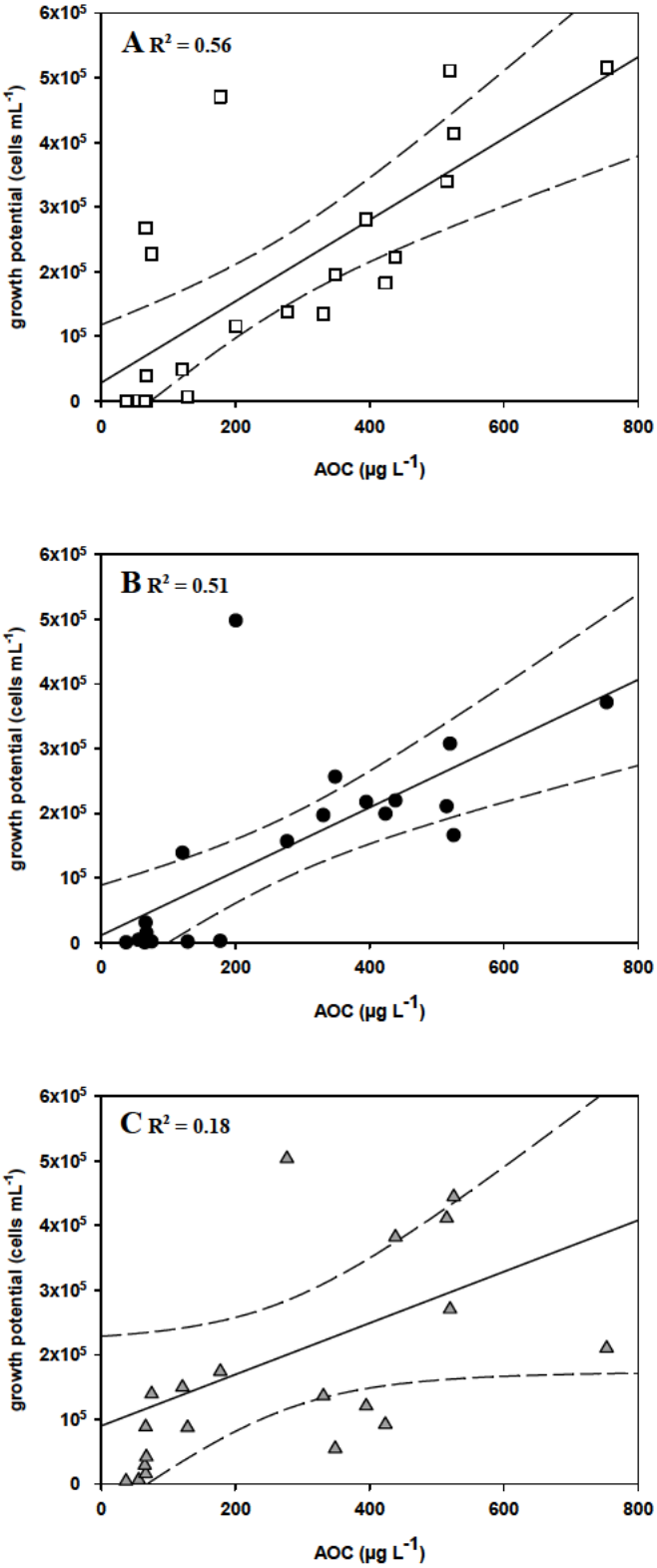

Figure S6.3. Correlation (R^2) between AOC and growth potential (net growth) of the three pathogens, *E. coli* O157 (A), *V. cholerae* (B) and *P. aeruginosa* (C). Due to the high AOC concentration the values from the raw wastewater were not included. The solid line shows the regression line, whereas the dashed lines represent the confidence intervals for 95 %.

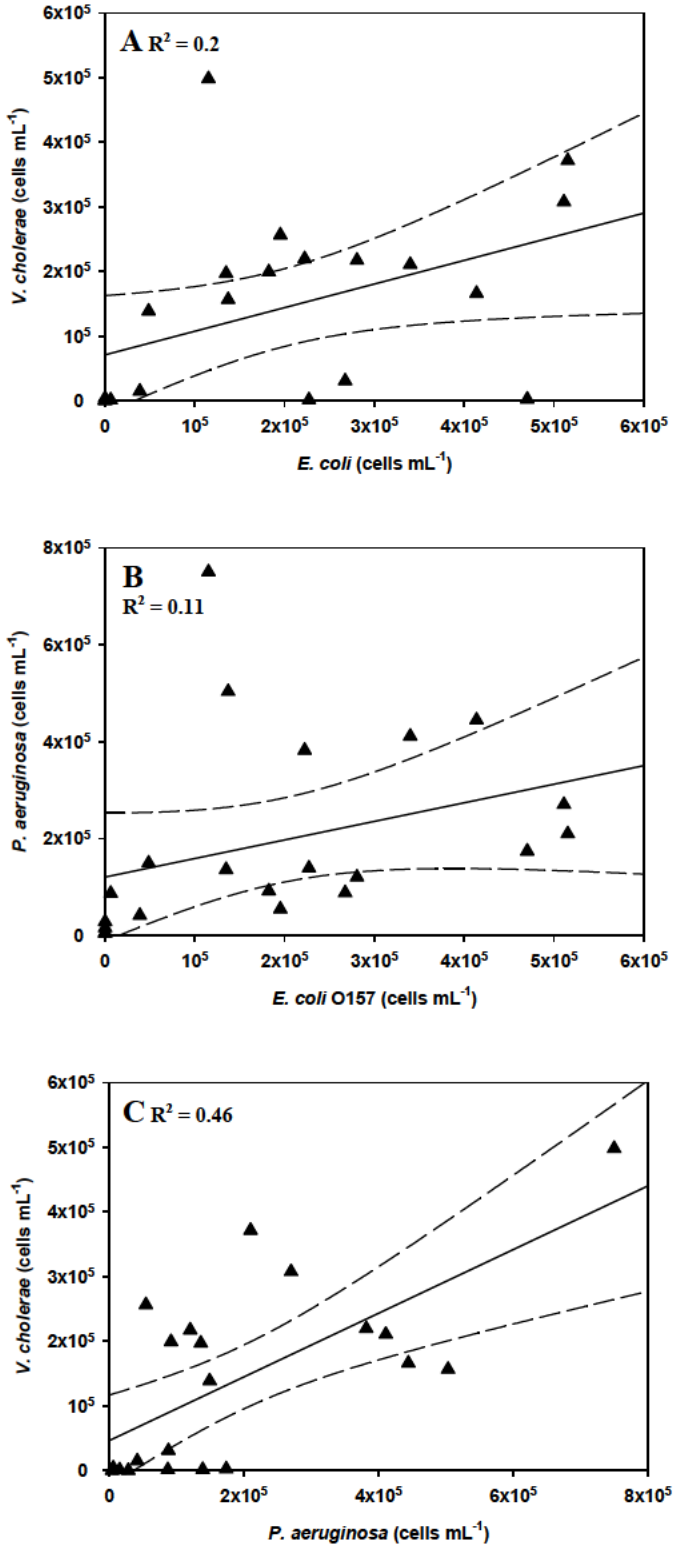

Figure S6.4. Correlations (R^2) between the growth potential (net growth) of the individual pathogens. *E. coli* O157 vs. *V. cholerae* (A), *E. coli* O157 vs *P. aeruginosa* (B) and *V. cholerae* vs *P. aeruginosa* (C) are shown. The solid line shows the regression line, whereas the dashed lines represent the confidence intervals for 95 %.

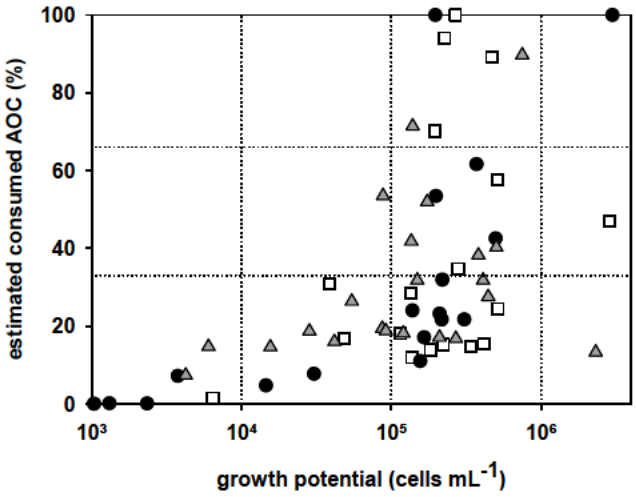

Figure S6.5. Overview of the obtained results from the developed pathogen growth potential assay. The growth potential (net growth) and the fraction of estimated consumed assimilable organic carbon of each pathogen in the different waters analysed (n = 22) is shown. *E. coli* O157 (□), *V. cholerae* (●) and *P. aeruginosa* (▲).

7. General Conclusions & Outlook

Background

Bacterial waterborne diseases occur worldwide. The abundance of waterborne pathogens in aquatic ecosystems is influenced by various factors illustrated in Figure 7.1. External sources, such as pathogen-shedding animals and wastewater, as well as *in situ* growth, lead to increased pathogen concentrations, whereas the "top down control", i.e. viral lysis and predation, dilution and die-off due to either a lack of nutrients or physicochemical stressors, reduce their numbers (Bettarel *et al.*, 2003; Winfield & Groisman, 2003). However, as outlined in chapter 1, behavior of pathogenic bacteria in water is only poorly understood and requires extensive investigations in order to enable adequate microbial risk assessment. The current microbial water hygiene concept is solely based on the detection of selected index organisms, usually *E. coli*. Presence of *E. coli* indicates recent fecal pollution and a risk of pathogen prevalence, whereas *E. coli* free waters are considered safe for consumption (SLMB, 2000). Consequently, the water hygiene concept suggests that any factor shown in Figure 7.1. controls the abundance of both index organisms and pathogens in a similar manner. It should be pointed out that index organisms are thought to be incapable to grow at the low organic carbon concentrations in the environment, but that their die off starts immediately when leaving the host. Hence, pathogen growth is not considered at all in the water hygiene concept.

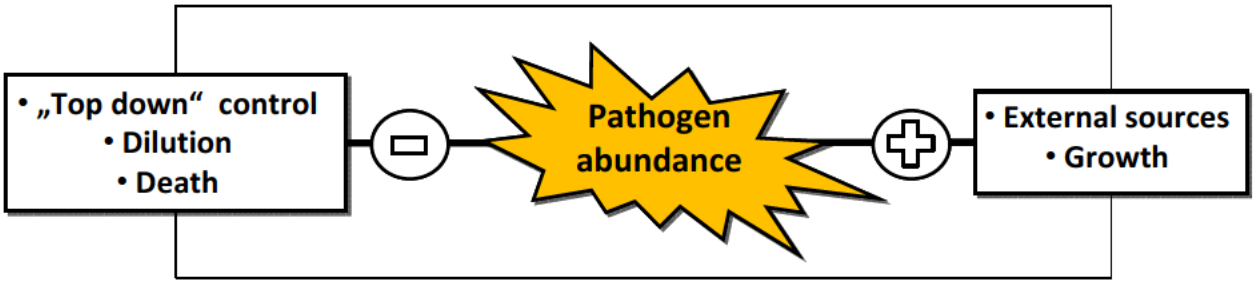

Figure 7.1. Major factors influencing the abundance of pathogenic bacteria in water.

However, "pathogenic bacteria" is a collective term commonly used for bacteria causing disease in humans, but the group comprises a variety of species all differing in their behavior in the environment. In fact, there are many pathogenic species, which are considered as autochthonous in specific aquatic ecosystems. Thus, for drinking water authorities it would be important to quantify all factors shown in Figure 7.1. for each pathogen individually in order to perform adequate microbial risk assessment to avoid spreading of waterborne diseases. In this respect, understanding their growth is of particular importance because it increases pathogen abundance and an elevated risk for infection. Furthermore, waters promoting growth of pathogens but die-off of the index

organisms are failing the water hygiene concept and are, thus, wrongly classified as safe for consumption.

Therefore, the aim of this thesis was to investigate and quantify growth properties of selected pathogenic bacteria and to identify factors governing their proliferation in different types of water (drinking water, surface water and wastewater) under nutrient-poor conditions. The obtained knowledge should contribute to the understanding on microbial growth under nutrient-poor conditions in general and provide a first step towards considering pathogen growth within microbial risk assessment.

Experimental set-ups and methods used to study growth at low nutrient/cell concentrations

Growth is defined as the consumption of substrates resulting in an increase of microbial cell mass (Stephenson, 1949). As outlined in chapter 1, the majority of reports on growth of pathogenic bacteria in water are based on experiments using wrong set-ups; bacteria were either inoculated into microcosms at way too high cell concentrations in order to study growth, or the water used for experiments was incorrectly considered to be sterile. In other reports this issue was approached by inoculating selected pathogens into complex, uncontrolled systems such as mesocosms containing untreated surface water or biofilters of pilot-scale water treatment plants where a multitude of factors are simultaneously affecting their behavior (e.g., Camper *et al.,* 1985; Lehtola *et al.,* 2007). Such experiments were considered to simulate *in situ* conditions, but rarely allowed to identify specific factors that explained the observations; therefore, obtained results were often difficult to interpret. On the other hand, studies based on investigations of pure cultures under carefully defined conditions (e.g., Ferenci, 1999; Wick *et al.,* 2002) were often criticized to be "artificial" and irrelevant to explain the growth and selection mechanisms occurring in the environment. The experimental set-up presented in this thesis, by growing pathogens on natural assimilable organic carbon (AOC) and highly diluted complex media *in vitro*, can be considered as an "in-between" approach. With this approach, it was possible to work with pure cultures in a controlled way under nutritional conditions similar to those bacteria encounter in the environment. Furthermore, it allowed increasing the complexity of the system in a defined manner towards competition in continuous culture. The experimental set-up allowed us to compare pathogen behavior with that of autochthonous aquatic communities. Since these bacteria are adapted to nutrient-poor conditions it is impossible to study their growth using conventional media at high concentration; for example, in our experiments only a minor fraction (< 10 %) of autochthonous bacteria were cultivable on

conventional R2 agar plates (Hammes *et al.,* 2009). Many of the obtained results could be explained by mathematical modelling, which provided a detailed insight into the growth dynamics.

Many methods for quantifying growth are available, but they often require high cell densities and the technological spectrum is more restricted in dilute (environmental) systems. Basically two types of methods are employed to monitor bacterial growth at low cell/nutrient concentrations, (1) those based on single cell analysis such as plating, microscopic or flow cytometric counting (e.g., Bowden, 1977), and (2) those measuring bulk parameters such as ATP or radioactive substrate incorporation (e.g., Fuhrman & Azam, 1982). In this thesis growth was monitored using flow cytometry-based techniques enabling analysis of multiple parameters simultaneously at the single cell level (Vives-Rego et al., 2000). It allowed fast bacterial enumeration, gave information on the cellular biovolume and visualized distinct subpopulations during growth. It was combined with fluorescent antibodies to specifically immuno-detect pathogens growing in competition with complex bacterial communities and, furthermore, it was used together with several stains targeting bacterial physiological properties (Berney *et al.,* 2008).

Both environmental as well as classic enteric pathogens can grow with natural AOC

In most aquatic ecosystems, heterotrophic bacteria are exposed to nutrient-poor conditions and usually organic carbon is the growth-limiting factor (Morita, 1993). Total AOC concentrations range from only a few µg L^{-1} up to several hundred µg L^{-1}, whereas individual substrate concentrations are in the low µg or even ng L^{-1} range (Münster, 1993; Egli, 1995). In this thesis, it was demonstrated that growth under such nutritional conditions is not restricted to autochthonous bacteria. Also enteric pathogens such as *E. coli* O157 can proliferate. Hence, the strict separation of pathogenic bacteria into two groups based on their ability to grow in water (see chapter 1; OECD/WHO, 2003) does not hold true. Growth in sterile surface water was also demonstrated for several other enteric bacteria such as *Salmonella typhimurium* or *Yersinia enterocolitica* (data not included in this thesis). The results suggest that a potential risk for growth in water can be expanded to many more pathogens than presently thought.

Growth is even possible at minute AOC concentrations

For none of the investigated pathogens a threshold concentration for growth was detected, but all cultures were able to grow even at a few µg L^{-1} of available AOC (chapter 4). To understand these results one has to take a closer look at the phenomenon called “mixed substrate growth”. The low concentration of individual substrates available for growth lead to a de-repression of catabolic

pathways and a multitude of different compounds can in this way simultaneously be taken up and metabolized by a cell; this nutritional behavior has been referred to as "mixed substrate growth" (Harder & Dijkhuizen, 1976). In other words, bacteria are not selectively picking individual carbon compounds from the total available pool, but are always prepared to simultaneously take up and metabolize numerous of the substrates which are present in their surroundings. Hence, the phenomenon of "diauxic growth", where a "good" carbon compound is inhibiting the utilization of another "worse" one, does probably rarely occur in a carbon/energy limited environment. For example, *E. coli* cells in carbon-limited complex medium chemostats at intermediate dilution rates were able to immediately take up and oxidize 43 different substrates (Ihssen & Egli, 2005). "Mixed substrate growth" was not only observed for *E. coli*, but has been reported for a multitude of other species as well (reviewed by Egli, 1995) and can, therefore, be considered as the norm during growth under low carbon concentrations. It allows a cell to establish a relatively decent carbon flux even at minute concentrations of individual substrates thereby circumventing threshold concentrations for growth. For example, estimated total available AOC concentrations in 500,000-times diluted LB medium are in the range of 3 - 5 μg L^{-1}, and concentrations of individual available substrates such as free amino acids are consequently even much lower (in the ng L^{-1} range). However, all cultures, even *E. coli* O157, were able to grow at this dilution. Unfortunately, our experimental set-up did not allow to work below these AOC concentrations and the existence of any threshold concentrations for growth of the tested pathogens cannot be ruled out. Different experimental set-ups such as growing bacteria in retentostats, where the fed substrate flux per cell is continuously reduced to the point where no growth is detected any more would allow studying this aspect and the physiology involved in more detail (Chesbro *et al.,* 1979; Tros *et al.,* 1996). Such experiments have been initiated, but so far no complete data sets are available.

Only a fraction of the total AOC is available for pathogen growth

The presence of AOC in a sample does not imply that growth of all bacterial species is possible; each AOC pool has its unique substrate composition and selects for growth of bacteria with matching catabolic capabilities; a certain bacterium can not grow with all substrates but is restricted to its specific substrate utilization spectrum, which is also reflected in the traditional bacterial taxonomy (van der Koiij, 1992; Sack *et al.,* 2009). Hence, only a part of the total AOC is available for growth of individual strains, which was the motivation for developing a bio-assay enabling to assess the AOC pool whether or not it supports growth of selected pathogens (chapter 6). With this assay, it was shown that the access to AOC compounds for individual pathogens is indeed distinct and considerably changing during water treatment. The relative fraction available for a particular

bacterium has a considerable impact on its kinetics and the resulting dynamics during competitive growth (see below).

Kinetics during growth with mixtures of substrates at low concentration

Microbial growth kinetics describes the relationship between the consumption of nutrients and the resulting specific growth rate (μ). The basic concept that the concentration of one growth-limiting substrate is dictating μ in a saturation-type relationship was already established in the 1940s by J. Monod (Monod, 1949) and is still by far the most used model (Pirt *et al.,* 1975). Additional concepts and modifications were proposed in the 1950s and 1960s, but since then the topic was considered to be a closed book (reviewed by Kovarova-Kovar & Egli, 1998).

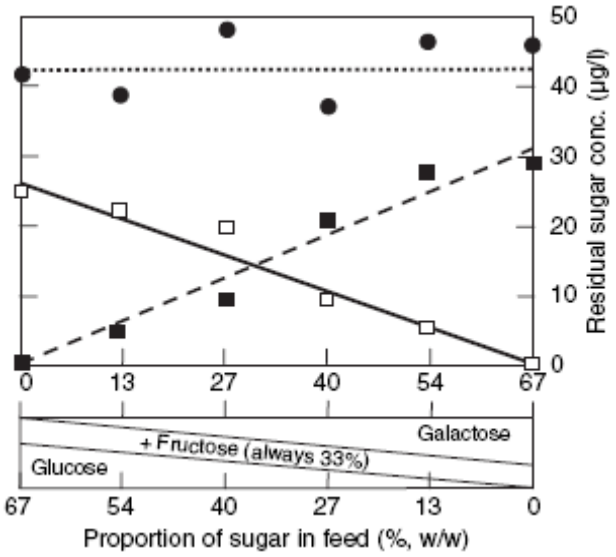

Figure 7.2. Residual steady-state substrate concentrations during growth of *E. coli* ML 30 with three sugars, namely glucose (□), galactose (■) and fructose (●), mixed at different ratios in the feed medium (adapted from Egli, 2009).

However, it is questionable whether these established concepts do also apply for growth in the environment where not a single compound is limiting, but "mixed substrate growth" prevails (discussed above). Based on the Monod model and reported Monod constants, maximum specific growth rate (μ_{max}) and saturation constant (K_s), the low individual substrate concentrations measured in aquatic environments for a range of common organic carbon substrates predict very low growth rates on single carbon sources. However, the utilization of different compounds simultaneously allows a bacterium to feed both its energy-generating and biosynthetic pathways from various directions, which promotes relatively high growth rates also at low individual substrate concentrations; they can integrate the carbon fluxes from individual compounds. It was demonstrated that during growth with mixtures of sugars in chemostat culture at a particular growth rate residual substrate concentrations are lower compared to growth on corresponding single

substrates alone (Lendenmann *et al.,* 1996; Figure 7.2.). Furthermore, this principle was confirmed by reports on several strains of *Pseudomonas aeruginosa*, which grew relatively fast with specific growth rates ranging from 0.03 to 0.08 on a substrate mixture at 15 °C, where each compound was present at a concentration of only one µg C L^{-1} (van der Kooij, 1982). The concept of "mixed substrate growth" also explains the high growth rates observed in our experiments, which demonstrate that this phenomenon is indeed determining heterotrophic growth in the environment on natural AOC. Figure 7.3. shows a compilation of determined specific growth rates of *E. coli* O157 and aquatic communities at different AOC concentrations.

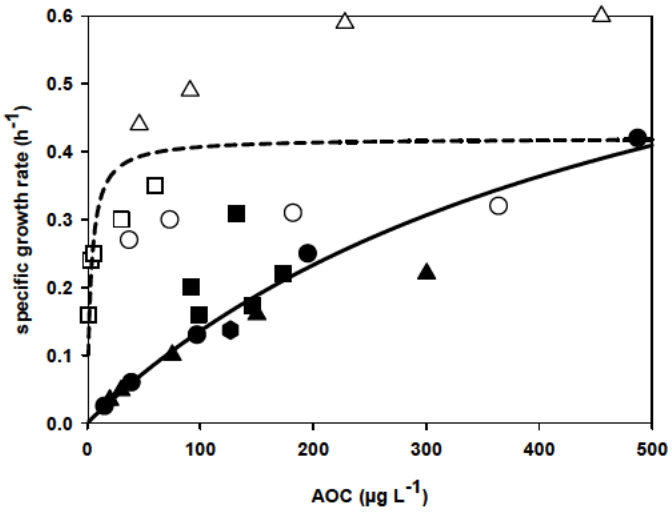

Figure 7.3. Specific growth rates of *E. coli* O157 (black) and aquatic bacterial communities (white) in relation to the AOC concentration. Diluted LB medium (▲, Δ; chapter 4), diluted wastewater (●, ○; chapter 6) and river water (■, chapter 2; □; data from Hammes & Egli, 2005). The lines represent the modelled results based on Monod kinetics. The values are adjusted to the amount of available AOC for the individual cultures.

The two populations cluster in distinct regions showing a classical "opportunist" versus "gleaner" relationship (Grover, 1990). *E. coli* O157 is here "the opportunist", adapted to high nutrient concentrations (μ_{max} = 0.83 h^{-1}; K_s = 510 µg available AOC L^{-1}), and the autochthonous bacterial community resemble "the gleaner", characterized by high specific growth rates also under nutrient-poor conditions (μ_{max} = 0.42 h^{-1}; K_s = 3.2 µg available AOC L^{-1}). Such a kinetic relationship between enteric and autochthonous bacteria has been suggested long ago (e.g., Jannasch, 1968) but has not been experimentally verified so far. The physiological properties behind this relationship are still unknown. Low K_s values (< 10 µg C L^{-1}) were also reported for bacterial strains isolated from drinking water growing on "single" carbon compounds (van der Kooij, 2002; Magic-Knezev & van der Kooij, 2006). It was proposed that autochthonous bacteria display high affinity uptake mechanisms enabling their high growth rates (Button, 1998), but the underlying molecular mechanisms have not been explored in the laboratory yet, mainly due to the difficulties to culture representatives at high enough cell densities allowing physiological investigations. In chapter 4 it was demonstrated that the kinetic properties of an "environmental pathogen", namely *P.*

aeruginosa, were distinctly different from those of enteric pathogens during growth at low nutrient concentrations. This might be the explanation for the environmental fitness and frequent detection of this organism in water. However, also for *E. coli* the expression of high affinity uptake systems and selection for low K_s-mutants during long-term cultivation on single carbon sources are described, which considerably improve their kinetic properties (Wick *et al.,* 2001). In this context, it would be interesting to investigate adaptation of *E. coli* during long term cultivation on natural water. However, natural waters contain mixtures of substrates, which considerably reduce the selection pressure for improving the uptake kinetics of a single compound and it is questionable whether in the environment similar adaptation mechanisms occur as during growth with a single sugar (Franchini, 2006).

It should be pointed out that at given AOC concentrations, the derived specific growth rates were dependent on the type of water, which indicates the influence of AOC composition on kinetics (Figure 7.3.). Variations were more pronounced for the aquatic bacterial communities than for *E. coli* O157. Since in the experiments both the bacterial communities and the water used for cultivation, differed it is not possible to track the discrepancies on either growth properties or AOC composition. Nonetheless, this implies that kinetic values from literature have to be applied with extreme caution since every water contains an individual AOC composition and specific autochthonous bacteria, which are characterized by specific kinetic properties.

Mathematical prediction of growth

Mathematical modelling is increasingly used to describe bacterial growth in divers ecosystems. However, adequate modeling requires a basic understanding of factors controlling bacterial proliferation in a given environment. Ideally, *in silico* predictions are based on measured parameters and can be verified in the laboratory; simulations can then be used to visualize the dynamics underlying the experimental results. In addition, modeling can be a helpful tool to enable exploring mechanisms governing bacterial growth and competition, which are not or very difficult to investigate in the laboratory (e.g., Huisman & Weissing, 2001). It should be pointed out that such *in silico* results should not be taken as absolute facts but rather as suggestions for leading experimentation into a direction that allows investigating the proposed mechanisms.

As discussed previously, in the environment microbes do not grow on a single growth-limiting substrate but with a mixture of compounds. Hence, the Monod equation based on a single substrate should not be applied. The data of Lendenmann and coworkers (1996) suggest that during growth

with a mixture of sugars the total substrate concentration controls the growth rate in a similar manner as if cells grew on a single carbon source alone. In chemostats, the individual residual substrate concentrations were explained by their corresponding percentage of the total carbon substrates in the feed mixture (Figure 7.2.; Lendenmann *et al.,* 1996). This implies that a sum parameter such as the available AOC may be used to describe the kinetics of individual species in a given carbon-limited environment. However, other experiments under similar defined conditions indicated that this is not always the case since affinities for individual substrates can differ considerably, which introduces additional aspects (Kovarova-Kovar & Egli, 1998). Nevertheless, the results of chapter 4 and 5 demonstrate that it was possible to combine AOC with the Monod equation for describing bacterial growth and competition even for mixed bacterial communities. In this respect, it is important to consider AOC, i.e. the substrates readily available for growth, as the growth-limiting factor and not the total dissolved organic carbon (DOC) or biodegradable DOC (BDOC) as often suggested (e.g., Dukan *et al.,* 1996; Thingstad & Lignell, 1997).

The continuous culture experiments (chapter 5) indicate an additional level of complexity during growth in the environment on heterogenic AOC substrates. The spectrum of accessible compounds depends on the dilution rate, with fast-growing cells use fewer substrates simultaneously than slow-growing ones (Sepers, 1984; Egli, 1986). This implies that the substrate composition of the residual AOC in chemostat cultures is distinct to that from the feed medium, which could considerable influence competition dynamics, especially when competitors differ within this physiological/nutritional behavior. The mechanisms for growth on AOC are, hence, not yet fully understood and ask for additional investigations, especially considering balances and concentrations of individual substrates during growth. At the moment, such experiments are restricted by analytical limitations. Maybe recent developments for meta-analysis of individual substrates at low concentrations will enable new insights into this area and might allow the discovery of additional mechanisms governing growth of bacteria under complex nutritional conditions (Frazier *et al.,* 2005). Nevertheless, modeling can already be a useful tool in order to investigate growth and competition dynamics and to propose different scenarios (see below).

Considering pathogen growth within microbial risk assessment

The virtual absence of consideration of pathogen growth within microbial risk assessment is mainly based on the lack of knowledge on this topic (OECD/WHO 2003). The common view suggests that pathogen growth poses no risk because many, especially the classic enteric pathogens, are simply assumed to be unable to grow in water at low nutrient concentrations. As mentioned above the

current water hygiene concept does only consider die-off of pathogens in the environment as relevant. However, next to the results of this thesis there are a number of recent examples where growth of both environmental and classic enteric pathogens as well as proliferation of index organisms was demonstrated *in situ* (e.g., Torvinen *et al.,* 2005; Ishii *et al.,* 2006; Juhna *et al.,* 2007; Kirschner *et al.,* 2008), indicating that this topic requires more attention. This thesis give new insights into the factors governing growth of pathogenic bacteria in water and the associated dynamics and could be a first step for including pathogen growth into microbial risk assessment. Figure 7.4. gives a suggestion on how this might be approached. Two main levels are identified, which are considered of specific importance, namely (1) whether the water quality allows for growth, and (2) how pathogen growth is affected by competition with other autochthonous bacteria.

The first level – water quality allows for growth?

The three main parameters that have to be considered at the first level of assessing the risk of pathogen growth in water (Figure 7.4.) are (i) the presence of disinfection residuals and (ii) water temperature, parameters routinely measured during drinking water monitoring, as well as (iii) the availability of nutrients; the latter parameter can be analyzed with the pathogen growth potential assay presented in chapter 6 (Figure 7.4.). If any of these parameters does not allow proliferation of a particular pathogen there is no risk for its growth. Hence, this first level yields a "plus" or "minus" and is based on parameters that are easily quantifiable. It can be considered as readily applicable and could provide a first step within microbial risk assessment. However, it has to be mentioned that specific micro-environments (e.g., in biofilms or inside protozoa) may support growth of a particular pathogen, a fact that is not included in this approach (Steinert *et al.,* 2002; Torvinen *et al.,* 2004).

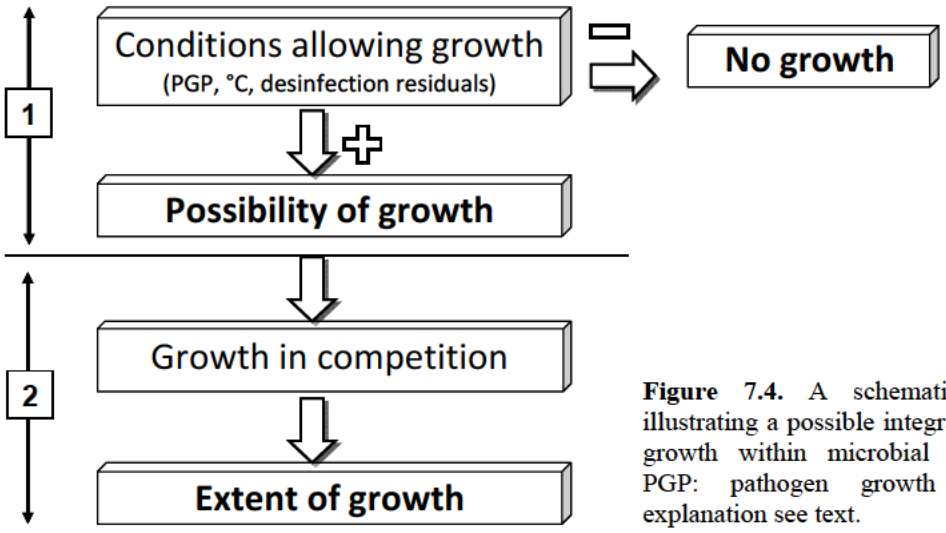

Figure 7.4. A schematic representation illustrating a possible integration of pathogen growth within microbial risk assessment. PGP: pathogen growth potential. For explanation see text.

The second level – competition with the autochthonous flora

The second level considers competition of a pathogen with other autochthonous bacteria for AOC. It is critical to elucidate the factors involved in competition processes to estimate the extent of pathogen growth. As previously outlined in chapter 5, only few studies exist so far, which are investigating this topic under "natural" nutritional conditions, i.e. bacteria are competing for a mixture of substrates at low concentrations. Clearly, this subject is complex and we are just starting to touch the underlying mechanisms. The second level in Figure 7.4. is, thus, not as well-defined as the first one, but contains considerable uncertainty. In chapter 5, the influence of three factors on the outcome of competition was investigated. It was demonstrated that increased AOC concentrations as well as rising temperatures are positively affecting the competitive fitness of *E. coli* O157. In addition, it was shown that the ratio between competitors is crucial; the more autochthonous bacteria being present and acting as "placeholders" the more difficult it is for *E. coli* O157 to scavenge nutrients and grow. In this respect, it should be mentioned (again) that not heterotrophic plate counts, but cultivation-independent methods have to be used in order to accurately quantify viable autochthonous cells (Berney *et al.,* 2008). Based on the three parameters investigated, which are all easily measureable *in situ*, it is possible to start identifying possible "hotspots" of pathogen growth within drinking water treatment and distribution. For example, endpoint disinfection without sustaining disinfection residuals (e.g., Juhna *et al.,* 2007) promotes uncontrolled bacterial re-growth in the distribution system and a risk for pathogen growth, especially at high nutrient concentrations and elevated temperatures. Hence, harsh disturbances within the autochthonous microbial community such as disinfection should be always followed by a treatment step where re-growth occurs in a controlled manner such as slow sand filtration, which then stabilizes the system again (Hammes *et al.,* 2010).

It should be pointed out that the parameters studied here are only a small selection of possible factors influencing competition and this topic requires further investigation. In this thesis it was demonstrated that determination of certain bacterial growth properties in pure culture enables prediction of the dynamics involved during competitive growth. Hence, investigations on growth parameters of bacterial cultures are valuable and can help to simplify the complexity. In this respect, mathematical modeling can provide a first step for theoretically exploring competition dynamics under different conditions in order to help designing specific competition experiments.

Outlook - competition experiments: some selected theoretical examples

The *in silico* scenarios on bacterial competition presented in Figures 7.5, 7.6 and 7.7 are based on the model developed in chapter 5 where competitive growth of *E. coli* O157 with a bacterial community derived from drinking water (for equations see Box 5.1). *E. coli* showed "opportunistic" growth kinetics (μ_{max} = 0.87 h^{-1}; K_s = 486.5 µg AOC L^{-1}), whereas the bacterial community was characterized as a "gleaner" (μ_{max} = 0.33 h^{-1}; K_s = 7.4 µg AOC L^{-1}). Furthermore only 53% of the AOC available for the community supported growth of the pathogen as well. In addition its yield, i.e. cells formed per consumed µg of AOC, was only around half of that of the bacterial community. The model is based on the Monod equation, but the single substrate, s, was replaced by the AOC available for a culture. Furthermore, residual steady-state concentrations were fitted and are not based on Monod. In this chapter, all alterations from the original model are based on the Monod equation.

Not everything is available for everybody

As outlined in chapters 4, 5 and 6 not every bacterial strain has access to the total AOC, but can only metabolize a specific fraction thereof. This implies that strains that can consume more different substrates have an advantage over those that have a more restricted spectrum since they can grow faster at a given AOC concentration (Egli, 1995; Lendenman *et al.,* 1996; Figure 7.3.). In this respect, it was demonstrated here that enteric pathogens, specifically *E. coli* O157, have a dual disadvantage because they have a lower substrate affinity and can only consume a fraction of the AOC available for autochthonous communities. *In silico* scenarios of the influence of AOC availability of *E. coli* O157 growing in competition with a bacterial drinking water community in continuous culture are shown in Figure 7.5. In the model the total AOC pool was accessed by the community, whereas the percentage of AOC available for *E. coli* O157 was changed to (1) 53 % (original data from chapter 5), (2) 10 % and (3) 100 %. At scenario 3 (10 % availability), the washout of the pathogen is close to the theoretical washout curve (μ = 0 h^{-1}) and, thus, only little growth of *E. coli* O157 would occur. However, if all AOC (100 %) was available for *E. coli* O157, growth of the bacterium would be considerably better and only a slow washout was predicted in this scenario. This implies that the bacterial community would need much more time to fill the complete nutritional niche. Hence, the results suggest that the relative AOC availability of a pathogen is a key factor to be considered during competition.

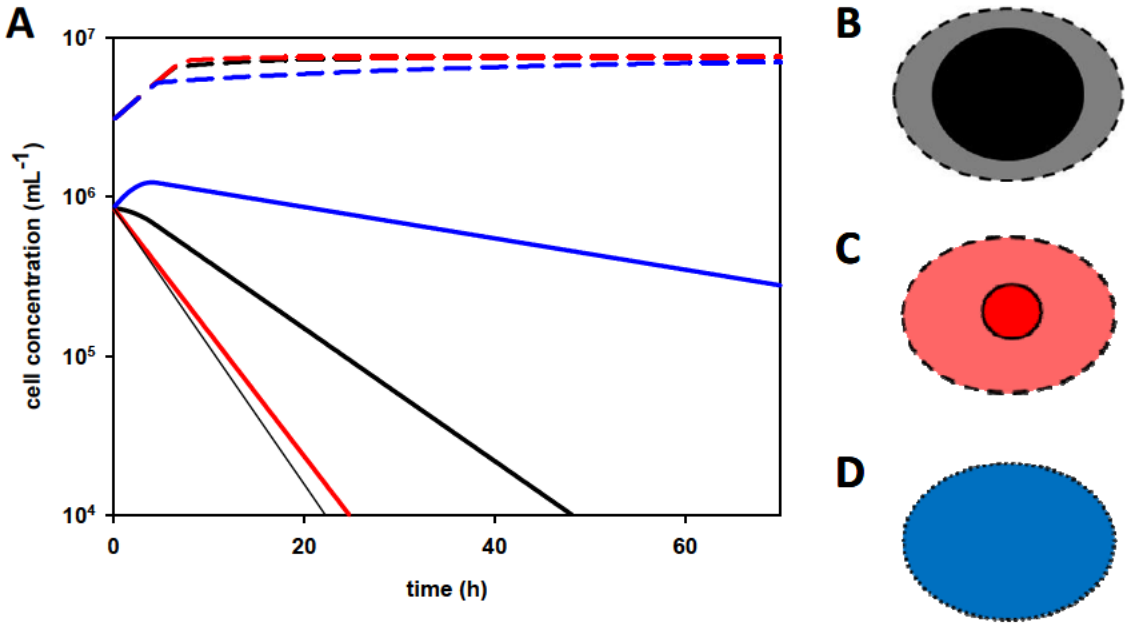

Figure 7.5. *In silico* scenarios (A) on the effect of AOC availability of *E. coli* O157 (solid lines) during competition with an aquatic bacterial community (dashed lines) in continuous culture (D = 0.2 h^{-1}; s_{in} = 820 µg AOC L^{-1}) using the model presented in chapter 5. The AOC available for the community, i.e. 100 %, was kept constant, whereas the fraction accessible for *E. coli* O157 was varied. Black lines: **original scenario from chapter 5, where 53 % of the AOC was available for *E. coli* O157;** red lines: **10 % availability** and blue lines: **100 % availability**. Schematic representations of the different scenarios are given in Panel B – D, where the light colored areas (dashed lines) represent the AOC pool of the community and the dark colored parts (solid lines) illustrate the substrate spectrum available for *E. coli* O157. At 100 % availability (D) the AOC pools are identical for both cultures. Thin solid line: theoretical washout curve (µ = 0 h^{-1}). The model is based on Monod kinetics presented in chapter 5 (*E. coli* O157: μ_{max} = 0.87 h^{-1}; K_s = 486.5 µg available AOC L^{-1} and bacterial community: μ_{max} = 0.33 h^{-1}; K_s = 7.4 µg available AOC L^{-1}) with adjustments to mixed substrate growth conditions - for detailed description see text (7.7 and chapter 5).

Substrate overlaps

In the scenarios presented in Figure 7.5., the AOC pool of *E. coli* O157 was considered to fully overlap with the one of the bacterial community. However, theoretically it is possible that the substrate spectra of the pathogen and the community do overlap only partly. "Mixed substrate growth" implies that bacteria growing in the environment are flexible on their substrate supply (Ihssen & Egli, 2005). Hence, if at a given dilution rate in chemostat culture some of residual substrate concentrations of *E. coli* O157 are decreased by competition due to a higher affinity of the community to those substrates, the pathogen could elevate its total residual substrate pool by increasing other parts of its residual AOC spectrum. Thus, the organism could compensate competition pressures by increasing the residual concentration of substrates, which are not available for the community, above the corresponding pure culture growth concentrations thereby preventing its washout. Figure 7.6. displays three theoretical scenarios, where substrate spectra of *E. coli* O157 and the aquatic bacterial community were set as (1) fully overlapping (original data), (2) 75 % overlapping and (3) 50 % overlapping. It can be clearly seen that the fewer substrates shared, the

higher the growth of the pathogen. In these scenarios it would be even possible for *E. coli* O157 to coexist with the bacterial community although competing for a considerably fraction of available substrates.

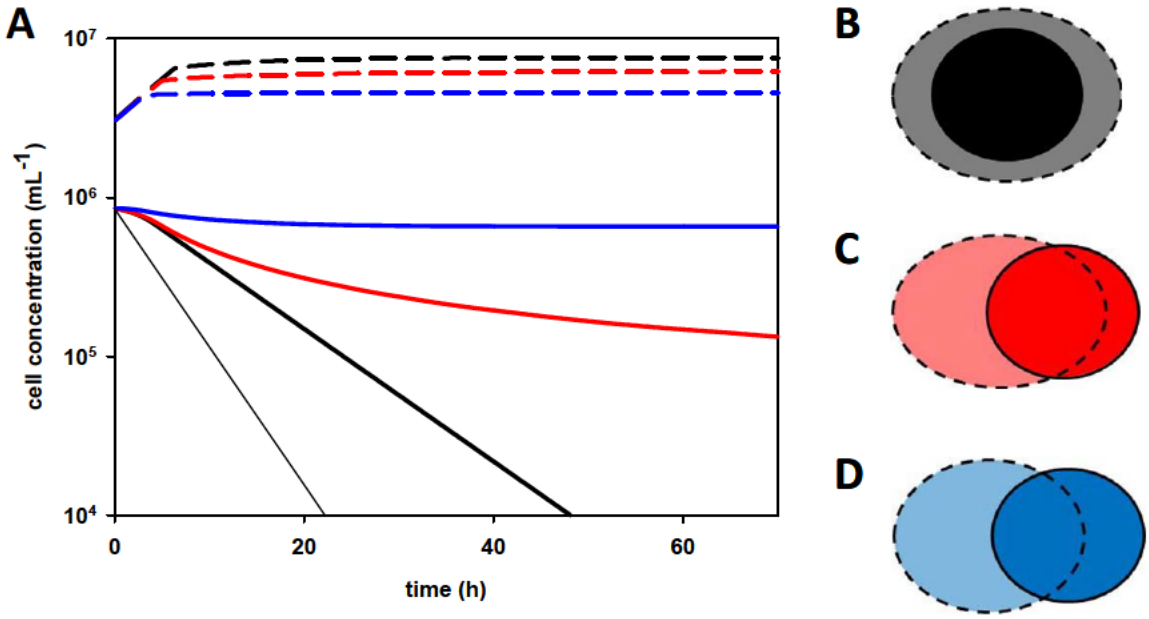

Figure 7.6. *In silico* scenarios (A) on the effect of substrate overlaps during competition of *E. coli* O157 (solid lines) with an aquatic bacterial community (dashed lines) in continuous culture (D = 0.2 h^{-1}; s_{in} = 820 µg AOC L^{-1}) using the model presented in chapter 5. The black lines represent the original measured data, where AOC pools of the two cultures **overlap to 100 %;** all AOC available for the pathogen was also available for the bacterial community. The red lines represent the predicted outcome if only 75 % of substrates consumable by *E. coli* O157 would also be available for the bacterial community **(75 % overlap)**. The theoretical result of a corresponding **50 % overlap** is shown by the blue lines. The total AOC concentration was kept constant. Schematic representations of the different scenarios are given in Panel B – D, where the light colored areas (dashed lines) represent the AOC pool of the community and the dark colored parts (solid lines) illustrate the substrate spectrum available for *E. coli* O157. Thin solid line: theoretical washout curve (µ = 0 h^{-1}). The model is based on Monod kinetics presented in chapter 5 (*E. coli* O157: μ_{max} = 0.87 h^{-1}; K_s = 486.5 µg available AOC L^{-1} and bacterial community: μ_{max} = 0.33 h^{-1}; K_s = 7.4 µg available AOC L^{-1}) with adjustments to mixed substrate growth conditions - for detailed description see text (7.7 and chapter 5).

Hence, if certain substrates are only available for *E. coli* O157, there is a considerable risk of growth even in the presence of autochthonous bacteria. Thus, the ability to cover the whole range of substrates available for pathogens can be considered as an important community function during drinking water treatment and distribution in order to limit growth of these unwanted bacteria. In stable systems, total substrate overlaps are most probably the normal case because autochthonous communities are well adapted to the present nutrients. However, disturbing events such as disinfection or selective grazing pressure could severely alter the community structure (Juhna *et al.,* 2007; Wang *et al.,* 2008) and in this way open a potential substrate niche for pathogen growth.

Nutrient fluxes

In natural waters, nutrient concentrations are often not constant over time but vary considerably. The impact of variation on competition outcomes has been investigated extensively, because it can lead to coexistence of different species even under pure and simple competition conditions, where all competitors compete for the same limiting substrate (Grover, 1990). Figure 7.7. shows three theoretical scenarios characterized by different nutrient supply rates, namely (1) continuous feeding (original data), (2) a pulse of 6 mg AOC L^{-1} every 24 h and (3) 2 mg AOC L^{-1} pulses very 7h. Scenarios (2) and (3) suggest a possible coexistence of the pathogen with the bacterial community. However, these examples do not represent typical scenarios as found in the environment but rather illustrate the dynamics of competition due to a variable substrate supply.

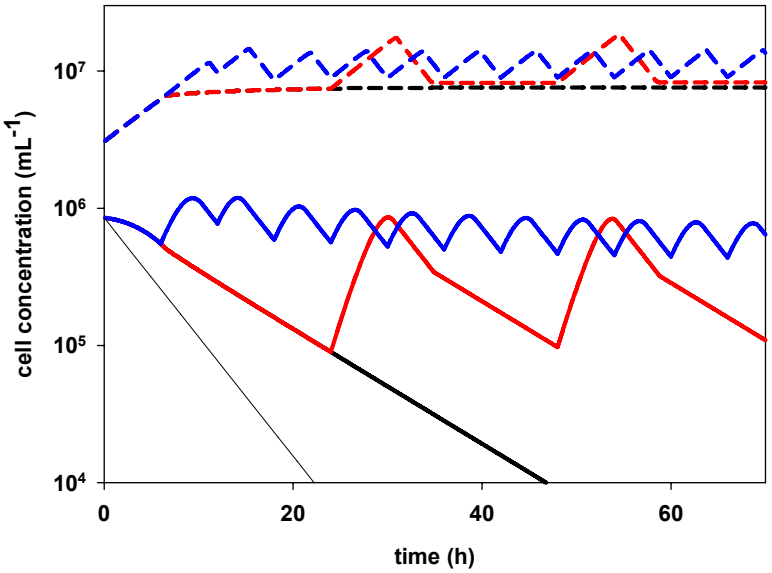

Figure 7.7. *In silico* scenarios on the effect of fluctuating substrate supplies during competition of *E. coli* O157 (solid lines) with an aquatic bacterial community (dashed lines) in continuous culture (D = 0.2 h^{-1}; s_{in} = 820 µg AOC L^{-1}) using the model presented in chapter 5. The black lines represent the original measured data, where a **continuous substrate supply** was applied. The red lines represent the predicted outcome for **additional pulses of 6 mg AOC L^{-1} every 24 h**, whereas the theoretical result for **additional pulses of 2 mg AOC L^{-1} every 6 h** is represented by the blue lines. Thin solid line: theoretical washout curve (µ = 0 h^{-1}). The model is based on Monod kinetics presented in chapter 5 (*E. coli* O157: μ_{max} = 0.87 h^{-1}; K_s = 486.5 µg available AOC L^{-1} and bacterial community: μ_{max} = 0.33 h^{-1}; K_s = 7.4 µg available AOC L^{-1}) with adjustments to mixed substrate growth conditions - for detailed description see text (7.7 and chapter 5).

The extent of AOC variations in water is quite distinct between ecosystems. Whereas stable man made-systems such as drinking water treatment plants display little variation within one treatment step (Hammes *et al.,* 2010), typical AOC substrates can vary considerably over the day in natural water systems such as surface waters. For example, dissolved free amino acids and carbohydrates can differ over several orders of magnitude during the day with maxima in the light and minima in the dark phase (Münster, 1993). Furthermore, it should be mentioned that only variations in the

concentration of AOC were considered, whereas in real systems the amount of AOC as well as its quality/composition will probably vary concurrently.

These selected examples demonstrate the complexities of bacterial competition in natural ecosystems and touch some of the many parameters involved. Furthermore, the individual scenarios discussed here should not be seen independent from each other, but they are interlinked and most likely simultaneously influencing the dynamics of bacterial competition. This demonstrates the present difficulties to predict competition outcome and, hence, to estimate the growth of single bacterial species such as pathogens in water. However, research should not shy away from such complexity but rather try to identify the most frequent scenarios and factors involved in order to understand the major dynamics. Thus, I hope that the results of this thesis encourage others to work on this topic and contribute to level 2 in Figure 7.4. for an improved microbial risk assessment.

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