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"The unseen majority": heterotrophic bacteria in freshwater, more than just small and non-cultivable

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Summary

The majority of bacterial members in the free-living microbial world is not accessible with conventional microbiological tools, such as plating. Members of this "unseen majority" are typically small in size and not easy or even impossible to cultivate. Knowledge on their growth properties is still largely inexistent, although lots of genetic information has been acquired recently using cultivation-independent methods. In this thesis, a novel cultivation approach, with natural freshwater as substrate and flow cytometry (FCM) as monitor tool, was applied to explore this "unseen majority".

It started with the investigation on filterable bacteria. Micro-filtration is a standard process for sterilization in scientific research, medical and industrial applications, and to remove particles in drinking water or wastewater treatment. It is generally assumed that filters with a 0.1 to 0.45 μm pore size can retain bacteria. In contrast to this assumption, we have regularly observed the passage of a significant fraction of natural freshwater bacterial communities through 0.45, 0.22 and 0.1 μm pore size filters. Here in this thesis, FCM enable us to show for the first time a systematic quantification of microbial filterability, especially their ability to pass through 0.1 μm pore size filters. The filtered bacteria were subsequently able to grow on natural assimilable organic carbon (AOC) with specific growth rates up to 0.47 h^{-1}. Bacterial communities that pass preferentially through all three pore size filters at significantly increased percentages were enriched using successive filtration-regrowth cycles on freshwater AOC. In all instances, the dominant microbial populations comprised slender spirillum-shaped *Hylemonella gracilis* strains, suggesting a shape-dependent selection during filtration.

To further study the factors that determine bacterial filterability, six different bacterial species of various sizes and shapes (*H. gracilis*, *Escherichia coli*, *Sphingopyxis alaskensis*, *Vibrio cholerae*, *Legionella pneumophila* and *Brevundimonas diminuta*) were tested for their filterability through sterile micropore filters. In all cases, the slender spirillum-shaped *H. gracilis* cells showed a superior ability to pass through sterile membrane filters. The results provide solid evidence that the overall shape (including flexibility), instead of biovolume, is the determining factor for the filterability of bacteria, while cultivation conditions also play a crucial role. Based on our findings, we recommend a re-evaluation of the grading system for

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sterile filters, and suggest that the species *Hylemonella* should be considered as an alternative filter-testing organism for the quality assessment of micropore filters.

The laboratory research on filterable bacteria was further scaled up to test the quantitative impact of industrial scale micro-filtration on native microbial communities. Two separate groundwater aquifers were tested. Up to 10% of the native microbial community in both aquifers was able to pass through the cartridge filtration units (0.22 μm pore size) installed at both aquifers. In addition, all samples (either with or without 0.22 μm filtration) showed significant growth after bottling and storage, increasing from initially $10^3 - 10^4$ cells/ml to an average final concentrations of $1 - 3 \times 10^5$ cells/ml. However, less growth was observed in carbon-free glassware than in standard PET bottles. Furthermore, our results showed that filtration and bottling can alter the microbial community patterns as observed with FCM. The selection of slender spirillum-shaped bacteria was again observed in one of the studied groundwater aquifers. This industrial scale study further confirmed our laboratory results, which suggested that the presently practiced micro-filtration cannot serve as an absolute barrier for the native microbial community. It also provided significant insight into the impact of filtration and bottling on microbial presence and growth in bottled water.

When investigating filterable bacteria with FCM, two distinctive clusters were observed in natural freshwater and drinking water microbial communities. The two clusters conform to the so-called high (HNA) and low (LNA) nucleic acid content bacteria according to their cell size and fluorescence intensity. To date, most reported studies focused on the relative abundance and *in situ* activities of these two microbial clusters. However, knowledge of their physiological properties does not exist. In this thesis, HNA and LNA bacteria were separated by micro-filtration and their growth properties were investigated. Three LNA bacterial isolates were enriched from different freshwater sources using extinction dilution and fluorescence-activated cell sorting. It is demonstrated here that LNA bacteria can be cultivated and are able to utilize natural AOC at different temperatures, ranging from 12 to 30°C. During growth, the main FCM parameters (i.e., fluorescence intensity and sideward scatter) remain distinct from those of typical HNA bacteria. The three LNA isolates were closely related to the *Polynucleobacter* cluster according to 16S rRNA sequencing results. Furthermore, morphology of the isolates was characterized in detail using electron microscopy and an extremely small cell volume (0.05 μm³) was observed for all the three

isolates. It is, to our knowledge, the first time that pure cultures of LNA bacteria have been isolated and characterized.

The growth properties of one LNA bacterium (isolate CB) were further compared with that of *S. alaskensis* (the model "oligotrophic ultramicrobacterium") in low-nutrient environments. Three cultivation media with different carbon quantities and qualities were used for the investigation. In general, the two bacteria displayed quite different growth properties. Isolate CB exhibited a higher maximum specific growth rate and achieved a higher final cell concentration in natural freshwater than in synthetic laboratory medium at a similar carbon concentration. In comparison, *S. alaskensis* reached a higher growth rate and final cell concentration in high nutrient synthetic medium. The cell volume of *S. alaskensis* increased, peaked and decreased during the course of batch cultivation, while such changes were hardly detectable for the isolate CB. Moreover, it was demonstrated how growth rate and final cell number of the two bacteria responded to different media carbon concentration and incubation temperatures. The difference between the growth properties of the environmental isolate and the laboratory model strain suggested that strains adapted to laboratory media and environment may exhibit a significantly different behaviour compared to strains growing in a natural environment.

The "unseen majority" of the microbial world is always referred to by various terms like "oligotrophs", "ultramicrobacteria", "uncultivable cells", etc. The three isolates reported in this thesis have been demonstrated to be both representatives of LNA bacteria, possessing typical "oligotrophic" growth characteristics, and to be very small bacteria ($0.05 \ \mu m^3$) with a cell size that falls into the category of "ultramicrobacteria/nanobacteria". Hence, I think that although the puzzle of "unseen majority" was approached by researchers in different ways, each of them influenced by his/her field, it appears that we are dealing with similar microorganisms but separated previously by different terminology. The cultivation approach employed and solid data reported in this thesis may shed light into the future direction of research on "unseen majority" of the microbial world.

Zusammenfassung

Der Grossteil der frei lebenden mikrobiellen Flora ist nicht zugänglich mit konventionellen mikrobiellen Methoden wie z.B. Plattierung. Diese „unsichtbare Mehrheit" besteht typischerweise aus sehr kleinen Zellen, die nicht einfach oder sogar unmöglich zu kultivieren sind. Kenntnisse über ihr Wachstumsverhalten sind grösstenteils noch nicht vorhanden, obwohl durch kultivierungsunabhängige Methoden in den letzten Jahren genetische Information über diese Mikroorganismen gesammelt wurde. Um diese „unsichtbare Mehrheit" zu erforschen, wurde in dieser Doktorarbeit ein neuer Kultivierungsansatz mit natürlichem Süsswasser als Substrat und Durchflusszytometrie (FCM) als Messinstrument angewandt.

Es begann mit der Untersuchung von filtrierbaren Bakterien. Mikrofiltration ist ein Standardprozess für die Sterilisation von Flüssigkeiten in wissenschaftlicher Forschung, medizinischer und industrieller Anwendung, sowie für die Entfernung von Partikeln bei der Trinkwasseraufbereitung oder der Abwasserbehandlung. Es wird allgemein angenommen, dass Filter von 0.1 bis 0.45 µm Porengrösse Bakterien zurückhalten können. Im Gegensatz zu dieser Annahme haben wir regelmässig beobachten können, dass eine signifikante Fraktion der natürlichen bakteriellen Süsswassergemeinschaft Filter mit 0.45, 0.22 und 0.1 µm Porengrösse passieren konnte. In dieser Arbeit zeigen wir zum ersten Mal eine systematische Quantifizierung der Filtrierbarkeit natürlicher bakterieller Flora, besonders ihrer Fähigkeit 0.1 µm Porenfilter zu passieren. Die passierenden Bakterien konnten anschliessend mit spezifischen Wachstumsraten bis zu 0.47 h^{-1} auf assimilierbarem organischem Kohlenstoff wachsen. Wir konnten bakterielle Gemeinschaften, die bevorzugt Filter aller drei Porengrössen passierten anreichern. Hierzu wurden aufeinanderfolgende Filtrations- und Wachstumszyklen eingesetzt. Der Prozentanteil filtrierbarer Bakterien stieg mit jedem Filtrationszyklus signifikant. In allen Fällen bestand die dominante mikrobielle Population aus dem spirillenförmigen Stamm *Hylemonella gracilis,* was darauf hinweist, dass eine formabhängige Selektion während des Filtrationsprozesses stattfand.

Um die Faktoren weiter zu untersuchen, die die Filtrierbarkeit der Bakterien bestimmen, wurden sechs unterschiedlichen Bakterienarten unterschiedlicher Grösse und Form (*H. gracilis, Escherichia coli, Sphingopyxis alaskensis, Vibrio cholerae, Legionella pneumophila*

und Brevundimonas diminuta) auf ihre Filtrierbarkeit durch kommerziell erhältliche sterile Mikroporenfilter gestestet. In allen Fällen zeigten die schlanken spirillenförmigen *H. gracilis*-Zellen die beste Fähigkeit sterile Membranfilter zu passieren. Unsere Ergebnisse liefern den soliden Beweis, dass überwiegend die Zellform (eingeschlossen der Flexibilität) und nicht das Biovolumen die Filtrierbarkeit der Bakterien bestimmt. Dabei spielen die Kultivierungsbedinungen ebenfalls eine ausschlaggebende Rolle. Nach unseren Erkenntnissen empfehlen wir eine Neubewertung des Einstufungssystems für Sterilfilter und schlagen die Art *Hylemonella* als alternativen Testorganismus für die Qualtitätsbewertung von Mikroporenfiltern vor.

Die Untersuchungen wurden anschliessend auf den quantitativen Einfluss industrieller Mikrofiltration von mikrobieller Flora in Süsswasser erweitert. Zwei verschiedene Grundwasserquellen wurden getestet. Bis zu 10% der mikrobiellen Flora beider Grundwasser passierten Filterpatronen (0.22 µm Porengrösse), die an beiden Grundwasserquellen installiert wurden. Ausserdem zeigten alle Proben (entweder mit oder ohne 0.22 µm Filtration) ein signifikantes Wachstum nach Flaschenabfüllung und Lagerung. Dabei erreichten sie, ausgehend von 10^3-10^4 Zellen/ml, durchschnittliche Endkonzentrationen von 1-3 × 10^5 Zellen/ml. Generell wurde weniger Wachstum in kohlenstofffreier Glasware als in Standard - PET - Flaschen beobachtet. Weiterhin zeigen unserer Ergebnisse, dass Filtration und Abfüllung die Zusammensetzung der mikrobiellen Flora verändern können, was wir mit FCM beobachten konnten. Auch hier wurde die Selektion des schmalen, spirillenförmigen Bakteriums aus einem der Grundwasser beobachtet. Diese industrielle Studie bestätigte weitgehend die Resultate der vorangegangenen Laborarbeit d.h., dass Mikrofiltration nicht als absolute Grenze für die mikrobielle Flora gilt. Sie gibt auch einen wichtigen Einblick auf die Auswirkung von Filtration und Flaschenabfüllung auf die Konzentration von Mikroorganismen in flaschenabgefüllten Trinkwasser.

Während der Erforschung von filtrierbaren Bakterien konnten mit FCM zwei unterschiedliche Gruppen von natürlichen mikrobiellen Süss- und Trinkwassergemeinschaften beobachtet werden. Diese beiden Gruppen können nach Anfärbung mit einem DNA bindenden Fluoreszenzfarbstoff aufgrund der Zellgrösse und Fluoreszenzintensität in Bakterien mit hohem (HNA) und niedrigem Nukleinsäureanteil (LNA) unterteilt werden. Die meisten bis jetzt veröffentlichen Studien legten Gewicht auf die relative Häufigkeit und in situ Aktivitäten dieser beiden Gruppen. Das Wissen über ihre physiologischen Eigenschaften ist jedoch sehr

beschränkt. In dieser Arbeit wurden HNA- und LNA- Bakterien durch Mikrofiltration getrennt und anschliessend ihre Wachstumseigenschaften untersucht. Aus unterschiedlichen Süsswassern wurden drei LNA- Bakterien angereichert und isoliert, wobei die Technik zur Verdünnung bis zur Extinktion und Zellsortierung durch Fluoreszenzanregung eingesetzt wurden. Wir zeigten, dass LNA- Bakterien kultiviert werden können und fähig sind natürlichen AOC bei unterschiedlichen Temperaturen im Bereich von 12-30°C für das Wachstum zu verwenden. Während des Wachstums blieben die wichtigsten FCM-Parameter (d.h. Fluoreszenzintensität und Seitwärtsstreuung) deutlich unterschiedlich von denen der typischen HNA- Bakterien. Die drei LNA- Isolate waren aufgrund ihrer 16S rRNA Sequenz nah verwandt zur Gruppe *Polynucleobacter*. Weiterhin wurde die Morphologie der Isolate detailliert mit Elektronenmikroskopie charakterisiert; alle drei Isolate wiesen extrem kleine Zellvolumina (0.05 µm^3) auf.

Die Eigenschaften eines LNA- Bakteriums (Isolat CB) wurden dann mit *S. alaskensis* (der Stamm gilt als Modellorganismus des Typs „oligotrophes Ultramikrobakterium") in Nährmedien mit niedrigen Nährstoffkonzentrationen verglichen. Drei Kultivierungsmedien mit unterschiedlicher Kohlenstoffmenge und Qualität wurden für diese Untersuchungen verwendet. Die beiden Bakterien zeigten recht unterschiedliche Wachstumseigenschaften, wobei Isolat CB ein höheres μ_{max} und höhere Endzellkonzentrationen in natürlichem Süsswasser als in synthetischem Labormedium bei vergleichbaren Kohlenstoffkonzentrationen erreichte. Das Zellvolumen von *S. alaskensis* nahm in der exponentiellen Wachstumsphase zu, erreichte ein Maximum, und nahm nach Eintreten in die stationäre Phase gegen Ende der Batchkultivierung wieder ab. Solche Veränderungen wurden bei dem Isolat CB nicht detektiert. Zudem wurde untersucht, wie Wachstumsrate und Endzellkonzentration durch unterschiedliche Kohlenstoffkonzentrationen und Inkubationstemperaturen beeinflusst werden. Der Unterschied zwischen den Wachstumseigenschaften des Umweltisolats und des Modellorganismus lassen vermuten, dass Stämme die sich an die Laborumwelt angepasst haben, möglicherweise nicht mehr das ursprüngliche Verhalten in ihrer natürlichen Umgebung aufweisen.

Die „unsichtbare Mehrheit" der mikrobiellen Welt bezieht sich immer auf verschiedene Begriffe wie „Oligotrophie", „Ultramikrobakterien", „nicht kultivierbare Zellen", etc. Die drei Isolate, die in dieser These beschrieben wurden, zeigten sowohl typische Eigenschaften von LNA- Bakterien mit typischen „oligotrophen" Wachstumseigenschaften, als auch mit sehr

kleiner Zellgrösse (0.05 µm^3). Auf Grund dieser Eigenschaften würden sie in den Bereich von „Ultramikrobakterien/Nanobakterien" fallen. Forscher haben bisher mit unterschiedlichen Ansätzen, innerhalb ihres Forschungsgebiets, versucht das Puzzle der „unsichtbaren mikrobiellen Welt" zu enträtseln. Daher denken wir, dass sich die verschiedenen jetzt propagierten Typen stark überschneiden, und dann die Terminologie wohl reduziert werden muss. Der Kultivierungsansatz der angewandt wurde und die soliden Daten dieser Arbeit zeigen wohl eine zukünftige Forschungsrichtung in die Welt der mikrobiellen „ unsichtbaren Mehrheit".

1. General introduction

The majority of the members in the microbial world is known as minute in size and not easy or even impossible to cultivate. In this work, unique features of free-living heterotrophic bacteria from freshwater environments were investigated such as cultivability, size relationships, taxonomic position, and filterability using a variety of tools including for example flow cytometry, denatured gradient gel electrophoresis and electron microscopy. For an introduction to the subject, literature available on this issue is reviewed and summarized.

The "unseen majority"

Prokaryotes are ubiquitous on earth. Although invisible to the naked eye, they are omnipresent and are very essential parts of ecosystems on earth. It has been estimated that the number of prokaryotes and the total cellular carbon on earth are 4-6 $\times$ 10^{30} cells and 3-6 $\times10^{17}$ g carbon, respectively (Whitman et al., 1998). This estimation was based on the assumption that most prokaryotes reside in three large habitats including seawater, soil, and sediment/soil subsurface (Whitman et al., 1998). Since aquatic microbes are the interests of this thesis, the distribution of prokaryotes in different aquatic biospheres is analyzed and shown in Figure 1.1. The open sea has the biggest share due to its large volume. In comparison to prokaryotes, there are "only" 10^{18} insects alive on earth, which is believed to be the most abundant species of animal kingdom.

Not only do microbial cells dominate the earth in numbers, they also play essential roles in biogeochemical systems. The importance of microorganisms has been reviewed by Rosswall (1982), Wainwright and co-workers (1991) and Arrigo (2005) for different ecosystems. For instance, bacteria represent around 50% of the particulate organic carbon (Cho and Azam, 1990) and they are the major biomass component (Schut et al., 1997) in marine systems. Furthermore, it is believed that almost half of the phytoplankton primary production is consumed by bacteria (Billen et al., 1990), which demonstrates the important role of bacteria in the consumption of primary production and nutrient cycling in the marine environment. The microbial world is also highly diverse. It is estimated that the microbial world is consisted of at least 10^6 species (Curtis et al., 2002; Harwood and Buckley, 2008). From this

enormous diverse microbial world, only about 6,900 species have been isolated and even less has been described.
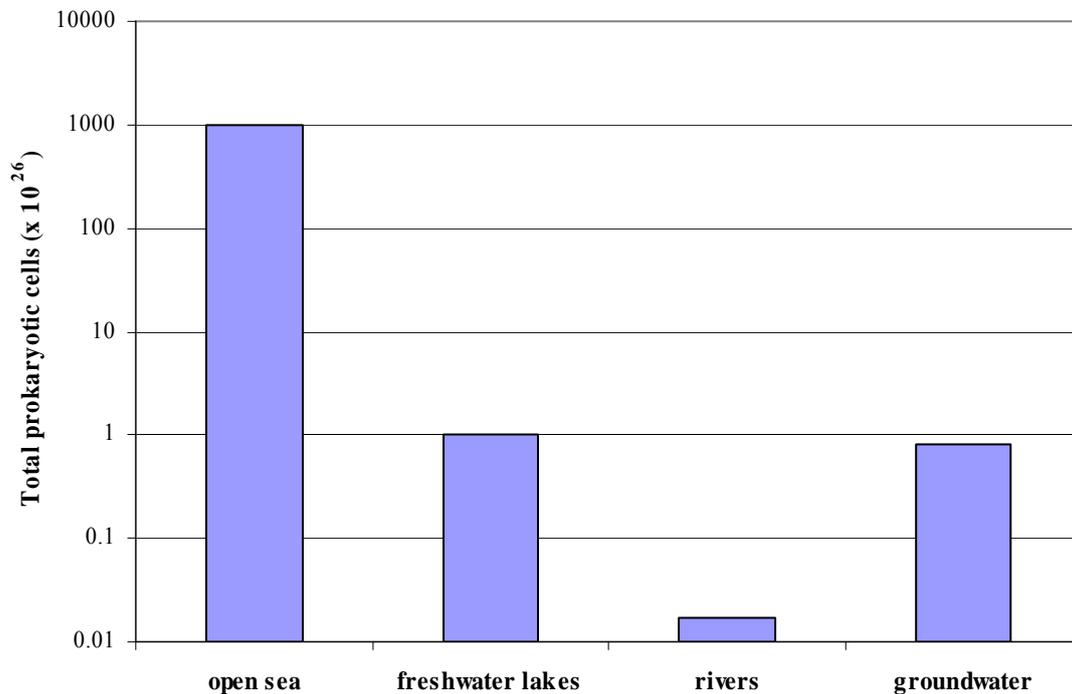
Figure 1.1 Prokaryotes abundance in aquatic environments. Figures calculated based on data from Gleick (1996) and Whitman et al. (1998).

In general, everything that wants to attract attention needs to been seen. Moreover, a unique appearance/behaviour helps for being easily recognized. This applies for the human society, for plants and animals, and also holds true for the microbial world. For long, microbiologists did focus on cultivable microorganisms, as they can be seen in various ways, e.g., colonies forming on plates, turbidity in test tubes. What has been neglected for a long time are the not readily "seen" part of the community: uncultivable microorganisms. The reason for the low cultivability can be attributed to at least two different aspects including physiological fundamentals and limiting detection methods, which are briefly introduced in the following sections.

Oligotrophic environments

The total amount of organic carbon present in aquatic ecosystems is approximately 3×10^{15} kg (Morita, 1993). However, when this organic carbon is distributed in the vast volume of aquatic bodies, the dissolved organic carbon (DOC) concentration in each system is extremely

low (Table 1.1). Also nutrient fluxes in aquatic environments are low. It has been reported that the flux of organic carbon in oligotrophic lakes does not exceed 0.1 mg C/l/day (Hood, 1970) and a similar flux was observed in ocean (Morita, 1993). The term "oligotrophic" was first introduced by Weber (1907) to describe the general low nutrient concentration in soil environments. Naumann (1919) adapted the concept and introduced the term into limnology (Morita, 1993). Recently, Cavicchioli and co-workers (2003) defined oligotrophic environments by two features: low nutrient flux (mg C/l/day; Poindexter, 1981) and low absolute nutrient concentrations (mg C/l; Morita, 1993). According to this definition, most natural aquatic ecosystems are oligotrophic. Especially the open sea, ground water and drinking water represent extremely oligotrophic environments. Despite the low level of nutrients in oligotrophic waters, microbial growth can be observed. It has been reported that planktonic microbial numbers persist in the magnitude of $10^4 — 10^6$ cells/ml in various aquatic environments (Table 1.1).

Table 1.1 Basic characterizations of aquatic environments, with respect to carbon and planktonic bacterial cell concentration.

	DOC (mg/l)	Cell concentration (cells/ml)	Cultivability (%)[a]	Reference
River	1-5	10^5-10^6	0.1-1	Amann et al., 1995; Whitman et al., 1998
Lake	1-6	10^5-10^6	0.1-1	Morita, 1993
Groundwater	0.1-1	10^3-10^4	0.001-0.01	Morita, 1993
Drinking water	0.7-1.4	10^4-10^5	0.001-2	Hammes et al., 2008; Siebel et al., 2008
Open sea	0.1-1	10^5-10^6	0.001-0.1	Yanagita et al., 1978

[a] cultivability is based on colony forming unit (CFU) measurements

Copiotrophs and Oligotrophs

<u>Definitions</u>

Bacteria living in such oligotrophic ecosystems (as discussed in the previous section) are often referred to as "dormant cells", "oligotrophic bacteria", "resting cells", "viable but non-culturable cells", or "starved cells" by different microbiologists (summarized in Morita, 1993; Table 1.2). In general, two groups of bacteria with different life-styles are recognized: copiotrophs (copio = plentiful) and oligotrophs (oligo = little/few). This division was derived from Winogradsky's classification of soil microflora (Schut et al., 1997). As copiotrophs form the majority of environmental isolates, they have been well studied and are often considered to represent the "normal" bacterial phenotype. In contrast, there is a confusing variety of definitions for oligotrophic bacteria, as can be expected for poorly studied organisms. Some researchers define oligotrophs as bacteria able to grow at nutrient concentrations of 5 mg C/l but not at concentrations of 7.5 g C/l (Yanagita et al., 1978). Other researchers define oligotrophs as those microbes that are able to grow on media with 1 to 15 mg C/l as well as on media with higher nutrient concentration (Kuznetsov et al., 1979). The latter type is also called facultative oligotrophs, as opposed to the former, obligate oligotrophs, that cannot grow at higher substrate concentrations in the range of g C/l. Furthermore, the obligate oligotroph was also defined as bacterium not capable of growth in 5 g/l and incapable of producing turbidity in medium containing 0.5 g trypticase/l (Ishida and Kadota, 1981). The various definitions used to characterize oligotrophic bacteria have been summarized by Schut and co-workers (1997). Hence there is little argument in the literature as how to define oligotrophic microbes. Boiling down all the suggestions and observations made by earlier researchers, the basic difference between obligate oligotroph, facultative oligotroph and copiotroph is illustrated in Figure 1.2. Microbiologists have neither agreed nor given experimental proof whether a true obligate oligotroph exists (Button et al., 1993; Morita, 1993; Eguchi et al., 1996). It was later argued that "obligate oligotrophy" best be understood as a transient characteristic only observed in cells that are taken directly from an extremely substrate-limited natural environment (Schut et al., 1997).
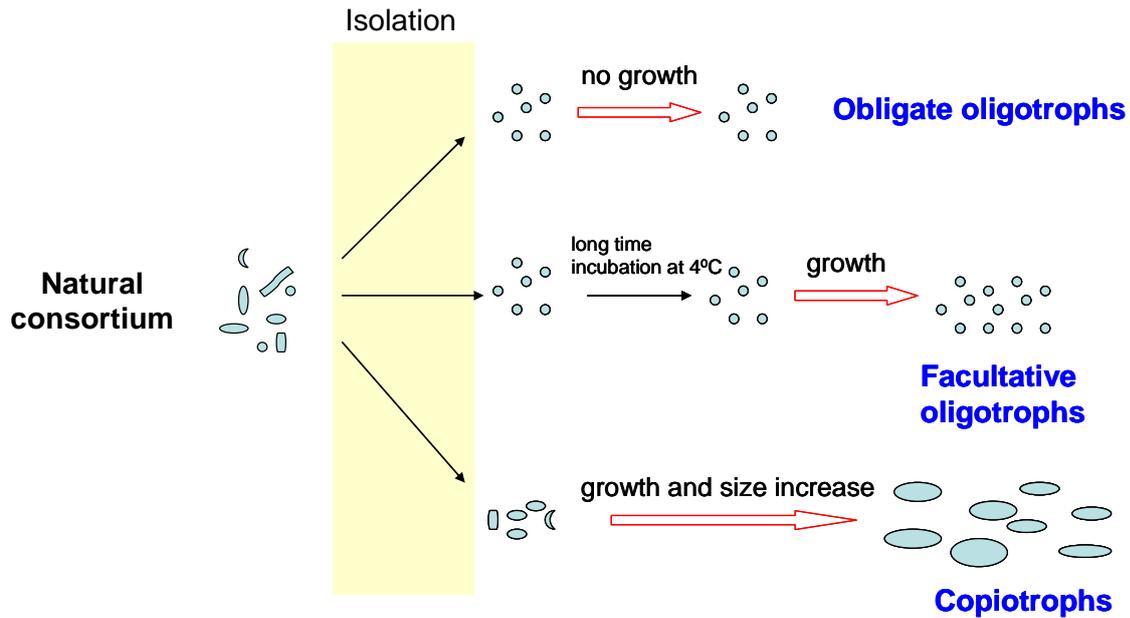
Figure 1.2 Schematic graph of the difference between "copiotrophs", "obligate oligotrophs" and "facultative oligotrophs". Red arrows represent exposure to laboratory conditions (high nutrient concentrations).

Up to now, there is no clear and consistent definition for so-called "oligotrophs". Various nutrient concentrations were used by different groups based on their understanding of the concept and their experimental condition used. Furthermore, the medium used for defining oligotrophic bacteria by researchers normally contain peptones, which has been criticized by Morita (1997) for its inability to support growth of all physiological types of bacteria in nature. If we consider the actual organic matter concentration in the environments (especially in aquatic environments) (Table 1.1), it is obvious that such definitions are arbitrary and clearly not based on the natural growth conditions bacteria encounter. In addition, one microbe can behave oligotrophic on one carbon source and copiotrophic on another (ZoBell and Grant, 1942; 1943). One needs to consider physical and chemical parameters for the growth of oligotrophic bacteria, to define the proper cultivation condition for them. Moreover, detection of microorganisms largely depends on the methods applied. Plating was often used in earlier studies to distinguish oliogotrophs from copiotrophs. However, no observable development of optical density or of colony formation is not equal to no-growth. It may just be unseen by our eyes. Hence, insensitive detection methods and improper cultivation media are the major obstacles for the study of bacteria living in nutrient-poor environments, or the

so-called "oligotrophs". In my judgement of the available literature, neither "oligotroph" nor "copiotroph" is fully suited for describing the physiological state and properties of microorganisms existing in the different natural compartments. There are probably groups of bacteria that are able to grow well and adapted easily to the laboratory media we designed, and hence can be studied well. The rest (actually the majority) is unknown and "labelled" for convenience as being "obligate oligotroph" or "oligotroph", which is largely the result of lacking proper cultivation and detection methods. To some extent, our attention is drawn too much to the "classification labelling" which distracts from the true nature of the bacteria we want to study. Often our inability to cultivate bacteria is taken as the inability of bacteria to growth.

Viable but non-cultivable (VBNC)

It has been observed that less than 1% of the bacterioplankton in aquatic samples can form a colony on agar surfaces of plates amended with commonly used growth media (Kogure et al., 1979; Roszak and Colwell, 1987; ZoBell, 1941). The cultivability of microbial cells in various aquatic environments was summarized in Table 1.1. In contrast to this extremely low cultivability, most bacterial cells in the environment (more than 50%) exhibit metabolic activity (Kogure et al., 1979; Rodriguez et al., 1992). Such cells without the ability to form colonies on agar media, are usually called "dormant" or "viable but non-cultivable (VBNC)" (Roszak and Colwell, 1987). Based on this definition, the obligate oligotrophs may seem to be, at least in part, equal the VBNC cells. However, the former emphasizes on the physiological nature of microbial cells while the latter describes a response of copiotrophs to certain conditions. The existence of VBNC bacterial cells was firstly reported by Dawe and Penrose (1978), who observed that the coliforms did not die off in seawater as reported before. Cells entering the VBNC state generally exhibit the characteristics of loss of plateability and a reduction in cell size (Linder and Oliver, 1989; Nilsson et al., 1991; Oliver et al., 1991). For example, *Vibrio* cells in stationary phase has a cell size of 1.5×0.7 μm, while those that have entered the VBNC state formed small cocci and had a diameter of only 0.8 - 1.0 μm and (Nilsson et al., 1991). Cells in the VBNC state are said to differ from starved cells in that the latter respond quickly to a change of environmental factors (e.g. nutrient availability) (Kjelleberg et al., 1987; Oliver, 1993) whereas the former are unable to do that immediately. A number of bacteria have been reported to enter the VBNC state under certain conditions (Amy and Morita, 1983; Oliver et al., 1991; Roszak and Colwell, 1987). The occurrence of

VBNC cells in oceanic waters has often been correlated with uncultivability of indigenous bacteria. It is presently clear that there is a relationship between starvation-survival and VBNC cells, but whether such cells play an important role in the open ocean has not been demonstrated conclusively (Oliver, 1993). The significance of VBNC state is far from fully known. There is even still an ongoing debate of the usage of the term VBNC (Kell et al., 1998; Oliver, 1993; Rahman et al,. 2001; Rowan, 2004). Meanwhile, new terms such as "active but not culturable", "not immediately culturable" and "as yet uncultured" have been proposed to describe this group of microbes (Barer and Harwood, 1999; Kell et al., 1998). Again, the terminology seems to cause confusion and mask the true nature and properties of the microorganisms we want to study. The cultivability here in the term VBNC again refers to growth in conventional rich media, which in my opinion cannot be taken as a criterion for bacterial ability to growth.

Physiology of the copiotrophs and oligotrophs

Extensive physiological studies have been carried out on various copiotrophs. *Escherichia coli* is one of the best-investigated bacteria with respect to its physiology, biochemistry and genetics (Neidhardt et al., 1996). Extensive work has been done on the adaptation of *E. coli* to starvation and nutrient limitation (Ferenci, 1996; Helling et al., 1987; Kurlandzka et al., 1991; Kovarova-kovar and Egli, 1998; Lendenmann and Egli, 1997; Notley-McRobb and Ferenci, 1999a,b; Rosenzweig et al., 1994; Senn et al., 1994). In comparison, due to the lack of pure isolates, only a few studies are available on the physiological aspects for oligotrophs. Three oligotrophic bacterial strains (*Aquabacterium* spp.) were isolated from biofilms of the Berlin drinking water system, with little physiological information reported (Kalmbach et al., 1999). To our knowledge, the most comprehensive understanding of the physiology of oligotrophs comes from a facultative oligotrophic bacterium *Sphingopyxis alaskensis*. This bacterium was isolated using extinction dilution method. An unknown mechanism enabled the cells to grow on rich medium after long-term incubation at stationary phase (6-12 months) in the dark and low temperatuer (4°C) (Cavicchioli et al., 2003; Figure 1.2). Strains of this species were isolated from three geographically separated locations: the Resurrection Bay (Alaska), the North Sea, and coastal waters near Japan using the same method (Cavicchioli et al., 2003).

Three unique characteristics make *S. alaskensis* a model oligotrophic bacterium: (1) a constant ultramicro-size (< 0.1 µm³) irrespective of whether it is growing or starving, (2) the

ability to utilize low concentrations of nutrients (mg C/l), and (3) the ability to simultaneously take up mixed substrates (Cavicchioli et al., 2003). Unlike the comprehensive physiological studies on *E. coli*, studies on *S. alaskensis* have been focused mainly on its response to different stresses. It was found that growing cells were remarkably resistant, being able to survive at a temperature of 56ºC, in 25 mM hydrogen peroxide, or in 20% ethanol. Growth rate control of stress resistance was found to be specific to carbon and energy limitation in this organism (Ostrowski et al., 2001). It was also reported that based on cell volume, *S. alaskensis* appears to have a higher concentration of ribosomes than *E. coli* (approximately double of that of *E. coli* with a growth rate of 1.5 h^{-1}) (Fegatella et al., 1998; Nystrom et al., 1992). These studies indicate that the bulk of the ribosome pool is not required for protein synthesis and that ribosomes are not the limiting factor contributing for the low rate of growth (Fegatella et al., 1998; Schut et al., 1997). It is reported that the facultative oligotrophic ultramicrobacterium (e.g. *S. alaskensis*) has a physiology that is distinctly different from that of typical copiotrophic bacteria, such as *E. coli* (Cavicchioli et al., 2003).

In conclusion, microbial oligotrophy has attracted a lot of attention. The industrial, medical aspects and their usage in biodeterioration and biocontamination have been reviewed by Wainwright and co-workers (1991; 1993). Scientists mainly focused on the response of bacteria (grown in laboratory rich media) to oliogotrophic conditions. However, with the fact that most natural environments are oligotrophic, it may be more appropriate to investigate this phenomenon in the opposite direction, namely the autochthonous bacteria adjust to copiotrophic environments. As discussed in the section above, little physiological information is available for "obligate oligotrophic" bacteria despite their importance in nature. Obviously, the main restriction and one difficulty in the study of oligotrophs is their poor culturability on plates which restricts at the same time the isolation of pure cultures and the inability to obtain reasonable cell densities for experimental investigation.

Searching for the smallest metabolic cell

The majority of bacterial cells observed in the environments are small, and smaller than the strains that can be cultivated in the laboratory. Based on this observation, it has been proposed that one of the survival strategies of bacteria in oligotrophic environments is to minimize cell

size. This increases the ratio of cell surface to cell volume and hence increases the space for nutrient transportation. For example, a significant relationship between decreased cell size and increased survival of bacteria in the deep sea was reported (Tabor et al., 1981). The typical facultative oligotroph, *S. alaskensis,* has also an ultra-micro cell size (< 0.1 μm^3). In the past 10 years, the topic of small bacteria has received more and more attention from microbiologists with the detection of small cell-like forms, in Mars meteorites (McKay et al., 1996), deep subsurface samples (Uwins et al., 1998) and human kidney stones (Kajander and Ciftcioglu, 1998). The emergence of the so-called "nanobacteria" (McKay et al., 1996; Kajander and Ciftcioglu, 1998) opened the debate on how small a metabolising cell can be and their importance in various research fields.

"Ultramicrobacteria"/"nanobacteria"

The size of microorganisms varies considerably. Cells with volumes of 0.02 – 180 000 000 μm^3 (Cavicchioli and Ostrowski, 2003) and diverse shapes (Cabeen and Jacobs-Wagner, 2005) have been isolated. The changes of bacterial volume and diameters with two representative shapes are demonstrated in Figure 1.3. It is clear that the cell volume changes much quicker than cell diameter. For example, when the cell diameter decreases 1/2, the cell volume reduces about 8 times depending on the cell shape (Figure 1.3). It is reported that the volume of bacterial cells is an indicator of their physiological status of bacteria, since starved cells are smaller than those grown in rich medium (Moriarty and Bell, 1993). Bacteria with a diameter of 300 to 500 nm are common in oligotrophic environments (Knoll, 1999). On average, the mean cell volume for lake water bacteria was about 0.03 μm^3, varying from 0.01 to 0.2 μm^3 (Cole et al., 1993). Similarly, bacteria with cell volume between 0.01 to 0.1 μm^3 dominate the microbial communities in marine environments (e.g., Gasol et al, 1995; Lee and Fuhrman, 1987; Simon and Azam, 1989). These small bacteria constitute the major biomass in aquatic ecosystem and play key roles in the biogeochemical cycling of nutrients. Often they are referred to as "ultramicrobacteria" or "nanobacteria" in the literature.

It is difficult to judge and compare the accuracy with the measurements of size, the factor used to characterize various small cells. It is likely that the terms "ultramicrobacteria" and "nanobacteria" are used to describe the same structures depending on the conditions of their discovery and the preference of researchers who discovered them (Schut et al., 1997; Kajander and Ciftcioglu, 1998; Velimirov, 2001; Martel and Young, 2008). The pioneering

work on "nanobacteria" was done by Folk (1993), who has demonstrated the presence of tiny spherical structure (50 - 200 nm) in geological materials. However, his finding did not draw much attention from microbiologists. It is the group of Kajander and Ciftcioglu (1998) who brought the term "nanobacteria" into the microbiological community. They showed that there are "nano"bacterial forms in human and cow blood and their correlation with calcification-related health problems. But these researchers received lots of criticism, most of it was related to the true viability of nanobacteria (e.g. Maniloff, 1997; Psenner and Loferer, 1997; Hamilton, 2000; Velimirov, 2001). Hamilton (2000) proposed to use a working hypothesis to define "nanobacteria" as "extremely small cellular forms, widespread in nature and closely associated with the formation of inorganic precipitates and geological strata". Still, what we do not know is whether the observed cells are "living units" and also whether or not this has to be associated with inorganic precipitation phenomenon.

| $D = 2\ \mu m$ | $D = 1\ \mu m$ | $D = 0.5\ \mu m$ | $D = 0.3\ \mu m$ | $D = 0.2\ \mu m$ |
| $V = 4.19\ \mu m^3$ | $V = 0.51\ \mu m^3$ | $V = 0.07\ \mu m^3$ | $V = 0.01\ \mu m^3$ | $V = 0.004\ \mu m^3$ |

$W = 1\ \mu m$	$W = 0.5\ \mu m$	$W = 0.25\ \mu m$	$W = 0.15\ \mu m$	$W = 0.1\ \mu m$
$L = 2\ \mu m$	$L = 1\ \mu m$	$L = 0.5\ \mu m$	$L = 0.3\ \mu m$	$L = 0.2\ \mu m$
$V = 1.29\ \mu m^3$	$V = 0.17\ \mu m^3$	$V = 0.02\ \mu m^3$	$V = 0.005\ \mu m^3$	$V = 0.001\ \mu m^3$
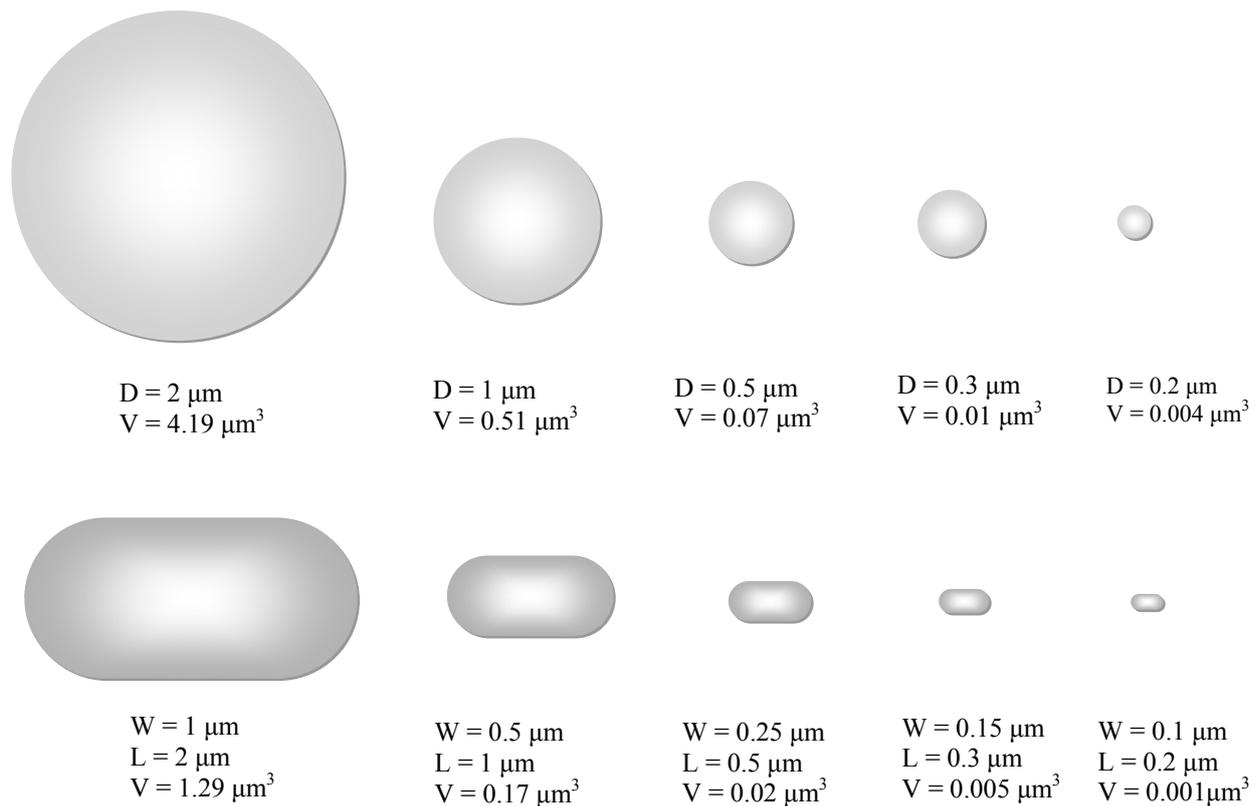
Figure 1.3 Schematic presentation of bacterial size changes with different shapes (coccus vs. rod). D represents the diameter of coccus, W for the width of rod, L for the length of rod, and V for the volume of the cell.

In contrast to "nanobacteria", "ultramicrobacteria" are slightly better defined, although still with conceptual shortcomings. The term "ultramicrobacteria" was first adopted by Torella and Morita (1981) to describe small bacteria isolated from seawater that formed "ultramicrocolonies" on agar plates, and grew very slowly in the presence of high concentrations of nutrients. It has been modified by Schut and co-workers (1997) to include microorganisms that have a cell volume of less than 0.1 μm^3 and retain this volume irrespective of growth conditions. In addition to the small cell volume, "ultramicrobacteria" also appear to have a small genome size (approximately 1 to 2.5 fg of DNA per cell), which is less than 50% of a single copy of the genome of *E. coli* (Savageau, 1983). "Ultramicrobacteria" appear to be most prevalent in oligotrophic environments, such as the open ocean. Due to their small size, "ultramicrobacterial" cells have a high surface-area-to-volume ratio, which may enhance their ability to take up nutrients from the environment. It is reported that "ultramicrobacteria" represent the main marine bacterial component in terms of biomass and activity (Schut et al., 1997). To date, most knowledge on "ultramicrobacteria" is generated from marine bacteria. The most intensively investigated "ultramicrobacterium" is *S. alaskensis*, as described above. In comparison, the presence of "ultramicrobacteria" in freshwater and drinking water has been largely ignored. Meanwhile, due to the "uncultivability" of "ultramicrobacteria" (Kjelleberg et al., 1987), we still know little about their physiological characteristics. Although the definition of "ultramicrobacteria" is fuzzy, the significance of such small bacteria in biological cycling of nutrients is generally acknowledged by microbial ecologists.

Filterable bacteria

Among microbiologists, there is a general consensus that the minimum size of a metabolizing cell is likely to be approximately 200 nm in diameter (Hamilton, 2000). It is perhaps not entirely a coincident that this matches with the pore-size diameter of so-called "sterilization filters". The small size of a bacterium is always linked to its ability to pass through filters. The ability of bacteria to pass through filters with different pore sizes was used to differentiate various size classes of bacteria. In microbiology, the term "filterable bacteria" refers to those that have the ability to pass through filters. It is commonly observed in literature that "ultramicrobacteria" are defined based on their ability to pass through 0.45 μm filters (Oppenheimer, 1952; Tabor et al., 1981; Schut et al., 1997). In some references, the filterable

bacteria and ultramicrobacteria are even inter-changeable (Anderson and Heffernan, 1965; Tabor et al., 1981; MacDonell and Hood, 1982).

The presence of filterable bacteria has a direct implication in practice. Temperature-sensitive solutions, which cannot be sterilized by autoclaving, are usually sterilized by filtration through sterilized membrane filters with pore sizes of 0.2 μm (Madigan et al., 1999). This method is generally believed to remove all microorganisms (except viruses) from the filtered solutions. However, the presence of filterable bacteria makes the safety of filtration sterilization questionable. The observation of filterable bacteria started as early as the 1930s. The pore-size used back then was 0.45 μm or larger. The filterable bacteria were regarded as a biological stage in the bacterial life cycle, especially for pathogenic bacteria (Sherman and Safford, 1931). A few reports from that time suggest that some bacteria are able to pass through these filters (Laidlaw and Elford, 1936; Sherman and Safford, 1931; Winslow, 1932; Zinsser, 1932). Later, it was reported that *Pseudomonas pickettii* can penetrate a 0.2 μm filtration system and contaminated the "sterile" commercial sodium chloride solution (Anderson et al., 1985). More recently, species of *Vibrio, Pseudomonas* and *Listonella* were found to be present in the 0.2 μm seawater filtrate (Hahn, 2004; Hood and MacDonell, 1987; Vybiral et al., 1999). These findings, however, have relied on the plating technique (Hood and MacDonell, 1987; Janssen et al., 1997), which leads to the selection of copiotrophs rather than oligotrophs, with the latter may be the major component in the filterable bacterial communities. Hence, the number of bacterial cells passing through filters may be largely underestimated. Again, our knowledge is limited on cultivability of microorganisms.

How small can a bacterial cell be?

The debate on "nanobacteria/ultramicrobacteria" leads to the question of how small a bacterial cell can be. The lower limit of the cell size is determined by various factors, such as number of protein and RNA molecules needed for minimal function, size of genome and corresponding ribosomes for expression, just to name a few. It has been intensively discussed by researchers from different fields. It seems the minimum volume enabling independent life for a cell is between 0.01 to 0.06 μm^3 (Koch, 1997; Maniloff, 1997; Psenner and Loferer, 1997). It has been generally agreed that a sphere with a diameter of 250 ± 50 nm should be the reasonable lower size limit for a free-living, self-sufficient microbial cell (Knoll, 1999; Figure 1.3). This lower limit was calculated based on that the genome codes for all proteins

and it is largely dependent on the number of non-ribosomal protein species (De Duve et al., 1999). The known smallest genome is that of *Mycoplasma genitalium*. The genome size appears to be controlled by cell volume because as the cell volume decreases, the fraction of volume occupied by genome increases dramatically. For instance, the *E. coli* genome contributes only to 1% of the cell volume, while in the case of *M. genitalium*, this fraction increases to 10% (Lawrence, 1999; Moore, 1999). Even the smallest genome known to date (*M. genitalium*) – about 470 genes - is not completely composed of essential genes. It is suggested by comparing known sequenced genomes, that only 256 genes are required for support the function of a bacterial cell (Mushegian and Koonin, 1996). Furthermore, if rapid gene transfer holds true, a cell may not need all the essential genes for growth. Nevertheless, the theoretical calculation could be used as a first guideline for the search of the smallest cell.

In general, there are various trends and conflicting results concerning the smallest size of bacteria. On one hand there is no evident proof for the viability of "nanobacteria" or their identity as living units (or prokaryotes). On the other hand, "ultramicrobacteria" are not necessarily representing the smallest prokaryotes. Furthermore, considering the low cultivability of "ultramicrobacteria", our knowledge is largely limited by the detection methods and availability to obtain representative isolates.

The advances and limitations of methodology

The methods for studying microorganisms are intimately linked to the knowledge acquired so far on microorganisms. Every significant leap in microbiological knowledge was accompanied by technological advances. The understanding of metabolic potential of microorganisms has been largely hampered by the inability to isolate pure species. A few commonly-used methodologies are discussed below.

Cultivation by plating

Since microbiology has established itself as a science in 1850, laboratory synthetic media for microbial cultivation were applied by microbiologist to accelerate microbial growth and particularly in the hope of fast detection of pathogenic bacteria. However, researchers have

estimated that less than 1% of the microorganisms present in natural environments can be cultivated using such conventional ways (Table 1.1). Still, one cannot deny the value of cultivation methods in the history of microbial studies, and today it is still vital for many discoveries of microorganisms. Most physiological studies still require pure cultures at high cell density and one also needs to grow strains up to visible colonies for further phylogenetic and genetic characterization. This is probably the reason why *S. alaskensis* is relatively well described since it is a facultative oligotroph that can be cultivated in conventional media (Schut et al., 1997).

Among the conventional cultivation approaches, growth on complex media, including the widely used heterotrophic plate counting (HPC), has been the key method for study bacteria in the past 150 years. Medium composition, incubation time and temperature are very significant variables for HPC (Olsen and Bakken, 1987). For enumeration and isolation of oligotrophs, the classical most probable number (MPN) enumeration method was widely applied (e.g., Button et al., 1993; Ishida and Kadota, 1981). The method consists of diluting an environmental sample in low nutrient medium and incubating for long periods of time (from weeks to months). The problem associated with the MPN methods is the lack of proper detection method for the growth in diluted medium (Fry, 1990). Plating cultivation methods were also gradually improved by modifying the traditional approaches in order to isolate those microbes that previously escaped cultivation. For example, with low-nutrient medium and increased incubation times, Janssen and co-workers (2002) were able to isolate *Acidobacteria*. Furthermore, signalling compounds have also been reported to aid the cultivation of aquatic microorganisms (Bruns et al., 2003). Despite these improvements, most of the natural microorganisms still escape these cultivation attempts. Our knowledge is largely limited by the few species that can form colonies on plates under laboratory conditions.

Imaging technology

He/she believes what one sees. Direct visualization enables us to have a clear image of microbial cells. The commonly used imaging methods include light microscopy, epi-fluorescence microscopy and electron microscopy. Imaging techniques have been applied to achieve enumeration of microorganisms (e.g., Sekar et al., 2003), to determine cell size (e.g., Dubochet et al., 1983), to estimate biomass (e.g., Loferer-Kroßbacher et al., 1998), to characterize their morphology (e.g., Sàra and Sleytr, 2000), and also to further study their

interaction with surfaces (e.g., Razatos et al., 1998). Epi-fluorescence microscopy is mainly used for quantitative measurements of the size and composition of microbial communities. It also forms an important component of some molecular techniques, such as fluorescence in-situ hybridization (FISH). FISH has been widely used to describe bacterial community composition (e.g., Amann et al., 1995; Cottrell and Kirchman, 2000; Lebaron et al., 2001; Pernthaler et al., 1998). One of the limitations of the technique is the relative wide variation of the effectiveness of the detection of target cells (reviewed by Bouvier and Giorgio, 2003). The electron microscopy has been used primarily for gathering descriptive information, and it is not a technique for quantitative data, except for morphological measurements. Nevertheless, electron microscopy has been a powerful tool for studying bacterial surfaces (reviewed by Beveridge and Graham, 1991) and facilitated the development of biofilm research (reviewed by Costerton et al., 1987). The main limitation of microscopic techniques is that they are in general time consuming and labour demanding.

Nucleic acid based technology

The development of DNA sequencing and analysis have enabled us to "see" much more of the microbial community from the environments and have revolutionized the study of microbial evolution and ecology. The recent review concluded that about 52 phyla can be discerned based on phylogenetic analysis (Rappé and Giovannoni, 2003), compared to the 12 originally proposed by Woese (1987). The knowledge on uncultivable microbial diversity also increased dramatically with the development of molecular techniques. For example, in the year 2002, approximately 9,500 environmental 16S rDNA clone sequences were published in GeneBank. At the same time, numerous phylogenetic trees sprung into the literature since 1980s. How to interpret these data has become a critical question.

Keeping in mind the bias involved in the PCR-based methods, one should be careful to claim that sequencing results reflect the whole microbial diversity in natural ecosystems. Steps such as DNA extraction, primer-binding, etc., during the PCR protocol, all give a certain degree of bias to the final result. Besides, cloning steps can also generate bias. Nevertheless, the PCR-based techniques enable microbiologist to engage researches deeply in the field of microbial diversity. Despite all its opportunities, this approach cannot answer many of the questions that microbiologists want to know, such as what unique cell functions do these bacteria have? Or what biogeochemical activities do they contribute to the ecosystem?

Most recently, random shotgun sequencing of DNA from entire environmental microbial communities has been applied to recover the gene complement of uncultivated organisms and to determine the degree of variability within populations at the genome level (Tyson et al., 2004; Venter et al., 2004). One drawback of applying molecular techniques is that it is difficult to study the physiological properties of the microorganisms based on their phylogenetic relationship with others. Also, despite the sequencing of total genomes, the DNA level does not always translate to the functional level. Hence, the isolation and physiological studies are still needed and important for microbial research. Furthermore, there is a danger that the molecular based sequencing techniques are becoming the research goals by themselves and masks the scientific question for microbial studies.

Flow cytometry

Flow cytometry (FCM) was first developed in the 1960s and has been applied immediately afterwards in the medical field for analysis of mammalian cells. Microbiologists only began to use this tool in the late 1970s. The first papers on the use of FCM to study bacteria appeared in 1977 (Paau et al., 1977; Bailey et al., 1977). However, it did not get popular amongst microbiologists, mainly due to technical limitations since bacteria are much smaller than mammalian cells. It only started about 15 years ago that FCM was more frequently applied in microbiology. The use of flow cytometry in microbial ecology and microbial related industry has enormous potential and only started to be realized. The advantages of FCM have been summarized by Steen (2000). Its attraction is rapid analysis, accuracy, the generation of multi-parameters, single cell level information, and easily obtaining statistically relevant data sets (Paul, 1993; Hammes and Egli, 2005). Microbial cells can be detected by FCM irrespective of their cultivability, which overcomes the one major obstacle in the study of microbiology. Furthermore, with the possibility of cell sorting, further analysis on the single cell level is possible. The method will be further perfected with the continuously advancing development of stains. Nowadays, FCM has a relative wide range of applications, including studies of bacterial cell cycles, microbial monitoring in sea water, in drinking water and the assessment of antibiotic susceptibility (e.g., Li et al., 1995; Lebaron et al., 2002; Hammes et al., 2008).

In particular, FCM has been applied by ecologist to study different microbial communities. It was observed that after staining with DNA-binding dyes, microbial cells tend to cluster into distinct groups based on cellular fluoroscence and sideward scatter signal. At least two

fractions were repeatedly reported in literature: cells with high nucleic acid content (HNA) and cells with low nucleic acid content (LNA) (e.g., Gasol et al., 1999; Jellett et al., 1996; Jochem et al., 2001; Lebaron et al., 2002; Li et al., 1995). However, this distinction is a phenomenon typical for FCM and has so far not been verified with other techniques. For more detailed discussion on HNA and LNA, the reader is referred to Chapter 5. The application of FCM in aquatic microbiology was recently reviewed by Vives-Rego and co-workers (2000). Still, the usage and advantage of FCM is not yet well received by many microbiologists. The potential of FCM for microbiology is still far from being fully utilised.

Cost is probably the biggest limitation of FCM when researchers consider including FCM into their studies. The first steps in handling FCM are relatively easy, however, control and validation of data are quite sophisticated. The criticism also raised is that for FCM it is difficult to verify the data obtained with other methods (Paul, 1993).

As discussed above, the advances and limitation in the study of prokaryotes (especially oligotrophs) are intimately linked with the techniques available. There is an urgent need for better cultivation methods for explore the "uncultivable" majority of cells inhabiting our planet. Microbiology requires more methods that allow researchers to mimic in the laboratory the natural environments that prokaryotes face, especially the conditions with low nutrient concentrations. Since each method has its advances and limitations, a combination of various methods could be the most reasonable way to achieve a better understanding of microbial life.

Objectives and structure of this thesis

In the preceding sections, I have documented the many questions still to be answered on the subject of oligotrophy and small bacteria, although plenty of reports are available in literature. Furthermore, I stressed the importance of proper methods and solid experimental data. Recently, a new cultivation method was developed using assimilable organic carbon (AOC) of natural origin as a growth substrate and FCM as a tool of quantification of growth (Hammes and Egli, 2005). This method provide us with a unique chance to explore the formerly "unseen majority". Based on this opportunity, the following objectives were set for this thesis:

(i)   to investigate whether those small uncultivable bacteria are omnipresent in aquatic environments and to isolate and study some members of this group.

(ii)   to explore the phenomenon of oligotrophy in more detail.

In this thesis, I tried to resolve the puzzle by relatively conventional approach (filtration) in combination of novel techniques including FCM and molecular analysis. My work has led me to some unforeseen roads such as filterability of microbial cells, bacterial shape and filterability relationship etc. The unexpected findings opened up an even more exciting research area and added significant part to the theme of the thesis.

This thesis first covered the presence and growth of filterable bacteria in natural freshwater (Chapter 2). Seeing that filterability does not equate small cell volume, the factors determining the bacterial filterability was consequently analyzed (Chapter 3). To further verify the findings in laboratory, the filtration process was tested in an industrial scale study (Chapter 4). Since small bacteria (so-called LNA bacteria) was observed to be dominant in numbers in various freshwater environments (Chapter 2 and 4), they became the next focus of the thesis. The successful enrichment and characterization of "LNA" bacteria was documented in Chapter 5. The story was closed with a comparison growth study of the new "LNA" isolate and the well-studied *S. alaskensis*, and a discussion of the concept of "oligotroph" and "ultramicrobacteria/nanobacteria" (Chapter 6).

2. Quantification of the filterability of freshwater bacteria through 0.45, 0.22, and 0.1 µm pore size filters and shape-dependent enrichment of filterable bacterial communities

Abstract

Micro-filtration is a standard process for sterilization in scientific research, medical and industrial applications, and to remove particles in drinking water or wastewater treatment. It is generally assumed - and confirmed by accessing filtration efficiency by plating - that filters with a 0.1 to 0.45 µm pore size can retain bacteria. In contrast to this assumption, we have regularly observed the passage of a significant fraction of natural freshwater bacterial communities through 0.45, 0.22 and 0.1 µm pore size filters. Flow cytometry and a regrowth assay were applied in the present study to quantify and cultivate filterable bacteria. Here we show for the first time a systematic quantification of their filterability, especially their ability to pass through 0.1 µm pore size filters. The filtered bacteria were subsequently able to grow on natural assimilable organic carbon (AOC) with specific growth rates up to 0.47 h^{-1}. We were able to enrich bacterial communities that pass preferentially through all three pore size filters at significantly increased percentages using successive filtration-regrowth cycles. In all instances, the dominant microbial populations comprised slender spirillum-shaped *Hylemonella gracilis* strains, suggesting shape-dependent selection during the filtration process. This quantification of micro-filterable bacterial omnipresence in natural freshwater and their re-growth characteristics demand a change in the sterile filtration practice used in industrial and engineering applications as well as scientific research.

Introduction

Micro-filtration (0.1 — 0.45 μm pore size) is commonly used for sterilization of heat-sensitive pharmaceutical and laboratory solutions and also for the physical removal of microbial cells during the treatment of drinking water and wastewater (Eykamp, 1991). The notion of bacteria that pass through membrane filters with micro-size pores drew considerable attention in the 1930s (Laidlaw and Elford, 1936; Sherman and Safford, 1931; Winslow, 1932; Zinsser, 1932). All these reports referred to "filterable bacteria" as those that can pass through filters with 0.45 μm and larger pores. Some researchers regarded filterable bacteria as a new group of organisms (Laidlaw and Elford, 1936), while others suggested filterable forms as a biological stage in the bacterial life cycle (Klieneberger-Nobel, 1951; Sherman and Safford, 1931). Most of this work was done based on filtration experiments with emulsified cultures of pathogenic bacteria (Klieneberger-Nobel, 1951). However, the interests in this topic gradually subsided due to the lack of well-defined experimental conditions and suitable methods with which it was possible to culture and isolate filterable bacteria (Dienes and Weinberger, 1951).

Membrane filtration through 0.22 μm pore size is continuously considered and commercially traded as a sterile-filtering procedure. However, aquatic bacteria that are able to pass through filters with a 0.22 μm pore size have been recently reported which have renewed the overall interest in this field (Elsaied et al., 2001; Hahn, 2004; Hahn et al., 2004; Haller et al., 1999; Hood and MacDonell, 1987; Lopez and Vela, 1981). Most of these studies focused on the existence of such bacteria, whereas data on their quantification, growth ability and filtration efficiency remains rather limited (Lillis and Bissonnette, 2001; Young, 2006). Furthermore, to date, there is to our knowledge no information available on bacteria that pass through 0.1 μm pore size filters. An exception to this is a report on "nanobacteria" (Kajander and Çiftcioglu, 1998) where the ability of small particles, so-called "nanobacteria", to pass through 0.1 μm filters was predicted from their size. However, no definite proof of the biological nature of these "nanobacteria" was given and no experimental data on filtration was provided. Presently, the biological nature of "nanobacteria" remains an unresolved debate (Velimirov, 2001).

Most findings regarding filterable bacteria hitherto, including standard filter challenging tests, have relied on the heterotrophic plate count (HPC) method, or equivalent techniques, for enumeration and isolation of filterable bacteria. The shortcomings of this method for detection

of natural bacteria are well documented (Noble et al., 1991; Roszak and Colwell, 1987). It has been observed repeatedly that less than 1% of the bacterioplanktonic cells in aquatic samples can form a colony on agar surfaces (Roszak and Colwell, 1987; Kogure et al., 1979), despite the fact that most bacterial cells in the environment exhibit metabolic activity (Kogure et al., 1979; Rodriguez et al., 1992). Hence, based on HPC the number of bacterial cells passing through filters may be largely underestimated. Failure to detect filterable bacteria could result in a substantial overestimation of the hygienic quality of drinking water and other filtered solutions. Flow cytometry (FCM), which allows rapid and accurate enumeration of all cells regardless of their culturability, is a more suitable detection method than HPC, particularly when physical barriers such as membranes are examined in conjunction with autochthonous aquatic microorganisms.

In our laboratory we were interested in studying the growth of microbes with natural assimilable organic carbon (AOC). In order not to change the properties of AOC, 0.22 µm-filtered freshwater was used as media; however, regularly we observed bacterial growth in filtered water samples used as blanks (not detectable with HPC). This prompted us to investigate the efficiency of filtration in more detail using FCM. In the present study, we have used FCM in combination with fluorescence-staining and a regrowth assay based on natural organic compounds (Hammes and Egli, 2005) to quantify, cultivate and enrich filterable bacteria from different freshwater samples.

Materials and Methods

Sampling and filtration

Freshwater samples were collected in autoclaved glass bottles from eight different freshwater environments in Switzerland: Chriesbach River (Dübendorf), Lake Zürich (Zürich), Limmat River (Zürich), Lake Lugano (Lugano), Rhine River (Basel), Glatt River (Dübendorf), Lake Greifensee (Uster) and Sihl River (Zürich). Drinking water samples included commercially available bottles mineral water and tap water (Dübendorf). Samples were stored at 4ºC during transportation and all samples were processed within 8 hours after sampling. Commercially available pre-packed sterile syringe filters with 0.45, 0.22 and 0.1 µm pore size, respectively,

were used in this filtration study. Filters from different commercial brands (Millipore, USA; Pall, USA; Whatman, USA; Schleicher & Schuell, Germany; Sartorius, Germany) and of different types (cellulose nitrate; nylon; polyethersulfone; polypropylene; PVDF) were tested. Aliquots of 30 ml were filtered with a constant flow rate (600 ml/h). Total cell concentrations (n) were measured before (n_{before}) and after filtration (n_{after}). The filtration efficiency (FE) was calculated as the passage percentage of the bacterial community in the freshwater samples (Equation 2.1). All measurements were done in triplicate and from this the standard deviation was determined.

$$FE = n_{after} \ / \ n_{before} \times 100\%$$ Equation 2.1

Growth characterization of filterable bacteria

Chriesbach River water (30 ml) was filtered through a 0.22 μm pore size syringe filter (Millipore, USA). The filtrates were incubated at 30°C in muffled (and therefore sterile) 40 ml vials free of any assimilable organic carbon (AOC) (Hammes and Egli, 2005). Samples (1 ml) were taken at regular time intervals during 70 hours. Samples were analyzed with flow cytometry and adenosine tri-phosphate (ATP) as described below. The specific growth rate (μ) based on cell number increase for the filterable bacterial community in each sample was determined as following (Equation 2.2):

$$\mu = (Ln(n_t) - Ln(n_0))/\Delta t$$ Equation 2.2

where n_t, n_0 are the cell concentrations measured at three subsequent time points and Δt is the expired time interval between these points. Such kinetic investigations were carried out on filtered bacteria from all freshwater samples collected. Note that when describing bacterial growth, the division rate (k) is also frequently used. k differs from μ in a factor Ln 2 (k = μ/(Ln 2)) and for a discussion see Vital et al. (2007).

Enumeration of filterable cells

Samples (1 ml) were directly stained with 10 μL/ml SYBR® Green I stain (1:100 dilution in DMSO; Molecular Probes, CA, USA), and incubated in the dark for at least 20 minutes before measurement. Flow cytometric absolute cell counting was performed to determine the cell

concentration, using a PASIII flow cytometer (Partec, Münster, Germany) equipped with a 25 mW argon ion laser (488 nm) and volumetric counting hardware. Settings were as described previously by Hammes and Egli (2005) and the detection limit was about 200 cells/ml with an average standard deviation of 5%.

ATP measurement

ATP measurement was carried out using the BacTiter-Glo™ Luminescent Cell Viability Assay (Promega, MI, USA) and a luminometer (Glomax, CA, USA) applying the method for planktonic cells described by Berney and co-workers (2006). The results were obtained as relative light units and converted to ATP concentrations using a calibration line constructed with an ATP standard solution (Promega). The detection limit of the method was about 0.01 nM ATP with an average standard deviation of 10%.

Antibiotics resistance

Six commonly used antibiotics were tested for their inhibition of the growth of filterable cells, namely ampicillin (10 µg/ml), chlortetracycline (25 µg/ml), kanamycin (10 µg/ml), nalidixic acid (25 µg/ml), streptomycin (12.5 µg/ml) and tetracycline (25 µg/ml). Growth inhibition was tested using 30 ml of 10,000-times diluted LB media (full strength LB diluted in nano-pure water) as carbon and energy source. The 0.22 and 0.1 µm filtrates were inoculated in such media (the starting cell concentration was 10^4 cell/ml) and samples were then incubated for 4 days at 30°C until stationary phase was reached. The number of cells was measured before and after incubation using FCM.

Enrichment of filterable bacteria

Successive filtration-regrowth cycles were carried out with freshwater from four different sites (Chriesbach River, Lake Zürich, Lake Lugano and Rhine River). The original waters were tested for the filtration efficiency through 0.45, 0.22 and 0.1 µm pore size filters as described above (cycle 0). The 0.22 and 0.1 µm filtrates were then directly incubated for 7 days at 30°C where growth proceeded on the AOC present in the original water samples. At the end of the incubation period, each culture was again tested for filtration efficiency (cycle 1). The filtrates of each culture (0.22 and 0.1 µm, respectively) were then replenished with

heat-sterilized river water as new source of AOC for further regrowth. The initial cell concentration after AOC addition was about 5×10^3 cells/ml. After a further 7 days of incubation, filtration efficiency was tested again (cycle 2), and the filtrates were again supplied with freshly heat-sterilized river water. Such filtration-regrowth experiments were carried out for 4 identical cycles. Depending on the filter used, the enrichment cultures were referred to as 0.22 μm and 0.1 μm enrichment, respectively. Parallel regrowth cycles were carried out without the filtration steps. Such cultures are designated as control cultures. In brief, original waters (cell concentration in the range of $1 - 3 \times 10^6$ cells/ml) were inoculated in freshly heat-sterilized river waters, with the cell concentration adjusted to 5×10^3 cells/ml by diluting the waters about 1000 times (e.g. 15 μl original water into 15 ml freshly heat-sterilized river water). After 7 days of incubation at 30ºC, cultures were inoculated into freshly heat-sterilized river water to a cell concentration of 5×10^3 cells/ml and then proceed to another 7 days of incubation. Such cycle was repeated four times. At the end of each incubation period, filtration efficiency of the cultures was tested.

Microbial community analysis

Genomic DNA was extracted from the water samples by using the UltraClean Water DNA Isolation Kit (Mo Bio, Germany). Denaturing gradient gel electrophoresis (DGGE) analysis was performed using a DCode System (Bio-Rad, Hercules, CA, USA) as described using the forward primer P338F and the reverse primer P518r (Boon et al., 2002; Muyzer et al., 1993). PCR products were loaded onto 8% (w/v) polyacrylamide gels in 1x TAE (20 mM Tris, 10 mM acetate, 0.5 mM EDTA, pH 7.4). The polyacrylamide gels were made with a denaturing gradient ranging from 45% to 60%. The electrophoresis was run over night for 16 h at 60°C, 38 V. After electrophoresis, the gels were soaked for 30 min in SYBR green I nucleic acid gel stain (1:10,000 dilution; FMC BioProducts, Rockland, ME, USA) and immediately photographed.

The processing of the DGGE gels was done with the Bionumerics software 2.0 (Applied Maths, Kortrijk, Belgium). The calculation of the dendrogram is based on the Pearson (product-moment) correlation coefficient and the clustering algorithm of Ward. Microbial community diversity analysis was based on the method described by Mertens and co-workers (2005) by Lorenz curve analysis and the Gini coefficient. Rep-PCR genomic fingerprinting was performed to discriminate the different isolates on a strain level, with the BOX-primers

(Versalovic et al., 1994) and the BOX-patterns were analyzed by Bionumerics software 2.0 (Applied Maths) as described above.

Isolation and identification

0.1 and 0.22 μm filtrates from four different water samples (as stated in enrichment experiments) were cultivated on R2A (Oxoid LTD., Basingstorke, Hampshire, England) agar plates. Genomic DNA of single colonies was extracted using QIAGEN DNA extraction kit (QIAGEN, Courtaboeuf, France). The eluted DNA was stored at –20°C until further analysis. DNA was subsequently amplified using universal 16S rRNA primers EUB_9/27f (5'-GCA GAG TTT GMT CCT GGC TCA G-3') and EUB_1492/1512 (5'-ACG GYT ACC TTG TTA CGA CTT-3'). For the amplification of the target sequence from DNA extracted from pure cultures, the reaction mixture (50 μl) consisted of reaction buffer (final concentrations, 10 mM Tris-HCl, 2.5 mM $MgCl_2$, and 50 mM KCl [pH 8.3]), a 200 mM concentration of each deoxynucleoside triphosphate, 20 pmol of each primer, 500 ng of bacterial DNA, and 2.5 U of *Taq* DNA polymerase (Sigma-Aldrich, St. Louis, MO, USA). The amplification program was 94°C for 2 min; 30 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 2 min; and finally 72°C for 10 min. Target bands were excised from agarose gel and DNA was further purified using Geneclean Kit (Q.BIOgene, Irvine, CA, USA). Purified PCR products were sequenced by Microsynth (Balgach, Switzerland). Analysis of DNA sequences and homology searches were completed with standard DNA sequencing programs and the BLAST server of the National Center for Biotechnology Information (NCBI) using the BLAST algorithm (Altschul et al., 1997). Sequences of the isolated strains were deposited in GenBank under accession number DQ861287 to DQ861290.

Scanning electron microscopy

For scanning electron microscopy, 10 ml culture of an isolate identified during this study as *Hylemonella gracilis* (10^5 cells/ml) were filtered onto 50 nm pore size cellulose filters (Millipore, USA). Discs of 3 mm were punched out with a disposable tissue puncher (Stiefel Laboratorium GmbH, Germany). The filter discs were plunge-frozen in liquid propane. The filters were attached on a specimen holder in LN2. The holder with the filters was then transferred to the cold stage of the freeze-fracturing/freeze-drying device BAF 060 (Bal-Tec AG, Balzers, Lichtenstein) pre-cooled to -100°C. The filters were freeze-dried at -100°C for 5

hours at a vacuum of $5 \times e^{-7}$ mbar. Afterwards, the temperature was increased slowly to room temperature and the specimens were withdrawn from the machine. The filters were sputtered with 1 nm of platinum in an MED010 high vacuum sputtering device (Bal-Tec AG) and analyzed in a Leo1530 field emission SEM (Carl Zeiss, Oberkochen, Germany) using the Inlens SE detector.

Results

Filterable bacteria quantification and growth capacity

Filtration of ten water samples representing a broad range of water types revealed that a high percentage of the natural aquatic microbial communities pass through 0.45 μm pore size filters. On average, 50% filterability was recorded and the microbial communities from Zürich Lake represented an extreme example (87%) (Table 2.1). A considerable percentage (0.03 — 3%) of the communities was recorded to be able to pass through 0.22 μm pore size filters (Table 2.1). Furthermore, a small yet significant percentage (0.003 — 0.2%) of the communities can even pass through 0.1 μm pore size filters. Based on the original total cell concentrations in the water samples ($2 — 4 \times 10^6$ cells/ml) this implies a total cell concentration of as high as 10,000 cells/ml in a 0.22 μm filtrate and 1,000 cells/ml in a 0.1 μm filtrate. Similar passage percentages were recorded for bacterial communities in drinking water samples, despite the lower original cell concentration (Table 2.1). Different brands and types of single-layer syringe filters tested (cellulose nitrate, nylon, polyethersulfone, polypropylene, and PVDF) all produced similar results under the experimental conditions used in this study (data not shown). Various filtration flow rates (30 — 1,200 ml/h) were also tested and yielded no significant differences in filtration efficiency.

Table 2.1 Mean passage percentage (± standard deviation, n = 3) of freshwater bacterial communities through pre-sterilized commercially available syringe filters (0.1 — 0.45 μm pore size)

Sources	Total count in original waters (10^6 cells/ml)	Filter pore size			μ (h^{-1}) of 0.22 μm filterable bacteria
		0.45 μm	0.22 μm	0.1 μm	
Lake Zürich	2.50 ± 0.22	87.07% ± 3.21%	3.61% ± 1.07%	0.05% ± 0.01%	0.08 ± 0.01
Limmat River	3.62 ± 0.16	46.30% ± 0.32%	1.30% ± 0.08%	0.03% ± 0.005%	0.07 ± 0.01
Sihl River	3.71 ± 0.19	5.56% ± 0.28%	0.03% ± 0.003%	0.03% ± 0.005%	0.25 ± 0.03
Rhine River	2.61 ± 0.25	58.62% ± 3.69%	0.43% ± 0.05%	0.003% ± 0.0004%	0.20 ± 0.02
Glatt River	2.23 ± 0.08	49.88% ± 1.35%	0.08% ± 0.04%	0.05% ± 0.003%	0.11 ± 0.01
Lake Greifensee	2.41 ± 0.23	49.32% ± 6.87%	0.53% ± 0.09%	0.22% ± 0.02%	0.27 ± 0.01
Lake Lugano	2.77 ± 0.10	70.16% ± 4.91%	1.90% ± 0.43%	0.04% ± 0.01%	0.29 ± 0.03
Chriesbach River	3.21 ± 0.20	45.49% ± 1.17%	0.80% ± 0.02%	0.10% ± 0.02%	0.47 ± 0.05
Bottled drinking water	0.31 ± 0.02	16.94% ± 0.02%	0.12% ± 0.001%	0.065% ± 0.0008%	ND
Tap water	0.26 ± 0.01	45.93% ± 0.12%	0.65% ± 0.004%	0.06% ± 0.0004%	ND

ND: not determined

The growth of 0.22 μm-filtered cells in natural river water was followed with FCM monitoring of the total cell concentration as well as the adenosine tri-phosphate (ATP) content of the community (Figure 2.1). Glutaraldehyde (1%) was added to a control culture during the early exponential phase (15 h). Directly after the addition of glutaraldehyde, growth was inhibited evidenced by the total cell concentration which remained constant. In contrast, the untreated culture utilized natural river water assimilable organic carbon (AOC) and had a final yield of 1.5×10^6 cells/ml in stationary phase after 60 h (Figure 2.1). All the filterable communities from freshwater that we tested were able to grow on natural river water AOC with relatively high specific growth rates, ranging from 0.07 to 0.47 h^{-1} (Table 2.1). The specific growth rates for the 0.22 μm filterable bacteria in the two drinking water samples were not determined due to the low AOC levels of these waters (below 10 μg/l). Furthermore, six antibiotics, namely ampicillin (10 μg/ml), chlortetracycline (25 μg/ml), kanamycin (10 μg/ml), nalidixic acid (25 μg/ml), streptomycin (12.5 μg/ml) and tetracycline (25 μg/ml), were tested on the filtered communities. Two of them (tetracycline and chlortetracycline) inhibited growth.
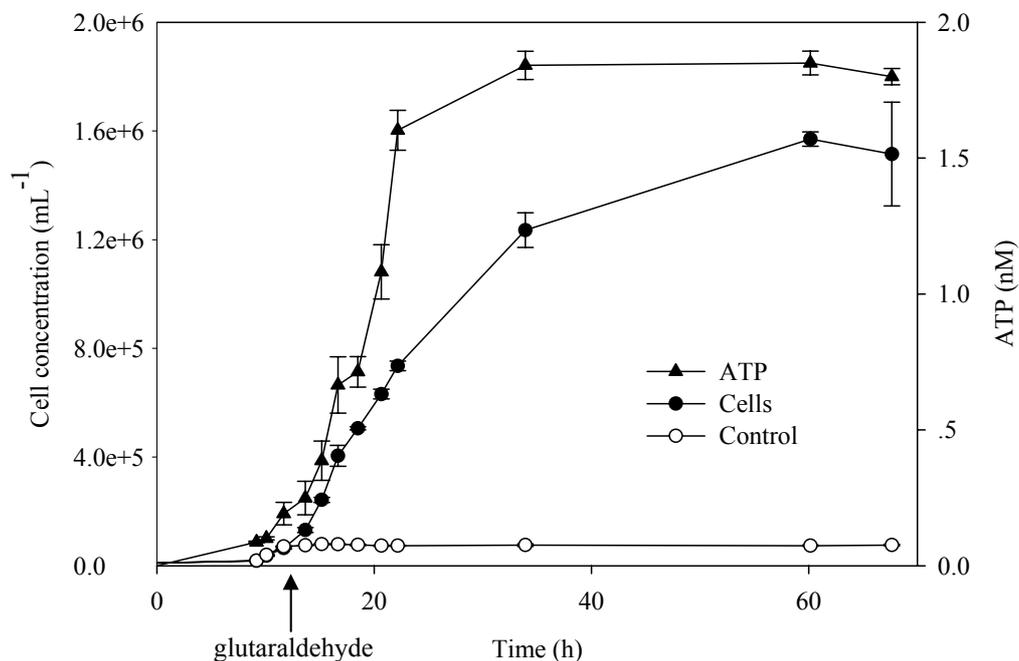
Figure 2.1. Batch growth of 0.22 μm filterable bacteria growing on natural AOC. Growth was measured with flow cytometric absolute cell counting and ATP analysis. Glutaraldehyde (1%) was added to the control at 15 h. Error bars represent the standard deviation of triplicate samples.

Enrichment of filterable bacterial communities

We designed a filtration and cultivation strategy to enrich specifically bacterial communities with high filterability using successive filtration-regrowth cycles (Figure 2.2). Typical results for the enrichment of such bacterial communities from freshwater samples are shown in Figure 2.3. For instance, a 10-fold (0.8 to 8%) increase in the percentage of cells passing through 0.22 μm pore size filters was observed already after one out of four cycles of filtration-regrowth for a microbial community from river water (Figure 2.3A). A similar remarkable increase (0.1 to 2%) was detected in the percentage passing through 0.1 μm pore size filters (Figure 2.3A). In contrast, no significant increase was observed for the control culture that went through regrowth cycles without filtration, which rules out the possibility of better filterability merely due to adaptation of the growing community to the carbon source or by temperature selection. We were able to enrich such communities with improved filterability from all four different water types tested (Figure 2.3B). In general, the passage percentage of the 0.22 μm enrichment culture through 0.22 μm pore size filters increased more than 10-times to the range of 5% to 10% (Figure 2.3B). Furthermore, the passage percentage of the same cultures through 0.1 μm pore size filters also reached a level of 2%, with one culture up to 8%, after four cycles (Figure 2.3B). For all enrichment cultures, more than half of the bacterial community that can pass through 0.22 μm pore size filters was also able to pass through 0.1 μm pore size filters (Figure 2.3B).
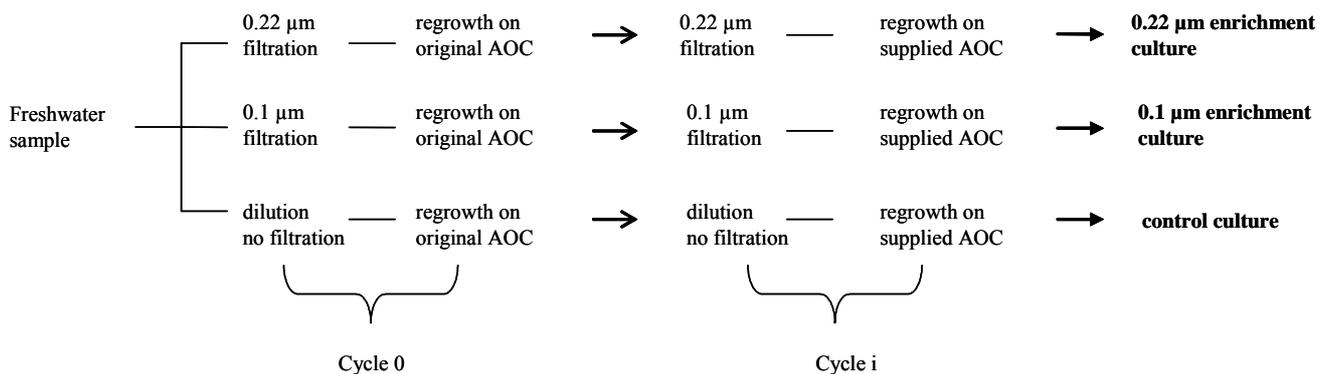
Figure 2.2. Schematic diagram of filtration-regrowth cycles for the selective enrichment of filterable bacteria. For cycle 0, bacteria were grown on AOC present in original water samples. For the following filtration-regrowth cycles (i = 1, 2, 3, 4), heat-sterilized river water was supplied as fresh AOC. All cycles were done in triplicate samples.

(A)

(B)
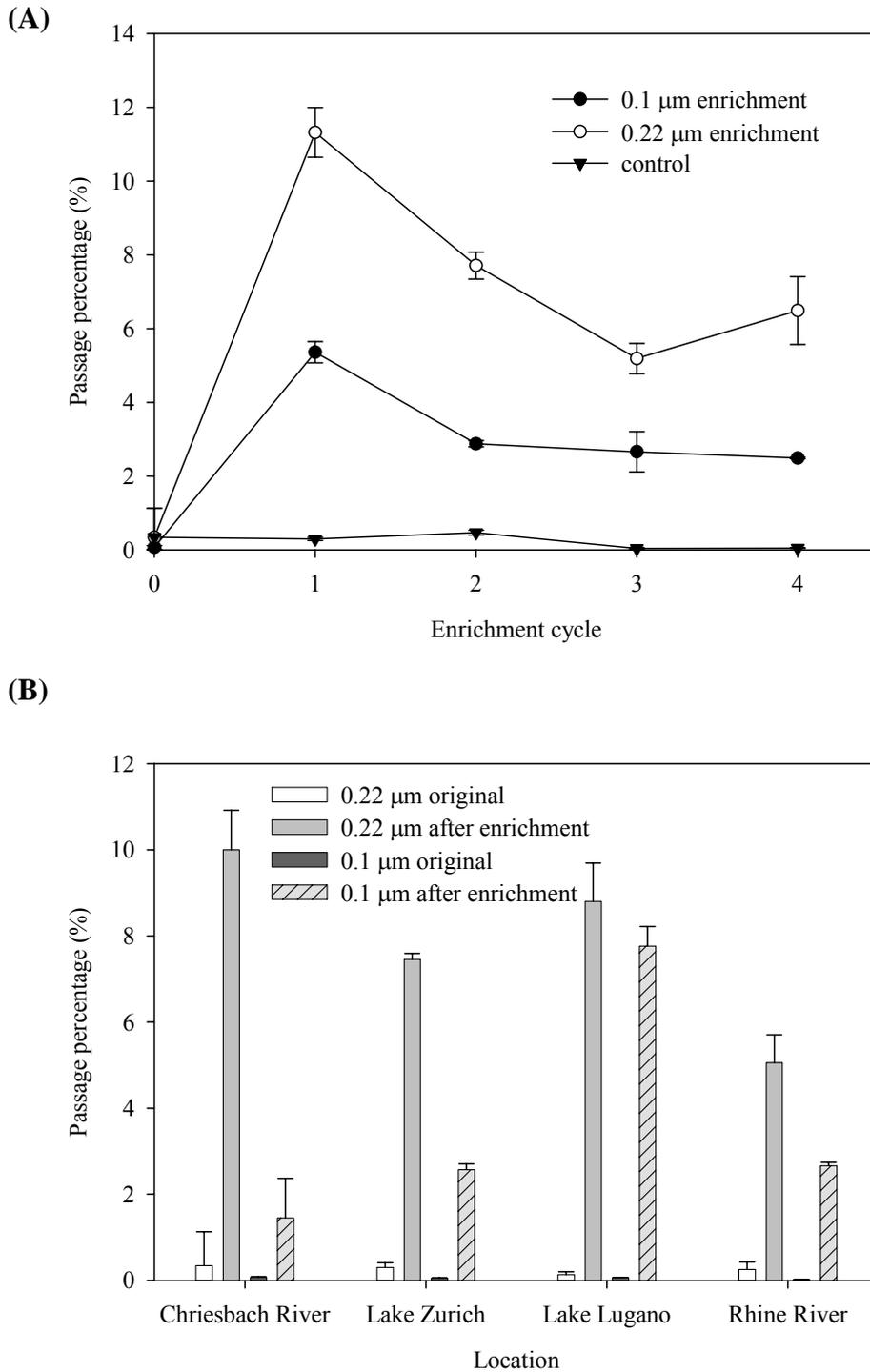
Figure 2.3. Enrichment of filterable bacteria through successive filtration-regrowth cycles. (**A**) Changes in the passage percentage of the 0.22 μm enrichment culture through 0.22 μm filters, the 0.1 μm enrichment culture through 0.1 μm filters and the unfiltered control culture through 0.22 μm filters. (**B**) Comparison of the passage percentage of 0.22 and 0.1 μm enrichment cultures through 0.22 and 0.1 μm filters from four different freshwater samples before and after enrichment. Error bars represent the standard deviation of triplicate samples.
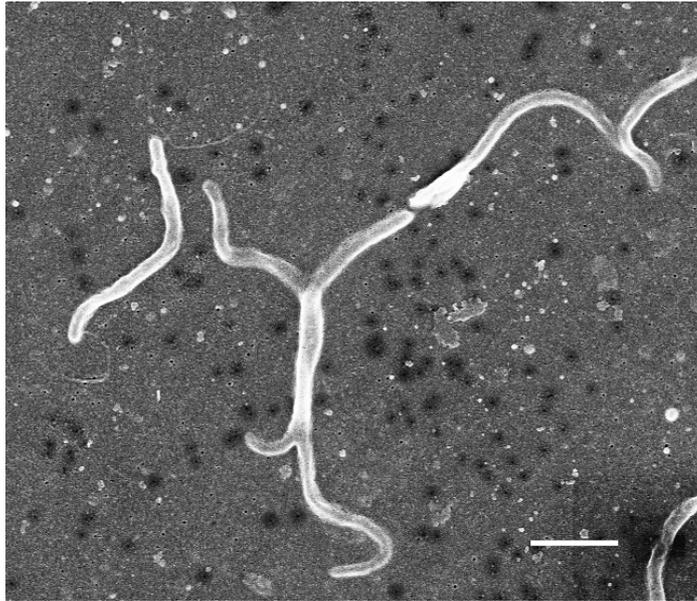
Figure 2.4. Scanning electron micrograph of *Hylemonella gracilis* isolate CB on a membrane filter (0.05 µm pore size). The strain was isolated in this study from freshwater and cultivated with natural AOC. Bar = 1 µm.

Biodiversity of filterable bacterial communities

Both epi-fluorescence and scanning electron microscopic examination of the enrichment cultures revealed a dominant spirillum-shaped bacterial population (Figure 2.4). We were able to isolate four strains with this morphology from both 0.22 and 0.1 µm filtrates of different lakes and rivers on R2A agar and they were all identified as *Hylemonella gracilis* (previously *Aquaspirillum gracile* (Spring et al., 2004) based on their 16S rRNA sequences (>99% similarity). Scanning electron microscopy revealed that the isolate of *H. gracilis* strain was a slender, spirillum shaped cell and with flagella at both ends (Figure 2.4). The average length of *H. gracilis* cells was about 5 µm. Denaturing gradient gel electrophoresis (DGGE) analysis of 16S rRNA gene fragments of water samples before and after enrichment confirmed the observed dominance of *H. gracilis* in enrichment cultures (Figure 2.5A). In addition to the dominance of *H. gracilis*, there were other bands with lower density present in enrichment cultures (Figure 2.5A). Three of these were also sequenced. The sequences indicated that those bacteria belong to the family of *Comamonadaceae* and the order of *Actinomycetales*.

(A)

Pearson correlation
(0.0% - 100%)

Rhine River   original
Lake Zurich   control
Rhine River   control
Chriesbach River   original
Chriesbach River   control
Lake Lugano   control
Lake Zurich   original
Lake Lugano   original
Chriesbach River   enrichment cycle 1
Chriesbach River   enrichment cycle 2
Chriesbach River   enrichment cycle 3
Chriesbach River   enrichment cycle 4
Lake Zurich   enrichment
Rhine River   enrichment
Lake Lugano   enrichment
H. gracilis isolate CB

(B)

Marker

Lake Zürich isolate

Lake Lugano isolate

Chriesbach River isolate

Rhine River isolate

(C)

Figure 2.5. Bacterial community and diversity analysis in all four freshwater samples before and after enrichment. (**A**) DGGE and clustering analysis of the water samples. The distance matrix of all the possible gel tracks within the DGGE pattern was calculated by using the Pearson correlation. Based on the values of the resulting matrix, a cluster analysis was performed and visualised as a dendrogram. (**B**) Rep-PCR of *Hylemonella gracilis* isolated from four different freshwaters. Band patterns indicate the isolates from four locations are different strains of *H. gracilis*, which eliminate the possibility of contamination of *H. gracilis*

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in water samples. (**C**) Lorenz curves based on DGGE analysis. Lorenz curves describe the species evenness within a community. The results show that filtered enrichment cultures have less evenness in diversity than the cultures in the original water.

The DGGE profiles of the enrichment cultures in comparison to its initial composition, illustrate the changes in abundance and diversity of bacteria during the filtration-regrowth cycles. One band becomes dominant in all 0.22 µm enrichment cultures for all four different water samples and this band corresponded with the *H. gracilis* isolate (Figure 2.5A). Species evenness was estimated by Lorenz curves (Versalovic et al., 1994). The more concave the curve, the less even the bacterial population. The enriched cultures showed a less evenly distributed community than the original culture, as the Lorenz curves of the former were more concave (Figure 2.5C). Based on the Lorenz curves, the Gini coefficients (G) were calculated, which clearly showed that the original samples (G = 0.73 ± 0.06) were significantly more even distributed than the enriched cultures (G = 0.40 ± 0.05). The unfiltered controls showed higher diversity than filtered enrichment cultures but they also exhibited a selection of certain bacterial communities, probably selected for by temperature and carbon type. A similar pattern was observed in all water samples tested (Figures 2.5A and C). Repetitive extragenic palindromic-PCR (Rep-PCR) genomic fingerprinting from the isolates and the enrichment cultures indicated that different *H. gracilis* strains were found in different water samples (Figure 2.5B).

Discussion

Filterability and quantification

Micro-pore-sized filters are extensively used by industry and the research community for either quantitative removal of bacteria from waste and drinking water, or to produce sterile products from medical and other liquid solutions. The current sterilization grade is rated by using the industry-accepted bacterial strain *Brevundimonas diminuta* (ATCC 19146) (ASTM, 2005). The standard testing method described by the American Society for Testing and Materials (ASTM) employs *B. diminuta* at a concentration of 10^7 cells per cm^2 of effective

filtration area. The entire filtrate is then filtered through an analytical membrane filter disc and organisms that were not retained by the filter being tested are enumerated according to the visible colonies developing on the analytical membrane when placed on a *B. diminuta* growth-supporting nutrient agar. Such methods have been employed by filter manufacturers and filter users for 15 years (ASTM, 2005). Based on this method, 0.22 µm pore size filters have been, and still are, recognized as standard sterilization filters. Furthermore, 0.1 µm pore size filters are commonly regarded to provide an absolute barrier to aquatic bacteria.

Reports on the existence of micro-filterable bacteria have a long history and go back to 1930s (e.g., Elsaied et al., 2001; Hahn, 2004; Haller et al., 1999; Kendall, 1931; Klieneberger-Nobel, 1951; Lopez and Vela, 1981; Zinsser, 1932). As far as we are aware, our results provided for the first time a systematic quantification of the amount of bacterial cells present in natural freshwaters and drinking waters that can pass through 0.45, 0.22 and 0.1 µm pore size filters (Table 2.1). In general, sterile syringe filters with 0.45 µm pore size provide only a limited, if any, barrier for aquatic bacterial communities. In fact, a considerable fraction of the bacterial communities in surface and drinking water is able to pass through filters with 0.22 and even 0.1 µm pore sizes. Particularly, filterability through 0.1 µm pore size filters has in our opinion never been quantified accurately before. Furthermore, based on our results, single layer syringe filters made of different materials give no significant difference in filtration efficiency. However, it should be noted that the filterability (expressed in percentage) is also a function of the experimental parameters (e.g., filtration volume, original cell concentration). In the present experimental setup, the challenge on filters is below the standard filter testing method requirement (i.e., 10^7 cells per cm^2 of effective filtration area), which excludes the possibility of bacterial passage due to over-loading of the filters. Moreover, the repeatability of the experiments suggests that the findings are not due to random filter damage, but in fact the actual filterability of the bacteria. The data convincingly demonstrated that micro-filterable bacteria are omnipresent in aquatic environments and this observation as such calls for a re-consideration of currently existing filtration sterilization standards.

Growth capacity

Reports on the existence of small, filterable bacteria have recently been criticized as artifacts and it was argued that the self-aggregation of dissolved organic carbon (DOC) may resemble the growth of bacteria and may be confused with bacteria when using fluorescent dyes for

detection (Kerner et al., 2003). Our observations, however, demonstrated not only a considerable part of the aquatic microbial communities pass through micro-pore size filters (Table 2.1), but also that the filtrates contained viable bacterial cells able to proliferate, rather than abiotic aggregations of DOC. Evidence of this is that their growth on AOC is paralleled by an ATP increase and that it can be terminated by the addition of glutaraldehyde (Figure 2.1). Glutaraldehyde is known as an agent that crosslinks proteins but has no effect on physical aggregation. In addition, the fact that two of the tested antibiotics were able to inhibit growth further confirms the microbial nature of the filterable community. The specific growth rates (μ) of the filterable bacterial communities are relatively high and comparable with natural bacterial specific growth rates on AOC in previous report (Hammes and Egli, 2005). The fast growth rates and short lag time suggest minimal, if any, physical damage of the cells during filtration process.

Enrichment and morphological selection by filtration

In the present study, the micro-filterable microbial communities were enriched and each filtration-regrowth cycle produced a community with significantly higher filterability (Figure 2.3). In general, a ten-fold increase of filterability was observed in all enrichment cultures. In fact, a single filtration-regrowth cycle already was sufficient to select for a bacterial community with high filterability as seen in Figure 2.3A. Furthermore, data from the filtration and cultivation experiments (Figure 2.3) rule out the possibility of "leaky filters" (Stockner et al., 1990) as an explanation for the occurrence of bacteria in the filtrates. In case of "leaky filters", random filtration efficiency would be observed instead of the significant increase presented in Figure 2.3. The fact that we can enrich a bacterial community with higher filterability underlines an important biological aspect of the filtration process i.e., that distribution of bacterial shapes present in the liquid solutions plays an important role in the filterability of the bacteria.

The filtration-regrowth cycles not only increased the filterability but also reduced the diversity of the filterable communities (Figure 2.5). Our results indicate that the filtration process resulted in definite selection of bacterial communities with a certain morphology (i.e., slender, spirillum-shaped cells). Previous reports using direct HPC and molecular methods demonstrated that the 0.22 μm filterable bacterial communities mainly comprised of taxa belonging to *Proteobacteria, Bacteroidetes* and *Actinobacteria* (Hahn et al., 2003; Vybiral et

al., 1999; Young, 2006). In all samples we examined, the dominance of *Hylemonella* spp. was recorded in the enrichment cultures (both 0.22 and 0.1 μm enrichments). Furthermore, different strains of *Hylemonella* spp. were found in different water samples (Figure 2.5), which eliminated the possibility of contamination and indicated the significance of the morphology for the observed enrichment of *H. gracilis* by micro-filtration. The presence of *Hylemonella* spp. in filterable bacterial communities was also reported in a few other publications (Anderson and Heffernan, 1965; Hahn et al., 2004; Haller et al., 1999; Shirey and Bissonnette, 1991), which further suggests some unique filterability features of this organism. *Hylemonella* spp. was isolated from 0.45 and 0.22 μm filtrates by heterotrophic plating in those studies. The present study demonstrated for the first time of *Hylemonella* spp. can also pass through 0.1 μm pore size filters. However, our results also indicate that not only *Hylemonella* spp. but also other bacteria than can pass 0.1 μm pore size filters and maybe selected for, depending on the cultivation conditions. In addition to *Hylemonella* spp., bands belonging to members of the family of *Comamonadaceae* and the order of *Actinomycetales* were also observed by DGGE in enrichment cultures (sequence data not shown).

The fact that passage through a membrane filter favored slender spirillum-shaped bacteria also suggests the potential passage of other bacteria with similar morphology. Spirillum-shaped bacteria are commonly distributed in natural aquatic environments (Anderson and Heffernan, 1965; Hahn et al., 2004), even including some pathogenic bacteria, e.g., *Leptospira* and *Treponema*. Rubin and colleagues (1980) reported the isolation of *Leptospira biflexa* in deionized water, which was sterilized by filtration through 0.22 μm pore size membrane filters and ten years earlier the ability of *Treponema pallidum* escaping the entrapment of 0.22 μm filters was also demonstrated (Chandler and Clark, 1970). Furthermore, we have observed a similar shape-dependent enrichment in a bottled drinking water pilot plant during 0.22 μm filtration (data not shown). Our experience, together with previous reports, emphasizes the need for thorough quality control of filter-sterilized reagents, particularly when used for medical applications.

Standard filter-testing procedures

The morphology of *H. gracilis* (Figure 2.4) also differs from what one expects for filterable bacteria. This is most notable when compared to the standard filter-testing bacterium *B. diminuta* that has the shape of a short rod and is known to be able to pass through 0.45 μm

filters. Conventional filter challenge tests employ *B. diminuta* pre-cultured in a growth medium containing a high nutrient concentration (7.5 g/l trypticase peptone and 2.5 g/l yeast extract) (ASTM, 2005). This cultivation approach leads to the formation of "fat and large" cells. However, such copiotrophic growth conditions are rarely encountered in natural aquatic environments and in drinking water, where biodegradable carbon concentrations are typically in the range of 50 to 400 μg/l (Hammes and Egli, 2005). Hence, the majority of the bacterial cells present in marine and freshwater habitats are small (cell volume < 0.3 μm^3) (Schut et al., 1997). Our observations suggested that spirillum-shaped bacterial cells cultivated with natural AOC (Figure 2.4) are much more meaningful candidates for filtration efficiency testing than *B. diminuta* cultivated with a laboratory medium. This calls for a revision of filter-testing and -grading methods and the overall procedure of using filtration for sterilization of liquids. The filter challenging procedures using *H. gracilis* together with technical details will be further investigated.

In conclusion, the data presented here clearly demonstrate and quantify the omnipresence of filterable bacteria in natural freshwater, and specifically highlight their ability to pass through 0.1 μm pore size filters. Furthermore, such filterable bacterial communities are shown to be able to regrow on the natural AOC with relatively high specific growth rates and the selective enrichment of such filterable communities underline the biological nature of this phenomenon. In this respect we expect that this report will have profound implications for preparation, production and handling of all liquid solutions processed by filtration.

Acknowledgements

3. Influence of size, shape and flexibility on bacterial passage through micropore membrane filters

Abstract

Sterilization of fluids by means of micro-filtration is commonly applied in research laboratories as well as in pharmaceutical and industrial processes. Sterile micropore filters are subject to microbiological validation, where *Brevundimonas diminuta* is used as a standard test organism. However, several recent reports on the ubiquitous presence of filterable bacteria in aquatic environments have cast doubt on the accuracy and validity of the standard filter-testing method. Six different bacterial species of various sizes and shapes (*Hylemonella gracilis*, *Escherichia coli*, *Sphingopyxis alaskensis*, *Vibrio cholerae*, *Legionella pneumophila* and *B. diminuta*) were tested for their filterability through sterile micropore filters. In all cases, the slender spirillum-shaped *Hylemonella gracilis* cells showed a superior ability to pass through sterile membrane filters. Our results provide solid evidence that the overall shape (including flexibility), instead of biovolume, is the determining factor for the filterability of bacteria, while cultivation conditions also play a crucial role. Furthermore, the filtration volume has a more important effect on the passage percentage in comparison with other technical variables tested (including flux and filter material). Based on our findings, we recommend a re-evaluation of the grading system for sterile filters, and suggest that the species *Hylemonella* should be considered as an alternative filter-testing organism for the quality assessment of micropore filters.

Introduction

Temperature-sensitive solutions, which cannot be sterilized by autoclaving, are usually sterilized by filtration through membrane filters with pore sizes of 0.22 μm (Eykamp, 1991). This method is generally believed to remove all microorganisms (except viruses) from the filtered solutions and is widely used in scientific research, medical and industrial applications. However, filterable bacteria (bacteria that can pass through micropore membrane filters) have been detected in various aquatic systems, e.g., marine water, freshwater, and drinking water (Anderson et al., 1985; Elsaied et al., 2001; Hahn, 2004; Haller et al., 1999; Hood and MacDonell, 1987; Lopez and Vela, 1981; Shirey and Bissonnette, 1991; Wang et al., 2007). Depending on the detection method and water source, different groups of filterable bacteria have been reported. Bacteria of the α- and γ-subclass of the *Proteobacteria* and the *Cytophaga-Flavobacterium-Bacteroides* were reported to be the major components in 0.2 μm filterable prokaryotic communities in marine water (Haller et al., 1999; Vybiral et al., 1999). In freshwaters, members of β-*proteobacteria* have been reported to be present in filterable prokaryotic communities (Hahn, 2004; Wang et al., 2007). Furthermore, filterable bacteria were reported to have various shapes, ranging from small cocci, short rods to spirilla (Hahn, 2004; Wang et al., 2007; Young, 2006).

The habitats, in which filterable bacteria are most commonly detected, are oligotrophic marine and freshwater environments, including drinking water. The biodegradable carbon concentrations in such environments are typically in the range of 50 to 400 μg/l (Münster, 1993). Sizes of bacteria are known to change with environmental conditions (Morita, 1997), and in oligotrophic environments most cells are significantly smaller than cells cultivated in the laboratory. One of the advantages to the bacterium of being small is an increase of its surface/volume ratio so that the proportion of transporters in relation to the cytoplasmic volume is higher. However, despite the oligotrophic natural habitat of filterable bacteria, the cultivation of standard filter-testing organisms for filter quality assessment is carried out in conventional nutrient-rich media according to American Society for Testing and Materials (ASTM, 2005). The standard filter-testing method employs a rod-shaped bacterium, *B. diminuta*, as the testing organism and the procedure requires that it is cultivated in a nutrient-rich medium with a dissolved organic carbon (DOC) concentration in the level of g-C/L (ASTM, 2005). This method of cultivation usually leads to the formation of bigger cells and

would hence result in an underestimation of the amount of bacteria grown under natural conditions that are potentially able to pass through micropore filters. Furthermore, employing a bacterium with one specific shape (rod) for testing micropore filters disregards the possible impact of cell shape on bacterial filterability.

In a previous report we have demonstrated that slender, spirillum-shaped bacteria were enriched during filtration-re-growth cycles (Wang et al., 2007). The results suggested that shape might be one of the key factors determining the filterability of bacteria. In the present study, various bacterial species were tested for their retention by filters with pore size in the range of 0.1 - 0.45 μm, specifically to determine the effect of cell shape and size on bacterial filterability. Moreover, some technical aspects (including volume, flux and filter material) of micro-filtration were investigated. Our results provide solid evidence of the crucial impact of cell shape and flexibility on bacterial filterability, and as such, call for re-evaluation of the current standard filter-testing method and sterile filtration practices in general.

Materials and Methods

Microorganisms and culture conditions

Five different bacterial species, i.e., *Hylemonella gracilis* isolate CB (Wang et al., 2007), *Escherichia coli* K-12 MG 1655, *Sphingopyxis alaskensis* RB2256 (Eguchi et al., 2001), *Vibrio cholerae* O1 (Biovar Eltor, serotype Ogawa, strain Nent 720-95), *Legionella pneumophila* sg1 (ATCC 33152) and *Brevundimonas diminuta* (ATCC 19146) were used in the current study. All bacterial strains (except *L. pneumophila*) were cultivated with natural assimilable organic carbon (AOC). For this, water from a shallow stream (Chriesbach, Dübendorf, Switzerland) was sampled with a 1 l Duran flask (Schott, Germany), autoclaved (20 min, 121°C) and filtered through sterile syringe membrane filters with a pore size of 0.1 μm (PVDF, Millipore, USA) to remove cells and precipitations. The sterilised river water was then used as the growth medium for the strains. Bacterial strains were inoculated separately into the medium with initial cell concentrations in the range of 0.5 - 1 × 10^4 cells/ml, and then incubated at 30°C for four days until stationary phase was reached. *L. pneumophila,* which was not able to grow on river water AOC, were cultivated in Charcoal Yeast Extract (CYE)

buffered medium for three days at 37°C until stationary phase was reached and then starved in sterile mineral water (Evian, France) for four days.

Passage percentage

Cultures from each bacterial species (30 ml, 2-5 $\times$ 10^5 cells/ml) were filtered separately through sterile syringe filters with 0.45, 0.22 and 0.1 µm pore size (PVDF, Millipore, USA) at a flux of 1333.3 l/(m²$\times$h). For pressing the water samples through filters, a syringe pump with a 60 ml Luer-Lok^{TM} Syringe (BD, Franklin Lakes, NJ, USA) was used in all experiments. The filtration procedure was performed under sterile conditions in a laminar flow bench. Filtrates were collected in 40 ml autoclaved glass vials, and 1 ml was used for enumeration. Measurement of bacterial cell concentrations before and after filtration was carried out with flow cytometry (see below). For each pore size, three separate filters were tested, and each filter was used only once. The percentage of cells not retained by the filter was expressed as the passage percentage as described previously (Equation 3.1; Wang et al., 2007).

Passage percentage (%) = TC_a / TC_b x 100%   Equation 3.1

Where TC_b stands for total cell concentration (cells/ml) measured before filtration and TC_a for total cell concentration (cells/ml) after filtration.

Technical aspects of filtration

Several technical aspects of filtration were tested using only *H. gracilis* isolate CB, cultivated as described above. To evaluate the effect of filtration volume on passage percentage, 60 ml of *H. gracilis* (5 $\times$ 10^5 cells/ml) was filtered through a 0.22 µm pore size sterile filter (PVDF) at a flux of 1333.3 l/(m²$\times$h). Samples (1 ml) of the filtrate directly below the filter were taken after every 5th ml, and the bacterial concentration was measured with flow cytometry as described below. To assess the effect of filter material on passage percentage, three different types of sterile membrane filters (polycarbonate, PVDF and cellulose acetate) with the same pore size (0.22 µm) were tested. For each filter, 30 ml of culture (5 $\times$ 10^5 cells/ml) was filtered at a flux of 1333.3 l/(m²$\times$h), and the total cell concentration in the filtrate was measured with flow cytometry (see below). The effect of flux on passage percentage was also

investigated. Fluxes of 26.7, 133.3, 266.7, 1333.3, 5333.2 l/(m^2×h) were used. For each flux, 30 ml of culture containing 5×10^5 cells/ml of *H. gracilis* was filtered through 0.22 µm sterile syringe filters (PVDF, Millipore, USA). The total cell concentration in the filtrates was enumerated. All enumeration was carried out with flow cytometry as described below. Filtration efficiencies were calculated as described above. All experiments were done in triplicate.

Total cell concentration measurements with flow cytometry

All samples (1 ml) were stained directly with 10 µL SYBR® Green I stain (1:100 dilution in dimethyl sulfoxide; Molecular Probes, USA), mixed and incubated in the dark for at least 15 minutes before measurement. Flow cytometry was performed using a PASIII flow cytometer (Partec, Münster, Germany) equipped with a 25 mW argon ion laser emitting at a fixed wavelength of 488 nm. A more detailed protocol of the detection and enumeration method was described by Hammes and co-workers (Hammes et al., 2008). Where necessary, samples were diluted prior to measurement in cell-free water, so that the concentration measured in the flow cytometer was always less that 3×10^5 counts/ml. The detection limit of the instrument is below 500 cells/ml (Hammes et al., 2008).

Scanning electronic microscopy

Stationary phase cultures of each bacterial species were filtered (15 ml, cell concentration in the range of $1 - 3 \times 10^5$ cells/ml) onto a membrane filter (GTTP, Millipore, USA) with a 0.2 µm pore size. The bacteria retained by the filters were then fixed with 1% glutaraldehyde for 30 minutes and washed with sterile river water (cell free). Small pieces (5×5 mm) of each filter were cut from the filters and mounted with double-sided scotch tape to a scanning electron microscopy (SEM) specimen stub. The samples were sputter coated with 10 nm gold and examined by SEM (Hitachi S-4800 FEG, Japan), operated at 5 kV Accelerating Voltage. The cell volume of the individual bacterial cells was calculated based on the measurements from SEM pictures (Equation 3.2) on the principle of a rod-shaped particle with round ends.

$$\text{Cell volume } (\mu m^3) = \frac{4}{3} \times \pi \times r^3 + \pi \times r^2 \times (L - 2r) \qquad \text{Equation 3.2}$$

where r represents the half of the smallest width and L represents the length of the bacterial cell. At least 30 cells for each bacterial species were measured and the average cell volume was used for comparison.

Results and Discussion

Bacterial size and shape comparison

In some of the literature, filterable bacteria were also defined as ultramicrobacteria, which have by definition a cellular volume of less than 0.1 μm^3 (Schut et al., 1997). It is a common misunderstanding that the size of bacteria (in terms of biovolume) relates to their ability to pass through filters. Hence, the choice of *B. diminuta* (biovolume = ca. 0.08 μm^3) as standard filter-testing organism (ASTM, 2005) is not entirely surprising. However, the results we reported here indicate that bacterial shape, rather than their absolute size, is the key factor determining the ability of bacteria to pass through filters.

Six bacterial species with various shapes and sizes were compared for their filterability through sterile syringe filters with 0.1, 0.22 and 0.45 μm pore size, respectively (Table 3.1). Two main factors that influence bacterial filterability are cell size (biovolume) and cell shape. One assumes generally that the smaller the cell's biovolume, the higher its filterability. However, our results showed that this is not always the case. *H. gracilis*, which had a relatively large biovolume (about 0.12 μm^3), has a clear advantage in passing through sterile membrane filters of all pore sizes (Table 3.1). In contrast, other bacterial species showed much lower filterability. *E. coli* K12, a well-known laboratory strain, showed the lowest filterability (passage percentage below 0.2% for all the filters tested) among the six bacterial species investigated, although it is not the biggest organism in terms of biovolume (0.47 μm^3) (Table 3.1). A similar filterability as for *E. coli* was observed for *V. cholerae* (0.11 μm^3) and *L. pneumophila* (0.58 μm^3) (Table 3.1). In comparison, bacterial species with smaller biovolumes, i.e., *S. alaskensis* (0.07 μm^3) and *B. diminuta* (0.08 μm^3), showed a higher filterability through 0.45 μm pore-size filters, but there was no significant difference between all species (except for *H. gracilis*) in the passage percentage through 0.22 μm pore-size filters.

Table 3.1. Passage percentage of different bacteria through sterile membrane filter with different pore sizes. Data are presented as mean ± standard deviation (n = 3). Average cell volume was calculated according to the electronic microscopic measurements done in the current study.

	Shape	Smallest width (μm)	Cell volume (μm³)	Passage percentage		
				0.45 μm	0.22 μm	0.1 μm
Escherichia coli	rod	0.60	0.47	0.15% ± 0.01%	0.03% ± 0.01%	ND
Sphingopyxis alaskensis	rod	0.31	0.07	3.48% ± 0.85%	0.03% ± 0.01%	ND
Brevundimonas diminuta	short-rod	0.40	0.08	1.47% ± 0.28%	0.02% ± 0.03%	ND
Vibrio cholerae	rod	0.42	0.11	0.22% ± 0.01%	0.03% ± 0.02%	ND
Legionella pneumophila	long-rod	0.55	0.58	1.04% ± 0.52%	0.02% ± 0.01%	ND
Hylemonella gracilis	spirillum	0.20	0.12	46.51% ± 0.81%	7.33% ± 0.01%	0.20% ± 0.002%
Hylemonella gracilis fixed[a]	spirillum	0.20	0.12	0.51% ± 0.05%	ND	ND

[a] *Hylemonella gracilis* cells fixed by 1% glutaraldehyde

ND: not detectable (cell concentration is below detection limit of flow cytometry used in the current study)

S. alaskensis is regarded a typical ultramicrobacterium, which has a volume less than 0.1 μm^3 and retains this volume irrespective of growth conditions (Cavicchioli and Ostrowski, 2003). Hence, our results indicate that an organism classified as an ultramicrobacterium is not necessarily equal to a bacterium with high filterability. As the standard filter-testing bacterium, *B. diminuta* is supposed to provide assurance that the membrane filters meet the critical performance criteria (i.e., complete retention of *B. diminuta* at a concentration of 10^7 organisms per square centimeter of filter surface area) of a sterilizing filter (ASTM, 2005). However, as shown in Table 3.1, passage of *H. gracilis*, although it has a biovolume larger than *B. diminuta*, was detected in the filtrate of all such "sterilizing" filters. In general, the filterability of all tested bacterial species, regardless of their cell biovolume, was more than 10-times lower than that of *H. gracilis*.

It is likely that some of the bacterial cells shrink to some extent during preparation for SEM. Therefore, the actual cell volume could be slightly larger than what we calculated here. However, SEM images are still the best option available to calculate the cell biovolume accurately under the low cell density conditions (less than 10 cells/picture) in comparison with other microscopic methods. For example, with fluorescence microscopy, the fluorescent halo around the bacterial cells and/or location-specific binding of the fluorochrome make it difficult to estimate where the actual cell boundary lies.

The second factor that influences the bacterial filterability is cell shape. In the context of cell shape, there are two parameters that should be considered: the smallest cell width and the overall cell shape (i.e., coccus, rod, spirillum etc.). Our results showed that both parameters play important roles in determining bacterial filterability. The smallest cell width determines the basic potential of filterability. It is evident that if the smallest cell width is bigger than the filter pore diameter, it is highly unlikely for the cell to pass (Figure 3.1). We observed a negative trend between the smallest cell widths against the passage percentage through 0.45 μm pore size filters (Figure 3.2). For bacteria that possess similar overall shapes (small non-flexible rods), the smaller their smallest widths are, the better chance they have to pass through filters (Figure 3.2). Furthermore, the passage percentage of *H. gracilis* (46.5%) is more than 10-times higher than the passage percentage observed with other bacteria (1 - 5%), while its smallest width is less than 5-times thinner than other bacteria (Table 3.1, Figure 3.2). The reason for the substantial increase is ascribed to the unique overall cell shape (long, slender spirillum) of *H. gracilis*. Our results suggest that once the smallest cell width is small

enough to fit into the filter pores, it is the overall cell shape that determines the cell's passage through the filter.

The significance of overall cell shape in determining the filterability of bacteria is further illustrated visually in Figure 3.1. In this example, a *H. gracilis* cell is oriented with one side inside a pore, which visualizes their potential manner of passing through the pores (Figure 3.1a). All cells of *H. gracilis* observed with SEM showed similar positions, with either one or two ends inside filter pores (data not shown). Naturally, the cells that we observed here are those that did not manage to pass through the filter. The possible explanation for their orientation half-inside the filter pores may lie in the fact that such slender, long, spirillum-shaped bacterial cells (Figure 3.1a) align better in the water flow velocity vector field than rod-shaped cells (Figure 3.1c). We hypothesize that the former align perfectly length-wise with the water flow and thus hit the filter pore directly, while the latter tumble in the flow velocity vector field and hit the filter randomly. Furthermore, the flexibility of *H. gracilis* cells may be a major contributing factor to its superior filterability. It has been reported that under stretching-tension in glycerol-gelatin, *Spirillum* species were stretched up to three-times their original length without breaking; when the tension was released, the cell walls and cell contents were able to return to their original size and shape (Isaac and Ware, 1974). When *H. gracilis* cells travel through the filter pore channels, they should face a similar stretching-tension. Therefore, their flexibility possibly enables their passage through filter pores. To test the effect of flexibility, glutaraldehyde (1% end-concentration) was added to a *H. gracilis* culture. Glutaraldehyde is known to cross-link the free nitrogen of amino acids of proteins with aldehyde groups on both ends of the glutaraldehyde polymer and, therefore, significantly reduced the cell's flexibility/elasticity (Hutter et al., 2005; Sullivan et al., 2007). We have observed significantly reduced passage percentage of such fixed *H. gracilis* cells: only 0.5% passed through 0.45 µm filters and the number of bacterial cells which passed through 0.22 and 0.1 µm filters decreased from 7.3% and 0.2%, respectively, to below the detection limit (Table 3.1).
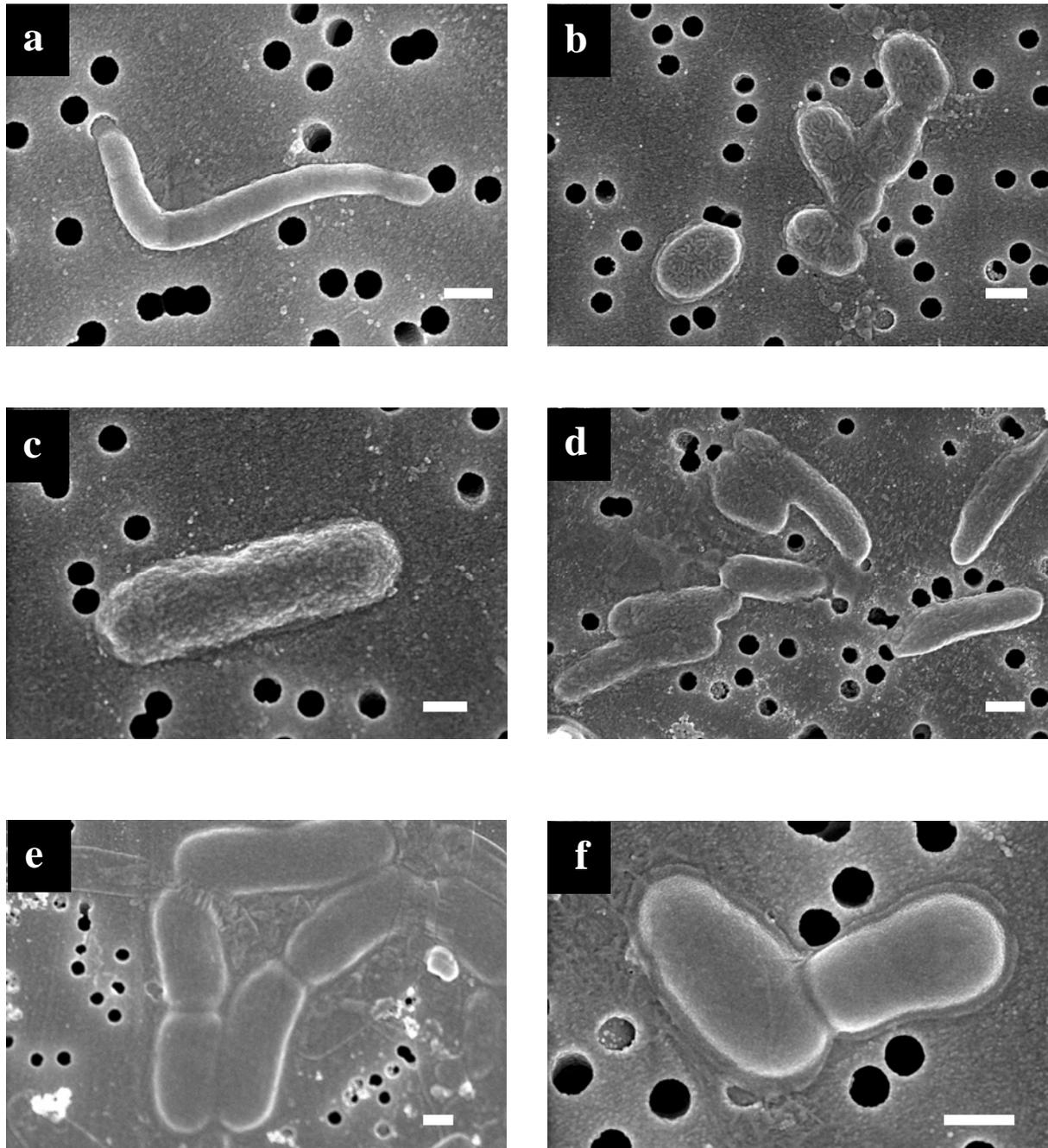
Figure 3.1. Scanning electron micrographs of six different bacterial species on the same type of membrane filters (polycarbonate, 0.2 µm). (**a**) *H. gracilis*; (**b**) *B. dimunita*; (**c**) *E. coli*; (**d**) *S. alaskensis*; (**e**) *L. pneumophila*; (**f**) *V. cholerae*. The scale bar represents 300 nm on each image.
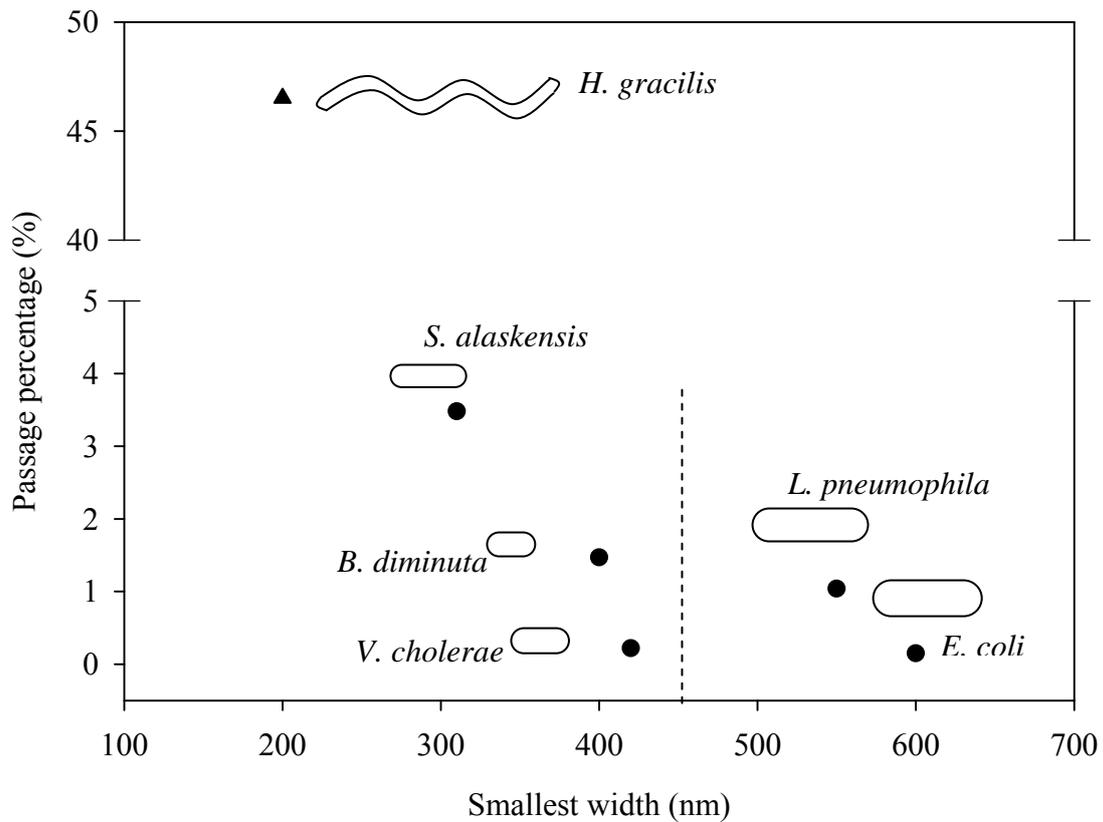
Figure 3.2. Correlation between the smallest width of bacterial cells (in the range of 150 to 700 nm) and their filterability through 0.45 μm pore size filters. The dashed line represents the diameter of the filter pore size on the x-axis. Drawings indicate the theoretical shape of the bacteria proportional to each other.

Among the six bacterial species tested, *H. gracilis* is the only one that has the ability to pass through filters with 0.1 μm pore size, which is commonly assumed to provide the absolute barrier to bacterial cells. It was previously speculated that so-called "nanobacteria" can also pass through 0.1 μm filters based on their size (Kajander and Ciftcioglu, 1998). However, no experimental data on their filterability was provided. To our knowledge, *H. gracilis* is the first bacterial species that has been shown to be able to pass through 0.1 μm pore size filters (Wang et al., 2007).

Technical aspects of filtration

In a previous report, we showed that cycles of filtration and re-growth resulted in the enrichment of the slender, spirillum-shaped bacteria of the genospecies *H. gracilis* from various freshwater samples (Wang et al., 2007). This genospecies showed superior ability of passing through membrane filters, which is confirmed by data presented in Table 3.1. Hence, the impact of some technical aspects (i.e., flux, filtration volume and filter material) on the passage of *H. gracilis* through 0.22 μm pore-size filters was examined in detail. The first mechanical variable tested was the volumetric effect. As shown in Figure 3.3, volume has a clear effect on the filterability of *H. gracilis*. A significant increase of passage percentage was recorded as a larger volume of culture was filtered (Figure 3.3a and b). Two different cell concentrations of *H. gracilis* and one cell concentration of *B. diminuta* were tested and a similar trend was observed. A possible explanation for this phenomenon is the change of bacterial concentration directly above the filter surface. At the beginning of filtration, the concentration directly above the filter surface is that of the original concentration in the solution. As the filtered volume increases, the concentration of bacteria above the filter surface increases due to retention of cells. Therefore, there are statistically more bacteria to hit the filter pores. Once the pores are saturated with bacterial cells, the passage percentage will from thereon decrease due to clogging. Such a clogging effect was reached after 50 ml for the high concentration culture (Figure 3.3a), while for the low concentration culture, saturation was not reached for the volumes tested. Interestingly, we have observed that for both concentrations, the passage percentage increased very slowly during the first 5 ml (Figure 3.3a). The reason for this phenomenon may lie in the fact that the smallest width of *H. gracilis* cells is very close to the pore size of the filter, which may cause a "lag phase" in time for the bacteria to "squeeze" through the membrane pore channel. In contrast, such a "lag phase" was not observed when *H. gracilis* cells were filtered through 0.45 μm filters (Figure 3.3b). It should be pointed out that the current study focused on the impact of technical variables encountered during real practice. The modeling of the filtration process is beyond the scope of the current study and a defined flow channel would be needed for this purpose.
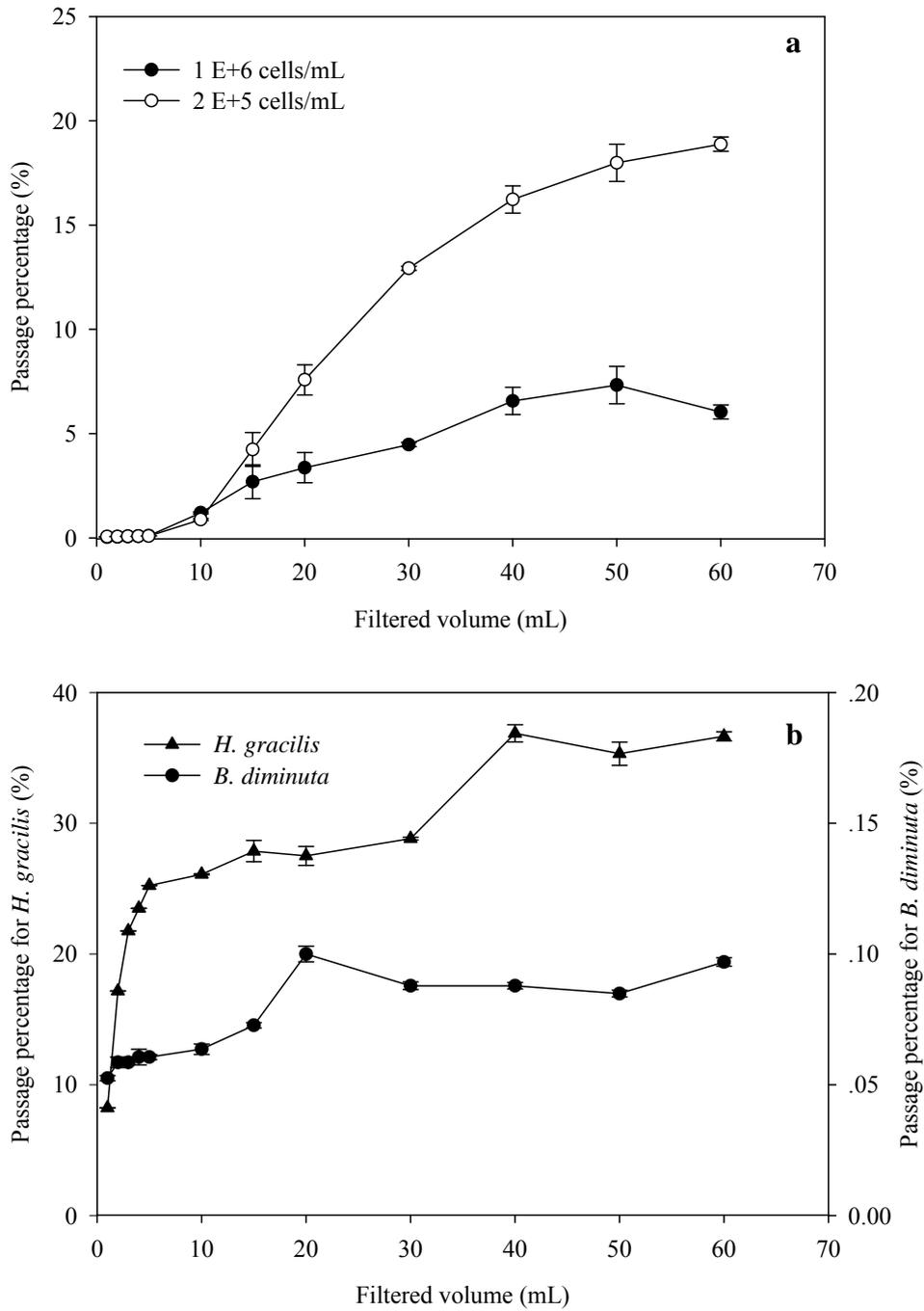
Figure 3.3. The effect of filtered volume on the passage percentage. (**a**) *H. gracilis* through 0.22 μm filters (two different concentrations of *H. gracilis* were tested); (**b**) Comparison between the *H. gracilis* and *B. diminuta* through 0.45 μm filters. Error bars indicate standard deviation on triplicate samples.
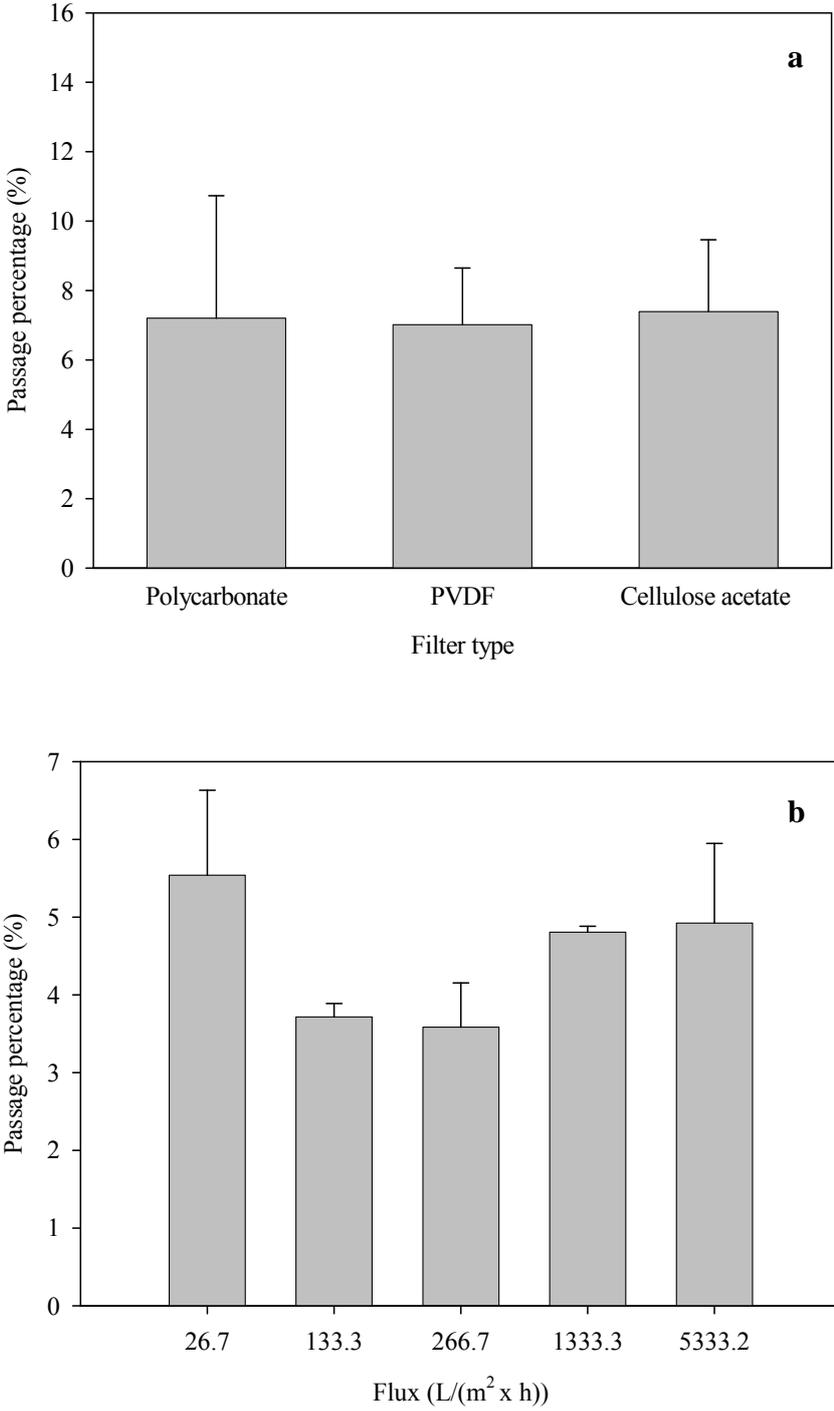
Figure 3.4. Technical aspects for the passage percentage of *H. gracilis* through 0.22 μm filters. (**a**) the effect of filter type; (**b**) the effect of flux. Error bars indicate standard deviation on triplicate samples.

Furthermore, filters of the same pore size-rating but made of different materials were tested (i.e., polycarbonate, PVDF and cellulose acetate). Filters made of polycarbonate have perfectly round pores while those made of PVDF and cellulose acetate have mesh structures. An average passage percentage of 7% was consistently observed with all three types of filters (Figure 3.4a), indicating there is no significant effect of filter material on passage percentage. Moreover, different fluxes were tested, ranging from 26.7 to 5333.2 $l/(m^2 \times h)$ (Figure 3.4b). The standard filter-testing method employs a flux of 0.5 to 1.0 GPM (gallons per min) per ft^2 of effective filtration area (≈ 2400 $l/(m^2 \times h)$) (ASTM, 2005), which is higher than most of the fluxes applied in the current study. The purpose of including an even higher flux (i.e., 5333.2 $l/(m^2 \times h)$) is to represent the flux that one may encounter during hand-filtration in real practice. Regression analysis was applied for accessing the difference among various fluxes. No significant difference in the results at various filtration fluxes were found ($p = 0.98$, $n = 5$).

Cultivation conditions used to grow bacteria for filter challenging tests

In the current study, all bacterial species (except *L. pneumophila*) were cultivated in a low nutrient concentration medium (autoclaved river water) with assimilable organic carbon concentrations in the range of 200 to 400 µg/l, which is comparable to the biodegradable carbon concentrations found in freshwater environments (Hammes and Egli, 2005; Münster, 1993). In contrast, the conventional filter challenge tests employ a growth medium containing a much higher nutrient concentration (7.5 g/l trypticase peptone and 2.5 g/l yeast extract) (ASTM, 2005). In general, microbes have the ability to adjust their biovolumes under different cultivation conditions (Morita, 1997). For instance, the typical cell size of *E. coli* under laboratory conditions is about 0.9 µm^3, with the smallest width of 0.8 µm (Cavicchioli and Ostrowski, 2003), which is significantly larger and fatter than that cultivated with nutrient-poor medium (Table 3.1 and Figure 3.1). It has been shown that several isolated filterable bacteria were no longer filterable after laboratory cultivation (Anderson and Heffernan, 1965). In this respect one should recall that most solutions to be filtered-sterilized would typically be oligotrophic: e.g. marine water for ecological studies (Li and Dickie, 1985), medical solutions (Rubin et al., 1980) and groundwater supplies (Lillis and Bissonnette, 2001). Our observations suggest that, for such applications, nutrient-poor media (with natural AOC) are much more meaningful cultivation media for filter validation testing than a laboratory nutrient-rich medium. Although the former media may not yet be standardized for industrial

applications, it does offer the opportunity to provide a suitable filter validation test for low-nutrient content liquid (e.g., drinking water, surface water etc.).

In conclusion, *H. gracilis* has shown superior filterability in comparison with other bacterial species tested. The data presented here clearly demonstrate the essential role of bacterial shape and flexibility for its filterability. Furthermore, nutrient-poor medium has proven to be a more suitable medium for cultivation of bacteria used for testing their filterability. Based on the results obtained in this study, we propose the usage of *H. gracilis* as an alternative standard filter-testing bacterium and recommend the application of filters with smaller than 0.1 μm pore size if absolute retention of bacterial cells is required.

Acknowledgement

4. The impact of industrial-scale cartridge filtration on the native microbial communities in groundwater

Abstract

Groundwater is a major source for bottled water, which is increasingly consumed all over the world. Some categories of bottled water can be subjected to treatments such as disinfection prior to bottling. In the current study, we present the quantitative impact of industrial scale micro-filtration (0.22 µm pore size) on native microbial communities of groundwater and evaluate subsequent microbial growth after bottling. Two separate groundwater aquifers were tested. Flow-cytometric total cell concentration (TCC) and total adenosine tri-phosphate (ATP) analysis were used to quantify microbial abundance. The TCC of the native microbial community in both aquifers was in the range of $10^3 - 10^4$ cells/ml. Up to 10% of the native microbial community was able to pass through the cartridge filtration units (0.22 µm pore size) installed at both aquifers. In addition, all samples (either with or without 0.22 µm filtration) showed significant growth after bottling and storage, reaching average final concentrations of $1 - 3 \times 10^5$ cells/ml. However, less growth was observed in carbon-free glassware than in standard PET bottles. Furthermore, our results showed that filtration and bottling can alter the microbial community patterns as observed with flow cytometry. The current study established that industrial scale micro-filtration cannot serve as an absolute barrier for the native microbial community and provided significant insight to the impact of filtration and bottling on microbial concentrations in bottled water.

Introduction

The bottled water market currently has an annual growth rate of 25% in North America, which is parallel to the growth of this industry all over the world (Warburton and Austin, 1997). "Natural Mineral Waters", "Waters defined by origin" (e.g., spring water), and "Prepared waters" are three different categories of bottled water. The first two categories allude to water that generally originate from groundwater aquifers. However, various treatment guidelines are applied in different countries. For example, according to the European Community Directive (1980), "Natural Mineral Waters" are not sterilized, pasteurised or otherwise treated to remove or destroy microorganisms. In contrast, "natural mineral water" in North America may be disinfected prior to bottling. Where allowed, micro-filtration is one of the major processes applied in the treatment of bottled water, either alone or as a part of the multi-barrier approach to insure complete microbiological safety of the finished product. Micro-filtration (0.22 μm pore size) is generally regarded as a method sufficient to remove all microorganisms except viruses. However, recent reports have shown that a considerable percentage of the native microbial community from various natural freshwater sources can pass through micro-pore size filters (Hahn, 2004; Wang et al., 2007). Thus it is important to evaluate the impact of an industrial filtration step on the microbial safety and general quality of bottled water.

Groundwater is a typical oligotrophic ecosystem with very low concentrations of natural organic matter and slow turn-over times. The microbial communities in those environments are generally present at low cell concentrations and in a starvation stage (Morita, 1997). However, once such a water is subjected to bottling, the microorganisms are able to re-grow due to increased temperature together with a possible minor increase of organic matter originating from the bottling and processing procedures. It has been reported that the microbial abundance in bottled water increases after bottling during 1-3 weeks of storage (Arms and Sutherland, 1999; Leclerc and Moreau, 2002).

The majority of microbiological studies on bottled water have been done using the conventional heterotrophic plate count (HPC) method. However, it has for long been known that only a small proportion of the metabolically active microorganisms present in drinking water can grow and be detected by the standard HPC technique (Allen et al., 2004). The long

history of HPC-testing used for validating water treatment processes is now challenged by new techniques, especially the determination of total microbial cell concentration using flow cytometry (FCM), which allows detecting and quantifying all microoganisms regardless of culturability (Hammes et al., 2008). Flow cytometry has a tremendous potential as a descriptive tool in the bottled water industry. Its successful applications for direct enumeration of the total cell concentrations in drinking water (Hammes et al., 2008) and surface water (Lebaron et al., 1998; Wang et al., 2007), detection of bacterial viability (Hoefel et al., 2003; Berney et al., 2006) and enumeration of specifically targeted cells with antibodies (Vital et al., 2007) have been reported.

Here we present the impact of industrial scale micro-filtration on the native microbial communities of groundwater. Flow cytometric total cell concentration and assimilable organic carbon concentrations were monitored for a one-year period from two completely separated sources of groundwater. The objective of the current study was to evaluate quantitative impact of a micro-filtration step on the concentration of the native microbial communities, and to investigate microbial growth after bottling.

Materials and Methods

Water sources and sampling

Two completely separate groundwater aquifers (GW I and GW II) were tested in the current study. Identical cartridge filtration units (Fluorodyne AB1DFL7WH4; 0.22 µm pore size), were installed at both locations. The units were validated for removal of *Brevundimonas diminuta* (10^7 cells per cm^2) according to ASTM F 838-05. The filtration units at both locations were operated at a constant flow rate of 800 l/h, and were cleaned and disinfected once every two weeks. Sampling started in October 2006; the first period of sampling was from October 2006 to January 2007, and the second period was from February 2007 to September 2007. During the second period, water samples from source GW II were taken from a different well connected to the same source. Samples (6 replicates for each source) were taken in commercially-use PET bottles (1.5 l; sterilized by gamma radiation) from the source water (upstream of the filtration unit) and filtered water (downstream of the filtration

unit). In the second sampling period, additional samples were taken in carbon-free glass bottles (see below) (3 replicates for both the source water and filtered water). Samples were transported in cooling boxes kept at low temperature (not exceeding 8°C). All samples were processed directly upon arrival (within 24 hours after sampling). 1 ml was taken from each replicate for total cell concentration measurements using flow cytometry (see below) and 0.5 ml was taken from each bottle for total ATP analysis (see below). After analysis, samples were incubated at 25°C without shaking for 21 days. After 21 days of incubation, all samples were analyzed again for total cell concentration and total ATP concentration.

Flow cytometry total cell concentration measurements

Flow cytometric measurements were done as described by Hammes and co-workers (2008). Microbial cells were stained with 10 μl/ml SYBR® Green I (1:100 dilution in dimethyl sulphoxide; Molecular Probes), and incubated in the dark for at least 15 minutes before measurement. When necessary, samples were diluted just before measurement in cell-free water, so that the concentration measured in the flow cytometer was always less that 2×10^5 cells/ml. Flow cytometry was performed using a CyFlow® space flow cytometer (Partec, Hamburg, Germany) equipped with a 200 mW argon ion laser, emitting at a fixed wavelength of 488 nm, and volumetric counting hardware. Green fluorescence was collected at 520 nm, and red fluorescence collected at 630 nm and all data were processed with the Flowmax software (Partec). The trigger was set on green fluorescence for all measurements, and data were collected as 2-parameter dot plots. Samples were measured in triplicate. Microbial cell concentration was also determined separately for two specific clusters (low (LNA) and high (HNA) nucleic acid content bacteria; see also Figure 4.4) as described earlier (Gasol et al., 1999; Lebaron et al., 2001). The detection limit of the instrument used in this study was about 200 cells/ml (Hammes et al., 2008).

Total adenosine tri-phosphate (ATP) measurements

Total ATP was determined as described in Berney and co-workers (2006) using the BacTiter-Glo™ reagent (Promega Corporation, Madison, WI, USA) and a luminometer (Glomax, Turner Biosystems, Sunnyvale, CA). The data were collected as relative light units (RLU) and converted to ATP (nM) by means of a calibration curve made with a known rATP standard

(Promega). ATP was measured in triplicate for all samples, and the detection limit of the measurement was about 0.01 nM ATP with a standard error of $8 - 10\%$.

Assimilable organic carbon (AOC) measurements

Borosilicate glass sampling bottles (250 ml) with glass caps were used for sampling. Sterile and carbon-free glassware was prepared as described previously (Hammes and Egli, 2005). Bottles were transported to both experimental sites in special cooling boxes three days before sampling. The AOC method used in this study was adapted from the method of Hammes and Egli (2005). In short, the cell concentration of the native microbial community was measured at T = 0, and this community was then allowed to grow in the water until stationary phase was reached and all AOC had been consumed. The final cell concentration at stationary phase (normally measured after 21 days incubation at 25°C) was then converted to an AOC concentration (Equation 4.1). A constant value of 1×10^7 bacteria per µg of AOC was used for the conversion of natural microbial community growth to AOC values (Hammes et al., 2006; Vital et al., 2007).

$$AOC\ (\mu g/l) = \frac{\text{final cell concentration (cells/l)}}{\text{conversion factor } (1 \times 10^7 \text{ cells}/\mu g)} \qquad \text{Equation 4.1}$$

Growth curves of the native microbial communities in PET bottles

Source and filtered water from both groundwater aquifers were incubated in PET bottles separately at 25°C. Samples (1 ml) were taken at regular time intervals for 21 days and analyzed with flow cytometry as described above. The specific growth rate (µ) based on cell number increase in each sample was determined as follows (Equation 4.2):

$$\mu = (Ln(n_t) - Ln(n_0))/\Delta t \qquad \text{Equation 4.2}$$

where n_t, n_0 are the cell concentrations measured at two subsequent time points and Δt is the expired time interval between these points.

Results and Discussion

Filtration efficiency

Filtration is one of the few treatments that can be applied to some types of bottled water, either alone or as a part of the multi-barrier approach to ensure complete safety of the finished product. Under such circumstances, cartridge filtration units with a 0.22 μm pore size are typically applied in bottled water industry. However, recent reports on the presence of filterable bacteria in natural freshwater environments necessitate a re-validation of this method (Wang et al., 2007). In the current study, the total microbial cell concentrations of samples taken from two separate groundwater aquifers were measured with flow cytometry (FCM) for a one-year period (Figure 4.1). The original microbial concentration in the source water of GW I was 3,000 cells/ml on average (Figure 4.1A). The filtered water from GW I had an average microbial concentration of about 300 cells/ml (Figure 4.1A), which is very close to the detection limit of the FCM used in this study. Hence, up to 10% of the native microbial community passed through the cartridge filtration unit. In contrast, groundwater from GW II had an average microbial concentration of 30,000 cells/ml, which is 10-times higher than GW I (Figure 4.1C). A similar passage percentage (1 — 10%) as that recorded for GW I, was observed for GW II. The passage percentage remained constant throughout the whole year of sampling. It has been previously reported that a significant part of the natural freshwater microbial community was able to pass through membrane filters with 0.22 μm pore size (Wang et al., 2007). The passage percentage observed in the current study (up to 10%) was even higher than the 1% passage percentage reported for 0.22 μm pore size pre-sterilized membrane filters (Wang et al., 2007). The reason may lie in the higher flow rates (800 l/h vs. 600 ml/h), the different filtration units used (cartridge vs. membrane filter) and operation scale (industrial vs. laboratory syringe filtration). In general, we could clearly demonstrate that the industrial scale micro-filtration is not a complete barrier for the microorganisms occurring in groundwater.
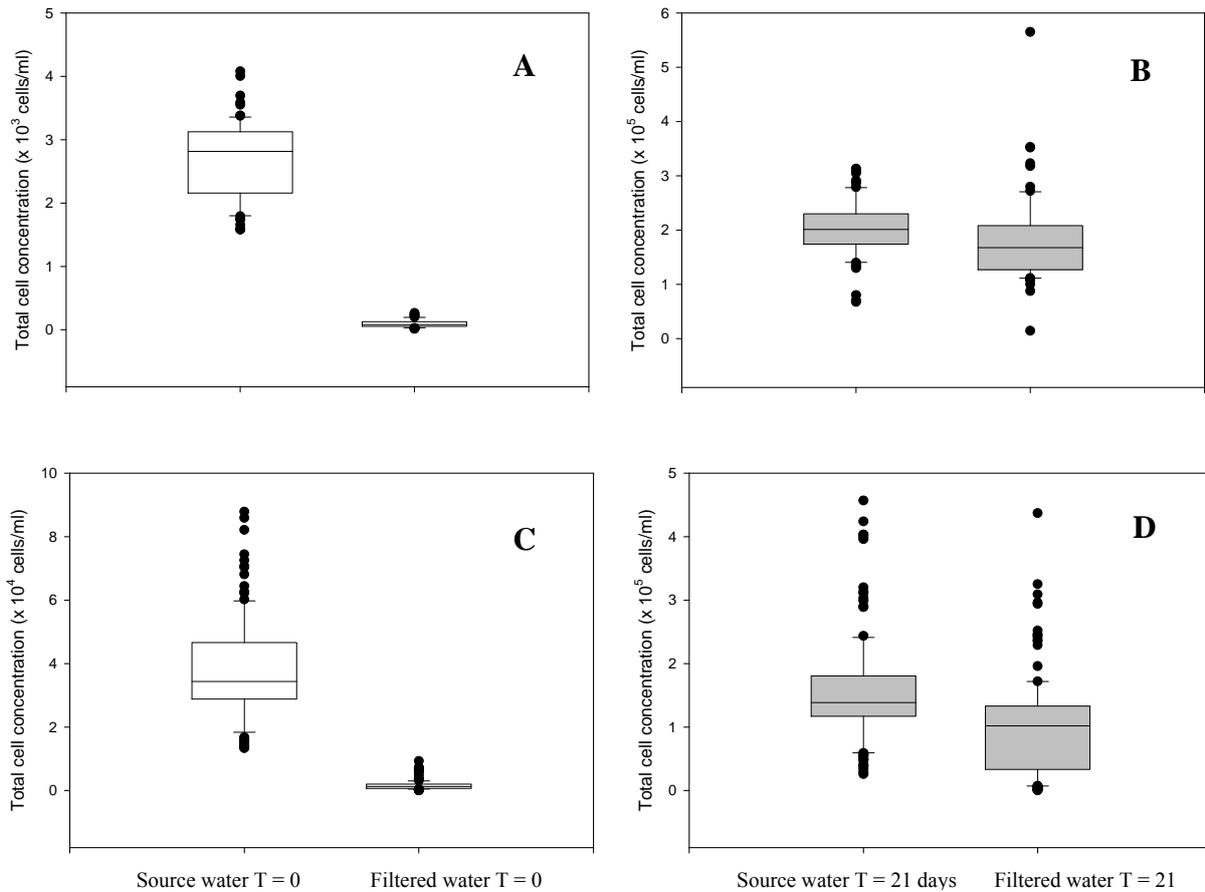
Figure 4.1. Box plot of flow cytometry microbial counts for one year of sampling at two groundwater aquifers (GW I (n = 78) and GW II (n = 138)) in the state received (T = 0) and after 21 days of incubation at 25°C (T = 21). Plot A represents for data from GW I at T = 0, B for GW I at T = 21, C for GW II at T = 0, and D for GW II at T = 21. Box bars represent the 75 percentiles, whisker lines represent the 90 percentiles and black dots represent the outlier values. The middle line in the box shows the median value of all data points.

Microbial growth after bottling

Groundwater is considered as a stable environment, with a balance of low nutrient (AOC) concentrations and low concentrations of microorganisms. However, the filtration procedure removed most of the native microbial community (~ 90%, Figure 4.1) but not the AOC (Meylan et al., 2007), leaving a niche open that can be occupied. Furthermore, bottling and

storage lead to a drastic change of the environment for the native microbial community, such as increased temperature and addition of organic carbon from the bottling materials and processing. Such changes disturb the balance of biological stability and hence stimulate the microbial growth in bottled water. In the current study, significant microbial growth was observed after 21 days incubation at 25°C in both source water and filtered water (Figure 4.2). The source water microbial community exhibited a higher specific growth rate than the filtered water microbial community (0.06 vs. 0.03 h^{-1}) (Figure 4.2). The reason may lie in that the filtration step only certain part of the microbial community to pass, which has lower growth rate (Wang et al., 2007). On average, the microbial concentration after incubation in the stationary phase was in the range of 1 - 3 × 10^5 cells/ml for the entire sampling period for GW I and GW II (Figure 4.1B and 1D). There was no significant difference between the net growth in the source water and that of filtered water samples (p = 0.5) in GW I. A higher variation was observed for GW II (Figure 4.1D), which is largely due to the change of the sampling well in the second sampling period. There have been several studies reporting that the HPC increased dramatically in bottled water after 1 to 3 weeks of storage (Arms and Sutherland, 1999; Leclerc and Moreau, 2002; Venieri et. al., 2006). Consistent with the previous studies, an increase in the total cell concentration was also observed during the incubation of groundwater samples tested in the current study. In general, flow cytometry-based total cell concentration data are up to two orders of magnitude higher than HPC because the latter is restricted to the culturability of microorganisms (Hammes et al., 2008; Siebel et al., 2008). Hence total cell concentration data usually show a more accurate picture of the growth phenomenon in groundwater after filtration and bottling. It is interesting to observe that statistically there is only a slight difference in the final microbial cell concentrations after incubation obtained from the source water and the filtered water after bottling in PET bottles. The filtration step only removes part of the microbial community and allows growth of the microorganisms that are able to pass through the cartridge filter.
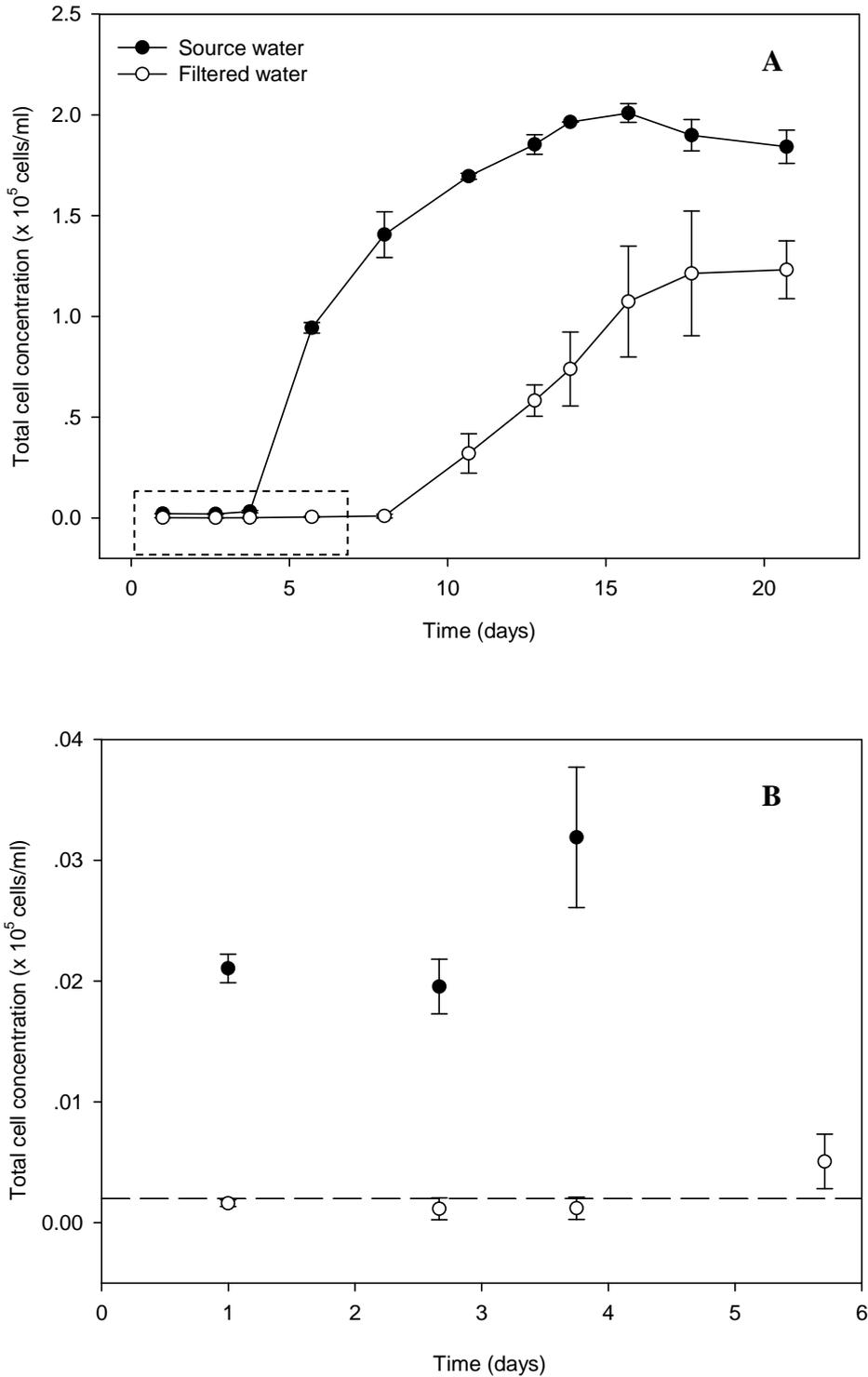
Figure 4.2. Growth of microbial communities in source water and filtered water from GW I incubated in PET bottles at 25°C (A). A detailed view of first four samples were shown (B) with detection limit of the flow cytometry indicated by dashed line. Total cell concentration was measured by FCM. Values shown are averages from triplicate samples. Error bars represent the standard deviation.

The AOC content of the water samples was calculated by determination of the microbial growth in AOC-free glass bottles (Hammes and Egli, 2005). GW I had an AOC content in the range of 5 – 15 µg-C/l and GW II was in the range of 1 – 10 µg-C/l. Based on the reported conversion factor between AOC and microbial cell concentration (Hammes and Egli, 2005; Van der Kooij, 2002), the AOC content in the two groundwater aquifers will support microbial concentration of $0.5 - 1.5 \times 10^5$ cells/ml and $0.1 - 1 \times 10^5$ cells/ml, respectively. In comparison with the total cell number in PET bottles, about 50% of the final microbial growth can be attributed to the AOC presented in the aquifers. The AOC concentration observed in the current study is considered to be comparable with the reported AOC level of drinking water. It has been reported that surface freshwater has a typical AOC level in the range of 50 to 200 µg/l (Hammes and Egli, 2005; Van der Kooij, 2000) and around 10 µg/l for drinking water (Van der Kooij, 2002). Furthermore, it was reported that the growth of natural microbial communities and also specific pure cultures in freshwater showed a positive correlation to AOC content (Van der Kooij, 2002; Vital et al., 2007).

ATP measurement is an alternative and complementary parameter to flow cytometric total cell concentration measurements for accessing microbial viability in aquatic systems (Siebel et al., 2008). Before incubation (T = 0) the ATP concentrations for both source and filtered water samples were below the detection limit (5.07×10^{-9} gATP/l) due to the very low cell concentrations in the water. After 21 days of incubation at 25°C (T = 21) the ATP concentration was in the range of $1.15 — 5.75 \times 10^{-8}$ gATP/l for both source and filtered samples. It showed a pattern similar to that obtained from microbial total cell concentration data (data not shown). An average cellular ATP-content of 1.70×10^{-16} gATP/cell was observed for GW I and 1.47×10^{-16} gATP/cell for GW II, which is comparable to previous reported cellular ATP contents for bacteria in drinking water (Hammes et al., 2008) and groundwater samples (Jensen, 1989).

Microbial community pattern on FCM

The presence of different microbial clusters was observed in the flow cytometric patterns of source and filtered water samples (Figure 4.3). More different clusters were observed after microbial growth and slightly different patterns were detected between source water and filtered water (Figure 4.3). It is interesting that the impact of filtration on GW I and GW II cluster patterns recorded after 21 days of incubation was quite different. For GW1, there was

a dominant single cluster on the FCM plot in the samples after filtration (Figure 4.3C), which is remarkably similar to the clustering of the filterable bacterium, *Hylemonella gracilis*, observed previously (Wang et al., 2007). Epi-fluorescence microscopic observation confirmed the slender spirillum-shape of the dominating cells, which were further identified as *Hylemonella* spp. based on 16S rRNA sequencing (data now shown). The clear dominance of spirillum-shaped bacteria indicated a possible selection of such bacteria during filtration process (Figure 4.3C). It has been reported that such slender spirillum-shaped bacteria have a clear advantage in passing membrane filters (Wang et al., 2007). The enrichment of certain bacterial species largely reduces the microbial diversity, which may lead to a relatively unstable biological state of the water and may even raise the chance of microbial contamination by unwanted strains (Leclerc and Moreau, 2002; Vital et al., 2007). In contrast, there was no such selection of spirillum-shaped bacteria recorded in samples for GW II (Figure 4.3F) and a clear selection of one specific microbial population has not been observed with either flow cytometry or epi-fluorescence microscopy. The reason may lie in the different microbial community composition in the two groundwater aquifers. However, although the microbial population remained heterogeneous, a clear cluster distribution difference was observed after filtration and 21 days of incubation for GW II (Figure 4.3 E and F). Therefore, filtration can have a selective effect on the microbial community, and may lead to a different community distribution after growth compared to that observed for the native microbial community. Within the scope of the current study, the population compositions were not further investigated.
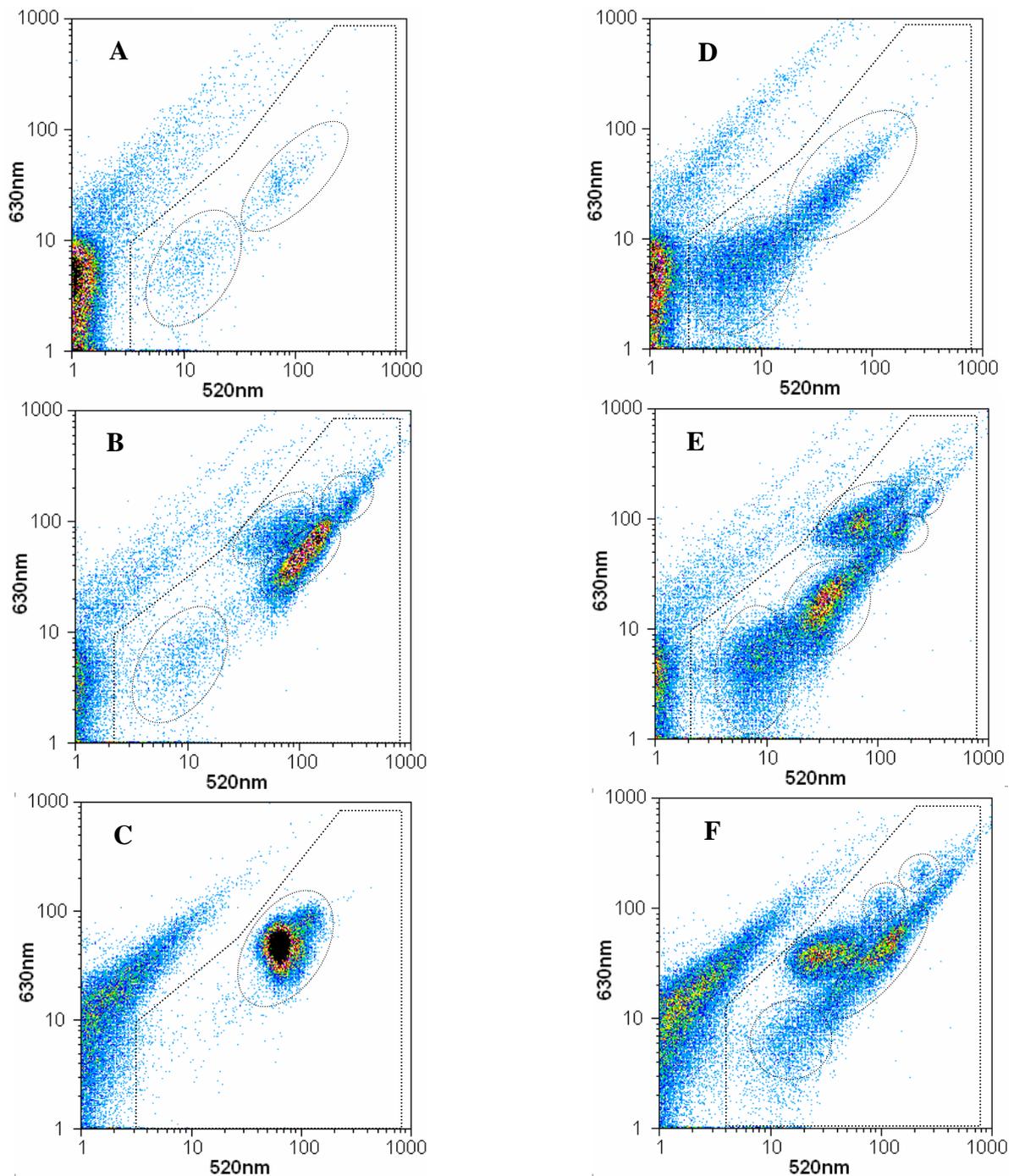
Figure 4.3. Effects of filtration and incubation (21 days at 25°C) in PET bottles on microbial communities' cluster patterns on flow cytometry dot plots. (A) GW I source water at T = 0; (B) GW I source water T = 21 days; (C) GW I filtered water at T = 21 days; (D) GW II source water T = 0; (E) GW II source water T = 21 days; (F) GW II filtered water T = 21 days. All samples were stained with SYBR Green I and analysed with FCM. Microbial communities and background are separated by the dashed gate. Different clusters are indicated by oval shapes.
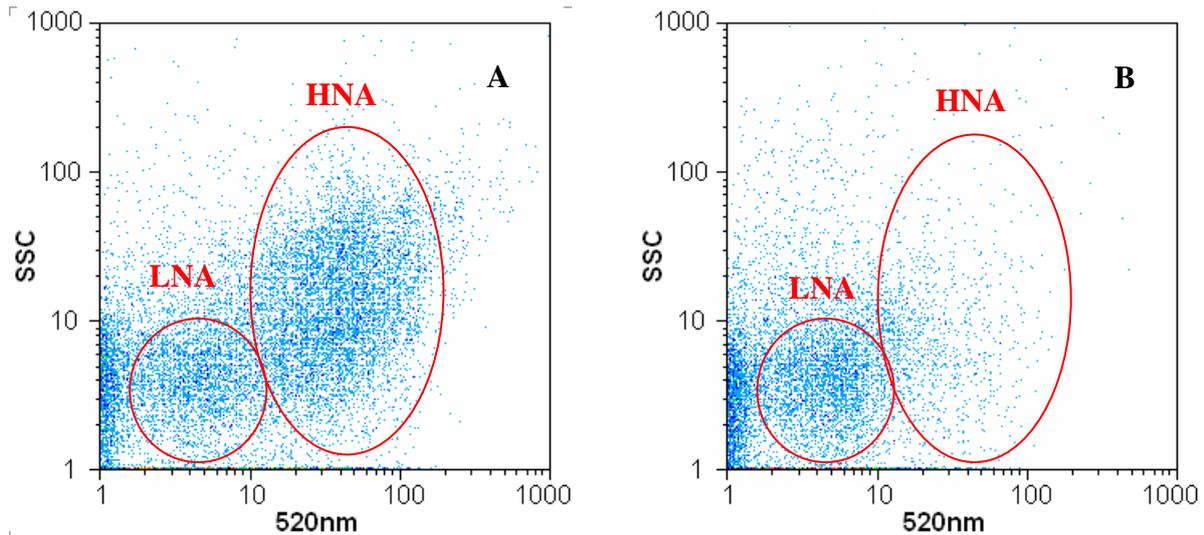
Figure 4.4. Effect of bottling materials on microbial communities' distribution after 21 days of incubation at 25°C. Representative flow cytometry dot plots of microbial communities from groundwater aquifer GW II incubated in PET bottle (A) and glass bottle (B), respectively. Samples were stained with SYBR Green I and green fluorescence (520 nm) against sideward scatter (SSC) was plotted. LNA stands for low nucleic acid content bacteria and HNA for high nucleic acid content bacteria.

Growth difference in PET vs. glass (AOC-free) bottles

It has been shown that bottling material had an influence on the growth of microorganisms in mineral water (Bischofberger et al., 1990). In the current study, the growth phenomenon of microorganisms in source water and filtered water after bottling was monitored in both PET and AOC-free glass bottles (non-commercial glass bottles) using FCM during 21 days of incubation. Significantly less microbial growth was observed in glass bottles (7.2×10^4 cells/ml for GW I and 1.0×10^5 cells/ml for GW II) than in PET bottles (1.8×10^5 cells/ml for GW I and 1.3×10^5 cells/ml for GW II) (Table 4.1). The results indicated a presence of small concentration of organic carbon in the PET bottles. In both PET- and glass-bottled samples, two defined clusters can be observed from flow cytometric patterns (Figure 4.4). These two clusters allude to the so-called low (LNA) and high (HNA) nucleic acid bacteria (Lebaron et al., 2001), which represent two classes of bacteria typically observed in aquatic environments (Gasol et al. 1999; Lebaron et al., 2001; Hammes et al., 2008). These two classes are distinguished in size and fluorescence intensity (Figure 4.4). It is claimed that HNA bacteria represent the active part of the microbial community and contribute to most of the total

microbial production (Gasol et al. 1999; Lebaron et al., 2001). However, few recent reports argued that LNA bacteria were not substantially different from HNA (Jochem, et al., 2004; Longnecker et al., 2005). The composition and function of the two groups remain to be elucidated.

Table 4.1. Distribution of HNA and LNA microbial populations in the original groundwater and after incubation for 21 days at 25°C in either glass or PET bottles. Data are listed as average value together with the standard deviation from 15 samples for GW I and 30 samples for GW II. Data are expressed as percentage, and secondly as total cell concentrations to give an indication of the growth of the two populations.

		GW I			GW II		
		T = 0	T = 21 glass	T = 21 PET	T = 0	T = 21 glass	T = 21 PET
Percentage (%)	HNA	65 ± 5	96 ± 2	98 ± 1	25 ± 4	20 ± 4	54 ± 3
	LNA	35 ± 5	4 ± 2	2 ± 0.1	75 ± 3	80 ± 2	46 ± 3
Cells/ml ($\times 10^4$)	HNA	0.1 ± 0.0	6.9 ± 1.4	18 ± 2.4	1.4 ± 0.2	2.0 ± 0.4	6.8 ± 0.7
	LNA	0.1 ± 0.0	0.3 ± 0.1	0.3 ± 0.1	3.7 ± 0.8	8.0 ± 0.2	5.9 ± 0.3

It is interesting to notice that after incubation for 21 days the percentage of LNA is much lower in the PET bottle samples than in glass-bottled samples of source water from GW II (Table 4.1). The reason may lie in the selection of HNA bacteria by the carbon source from the PET bottles. The fact that the PET bottles were pre-sterilized before use by gamma irradiation may increase the release of carbon by the PET bottle (data not shown). In general, the carbon released from PET bottle preferentially stimulates the growth of HNA bacteria from both groundwater aquifers (Table 4.1). The cell concentration for HNA bacteria in PET bottles was three-time higher than that in glass bottles (Table 4.1). In comparison, LNA

bacteria did not show further growth after bottling (Table 4.1). The results suggest that the bottling material can impose a change in microbial community composition in bottled water.

Conclusions

- The data presented here showed that industrial scale micro-filtration cannot serve as an absolute barrier for the native microbial community of groundwater. Up to 10% of the native microbial community from two separate groundwater aquifers was able to pass through an industrial cartridge filtration unit with 0.22 μm pore size.

- The microorganisms that passed through the filter could further grow after bottling and 21 days of storage at 25°C reaching a level of $1\text{-}3 \times 10^5$ cells/ml.

- Different flow cytometric patterns were observed in source and filtered water samples, indicating a population composition change caused by filtration and bottling. In particular, a dominant population identified as *Hylemonella* spp. was observed in filtered water samples in one of the two groundwater aquifers (GW I).

Acknowledgements

5. Enrichment cultivation and characterization of low nucleic acid (LNA) content bacteria from various freshwater environments

Abstract

Most planktonic bacteria are "uncultivable" with conventional methods, and flow cytometry (FCM) is one of the methods that have been applied to study these bacteria. In natural aquatic environments, bacteria with high (HNA) and low (LNA) nucleic acid content are commonly observed with FCM after staining with fluorescent dyes. While several studies have focused on the relative abundance and *in situ* activities of these two groups, knowledge of the growth of particularly LNA bacteria is largely limited. In the present study, typical LNA populations were enriched from three different freshwater sources using extinction dilution and fluorescence-activated cell sorting. We have for the first time demonstrated that LNA bacteria can be cultivated and are able to utilize natural assimilable organic carbon at different temperatures, ranging from 12 to 30°C. During growth, the main FCM fingerprints (i.e., fluorescence intensity and sideward scatter) remain distinct from those of typical HNA bacteria. Three LNA pure cultures were isolated which are closely affiliated to the *Polynucleobacter* cluster according to 16S rRNA sequencing results. Due to their small size, cells of the isolates remained intact in cryo-TEM and revealed a Gram-negative cell wall structure. The extreme small cell volume (0.05 μm^3) observed for all three isolates indicates that they are among the smallest free-living heterotrophic organisms known in culture.

Introduction

Planktonic bacteria are ubiquitous in aquatic environments and an average density of 10^6 – 10^7 cells/ml is typically observed in surface freshwater (Whitman et al., 1998; Wang et al., 2007). Not only are they present in large numbers, but they also play a critical role in the turnover of organic matter and nutrient cycling in aquatic environments (Billen et al., 1990). However, a large fraction of these planktonic cells are difficult (if not impossible) to study outside of their natural habitats. Due to the low nutrient concentrations that prevail in freshwater environments, these cells are typically smaller and less active than cells grown under laboratory conditions. Moreover, the absolute majority of these cells is only recognizable through cultivation-independent methods such as microscopy, denature gradient gel electrophoresis (DGGE), shotgun sequencing and radioactive labeling (e.g., Muyzer, 1999; Pernthaler et al., 2002; Rappé and Giovannoni, 2003; Tryson et al., 2004). However, the absence of cultivation often fosters a lack of understanding and acceptance.

Microbiologists have used various different names to describe members of autochthonous aquatic bacteria. Terms like "oligotrophs", "viable-but-not-cultivable (VBNC) bacteria", "ultramicrobacteria" have all been used by different groups along the history of microbial research (e.g., Oliver, 1993; Morita, 1997; Schut et al., 1997; Kajander and Ciftcioglu, 1998). With the emerging popularity of flow cytometry as a tool in aquatic research, two new terms have been assigned to planktonic bacteria visualized with this method. It started with the names of "Group I cells" and "Group II cells" proposed by Li and co-workers (1995), which were then renamed to "low-DNA (LDNA) bacteria" and "high-DNA (HDNA) bacteria" by Gasol and co-workers (1999), and again later modified to "low nucleic acid content (LNA) bacteria", and "high nucleic acid (HNA) bacteria" by Lebaron and co-workers (2001). LNA and HNA have since then been most widely used by researchers. This broad classification of the two groups is based on their different fluorescence intensity and sideward scatter signal detected by flow cytometry in combination with nucleic acid stains (see Figure 5.1A; Gasol et al., 1999; Lebaron et al., 2002). Fluorescence intensity is in this respect used as an indicator of apparent cellular nucleic acid content (Gasol et al., 1999; Lebaron et al., 2002) and sideward scatter signals have been applied as an index of cellular size (Lebaron et al., 2002; Felip et al., 2007). This classification is essentially method-specific; up to now, no other method has specifically recognised these distinct groups or organisms. Nonetheless, numerous studies

were published on the topic of LNA and HNA bacteria (Jellett et al., 1996; Marie et al., 1997; Zubkov et al., 2001; Lebaron et al., 2002; Jochem et al., 2004; Longnecker et al., 2005). The existing studies so far focused mainly on the relative abundance of LNA to HNA bacteria and their *in situ* activities. The two groups has widely been observed mostly in marine environments (Jellett et al., 1996; Marie et al., 1997; Zubkov et al., 2001; Lebaron et al., 2002; Jochem et al., 2004; Longnecker et al., 2005), but also in freshwater environments (Nishimura et al., 2005; Bouvier et al., 2007). In this respect, HNA bacteria are usually regarded as the active part of the microbial metabolic group whereas LNA bacteria were considered inactive (Lebaron et al., 2001; Lebaron et al., 2002; Servais et al., 2003; Tadonleke et al., 2005). However, there are also reports demonstrating conflicting results that LNA bacteria can also represent an active part of microbial communities in the sea (Zubkov et al., 2001; Longnecker et al., 2005), and in freshwater (Nishimura et al., 2005).

So far, all attempts to describe the phylogenetic differences between those two groups have resulted in contradiction. Some researchers reported that both groups are composed of the same dominant species (Servais et al., 2003), while others have opposed that they are phylogenetically different (Zubkov et al., 2002). Furthermore, scenarios have been reported that bacterial cells may exchange between those two groups (Bouvier et al., 2007). Nearly all the studies thus far reported were done by combining cell sorting, activity measurements and molecular methods. To our knowledge, no systematic cultivation or isolation study on LNA bacteria has been undertaken, which would, in our opinion, be extremely helpful to understand the nature and importance of these bacteria.

Hence, the aim of the present study was to (1) demonstrate that the LNA bacterial cells exist and are able to grow without changing their FCM fingerprints; (2) obtain LNA enrichment cultures and isolate LNA pure cultures; (3) then further characterize the pure cultures and provide substantial data on their growth properties, morphological and phylogenetic characteristics using a unconventional approach of cultivation with natural organic matter and flow cytometric monitoring. Here we report for the first time the successful enrichment and cultivation of LNA bacteria from freshwater. Their ability to grow at low nutrient concentrations and to maintain distinctive morphological characters in comparison to conventional HNA bacteria was systematically documented. We believe that the experimental approach and results presented in this study will open a new page on the investigation and understanding of autochthonous planktonic bacteria in aquatic environments.

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Materials and Methods

Glass vials free of assimilable organic carbon (AOC)

Sterile, carbon-free glassware was prepared as described previously (Hammes and Egli, 2005). In short, all glass bottles and caps were washed with detergent and then rinsed and followed by submerge in acid bath for at least 2 hours. The rinsed glass bottles were then muffled at 500°C for 6 hours before use. The caps were incubated at 60°C for 1 hour in 10% sodium persulphate, followed by rinsing with deionised water, and finally air-dried. The caps were freshly prepared for each experiment. After capping, all vials were incubated at 90°C for 2 hours for sterilization.

Sampling

Freshwater samples were collected in autoclaved Schott bottles (0.5 l) from six different water sources: Chriesbach stream (Dübendorf, Switzerland), Lake Greifensee (Uster, Switzerland), a small alpine stream (Luzern, Switzerland), tap water (Dübendorf, Switzerland), groundwater (Vittel, France) and wastewater treatment plant effluent (Dübendorf, Switzerland). All samples were kept at 8°C during transportation and processed within 24 hours after sampling.

Flow cytometry (FCM)

Flow cytometric measurements were done as described by Hammes and co-workers (2008). Bacterial cells were stained with 10 μl/ml SYBR® Green I (1:100 dilution in dimethyl sulfoxide; Molecular Probes, USA), and incubated in the dark for 15 minutes at room temperature before measurement. When necessary, samples were diluted just before measurement in cell-free water, so that the concentration measured in the flow cytometer was always less that 2×10^5 cells/ml. Flow cytometry was performed using a CyFlow® space machine (Partec, Hamburg, Germany) equipped with a 200 mW laser, emitting at a fixed wavelength of 488 nm, and volumetric counting hardware. Green fluorescence was collected at 520 nm, red fluorescence was collected at 630 nm and all data were processed with the Flowmax software (Partec). LNA and HNA cell populations were gated on the two-parameter dot-plot of green fluorescence (520 nm) against sideward scatter (SSC) and counted

separately (see Figure 5.1). The specific instrumental gain settings for these measurements were as follows: green fluorescence = 450, red fluorescence = 550, SSC = 300. This setting was used for all experiments in the current study. Geometrical mean (GMean) values for green fluorescence and SSC for both the LNA and HNA cell population were recorded. Samples were measured in triplicate. The standard instrument error on FCM measurement was always below 3%. The detection limit of the instrument used in this study was about 200 cells/ml (Hammes et al., 2008).

Continuous cultivation of autochthonous LNA bacteria in the bioreactor

Continuous cultivation of natural LNA bacteria at a dilution rate of 0.08 h^{-1} was performed in laboratory bioreactors with a working volume of 0.2 l. The temperature was maintained at 12.0 ± 0.1°C. Autochthonous LNA and HNA communities were separated by 0.45 μm filtration (Figure 5.1). Filtrate of the stream water (Chriesbach, Switzerland) (LNA fraction) was used as the culture for inoculation. Autoclaved river water (from the same source) was supplied as growth medium. The culture was continuously mixed at the speed of 100 rpm. The culture was operated in the continuous mode for 150 h. The theoretical washout line was calculated as follows:

$$C_t = C_0 \times e^{-D \times t}$$
<div align="right">Equation 5.1</div>

Where C_t is cell concentration in the reactor at time t, C_0 is the initial cell concentration at time 0, and D is the dilutions rate.

Samples from the reactor was taken at regular time interval and analyzed on FCM as described above. No wall growth was observed in the reactor. The sterility of the medium was checked regularly by FCM.

Enrichment of LNA bacteria

Water from a shallow stream (Chriesbach, Dübendorf, Switzerland) was sampled with a 1 l Duran flask (Schott, Germany), pasteurized (30 min, 60°C) and filtered through 0.1 μm pore-size sterile syringe filters (PVDF, Millipore, USA) to remove most particles. This sterilised

river water was then used as the growth medium for the cultures. LNA population were obtained by filtration through syringe filter with pore-size of 0.45 µm (Figure 5.1). The cells passing this filter were used for further enrichment experiments by extinction dilution or single cell sorting. Extinction dilution experiments were done in AOC-free glass vials containing 15 ml of sterile river water. The vials were inoculated with the filtrate to the dilution of 10^{-3} (100 cells/ml), 10^{-4} (10 cells/ml), and 10^{-5} (1 cell/ml) respectively. For each dilution, 24 vials were prepared (including 3 as blanks). All the cultures were incubated at 20°C for 14 days until stationary phase if there is growth. After incubation, 1 ml samples were withdrawn from each vial for all dilution series and analyzed with FCM as described above. Cultures containing only LNA populations were selected for further extinction dilution and re-growth. Such cycles were repeated until a stable LNA enrichment culture was obtained. The enrichments were used for further investigation. For the final step of achieving pure culture from the enrichments, further extinction dilution was carried out with inoculation concentration of 1 cell/vial.
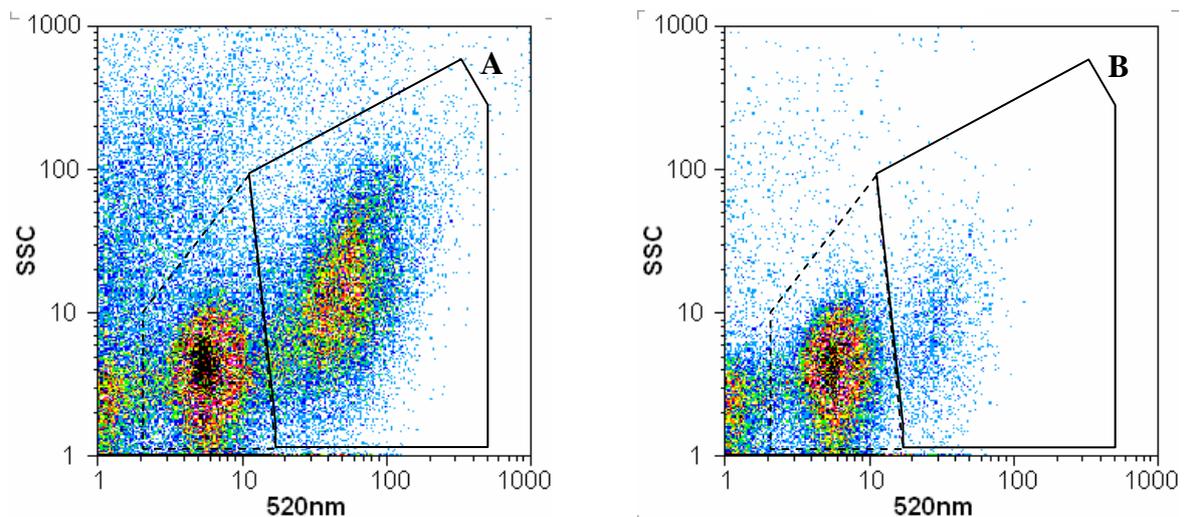
Figure 5.1. The separation of LNA and HNA bacteria was done by membrane filtration (0.45 µm pore size). A, total communities in water sample from Chriesbach stream (Dübendorf, Switzerland). B, 0.45 µm filtrate of the water sample. HNA bacteria were gated by solid lines and LNA bacteria by dashed lines. The filtrate contained mainly LNA bacteria.

In an alternative approach to obtain "pure" LNA bacterial populations, cell sorting was employed using a BD FACS Aria (Becton Dickinson, USA). Samples were stained with 10 μl/l SYBR® Green I (Molecular Probes). LNA bacteria were sorted into 96-well plates (50 cells/well) containing 150 μl sterile river water in each well. All the cultures were incubated at 20°C for 14 days until stationary phase if there is growth. After incubation, sample (50 μl) was withdrawn from each well and analyzed with FCM. Cultures containing a LNA bacterial population only were selected for further investigation.

These enrichment cultures were used for further investigation (Figure 5.2). For the final step of achieving LNA pure culture from the enrichment cultures, further extinction-dilution experiments were carried out with initial concentrations of 1 cell/vial. The terms used to describe the cultures in each step and the experimental design was illustrated in Figure 5.2.
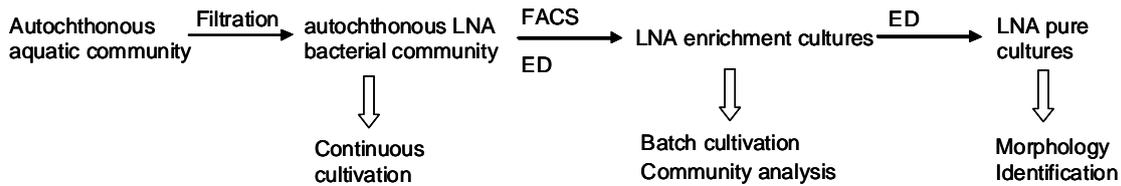
Figure 5.2.. Experimental flow chart, indicating terms used and experiments done in the current study. Filtration was carried out using syringe filter with 0.45 μm pore size. FACS stands for fluorescence-activated cell sorting and ED for extinction dilution.

Enrichment cultures cell size estimation by flow cytometry

The sideward scatter signal (SSC) was recorded as the geometrical mean (Gmean) of all data in arbitrary units (a.u.). The Gmean value was then converted to cellular biovolume using a calibration curve produced based on 5 bacterial strains including *Escherichia coli*, *Pseudomonas aeruginosa, Vibrio cholerae, S.alaskensis* and isolate CB (Equation 5.2). Bacterial volumes were measured with light microscope and the mean volume was calculated from at least 40 cells. The conversion equation is specific for the flow cytometry used in the current study.

$$\text{Cellular volume } (\mu m^3) = 0.0006 \times (\text{Gmean SSC})^{2.2} \qquad \text{Equation 5.2}$$

Adenosine tri-phosphate (ATP) measurements

Total ATP was determined as described in Berney and co-workers (2006) using the BacTiter-Glo™ reagent (Promega Corporation, Madison, WI, USA) and a luminometer (Glomax, Turner Biosystems, Sunnyvale, CA). The data were collected as relative light units (RLU) and converted to ATP (nM) by means of a calibration curve made with a known rATP standard (Promega). ATP was measured in triplicate for all samples, and the detection limit of the measurement was about 0.01 nM ATP with a standard error of 8 − 10%.

Batch growth characterization of LNA enrichment cultures

Three different LNA enrichments were cultivated in sterile river water (as described above) with initial cell concentration of 1×10^4 cells/ml at different temperatures ranging from 12°C to 37°C in 20 ml AOC-free vials (as described above). Samples (1 ml) were taken at regular time intervals until stationary phase was reached. Samples were analyzed with flow cytometry and ATP as described above. All experiments were done in triplicate. The specific growth rate (μ) based on cell number increase in each sample was determined as following (Equation 5.3):

$$\mu = (\text{Ln}(n_t) - \text{Ln}(n_0))/\Delta t \qquad \text{Equation 5.3}$$

where n_t, n_0 are the cell concentrations measured at two subsequent time points and Δt is the expired time interval between these points.

The AOC bacterial community obtained from Chriesbach stream was used as reference HNA enrichment (Hammes and Egli, 2005; Figure 5.3). This enrichment was used for comparison of growth characterization with LNA enrichment cultures. The comparison was carried out at 20°C. FCM and ATP measurements were done as described above.

Fluorescence in situ hybridization and catalyzed reporter deposition (CARD-FISH)

CARD-FISH was performed on LNA enrichment cultures according to the modified permeabilization protocol developed for freshwater bacterioplankton (Sekar et al., 2003). The

probes for *Bacteria*, *β-Proteobacteria*, *α-Proteobacteria*, *Cytophaga-Flavobacterium-Bacteroides*, *Actinobacteria* were used in the current study (Warnecke et al., 2005). Details of the procedure are documented by Pernthaler and Pernthaler (2007). Counterstaining of CARD-FISH preparations with 4,6-diamidino-2-phenylindole (DAPI) and mounting were performed as described previously (Pernthaler et al., 2002). Total bacterial abundances and the fractions of FISH-stained bacteria in at least 1,000 DAPI-stained cells per sample were quantified at a 1,000-fold magnification using an epi-fluorescence microscope (Leica, Wetzlar, Germany).
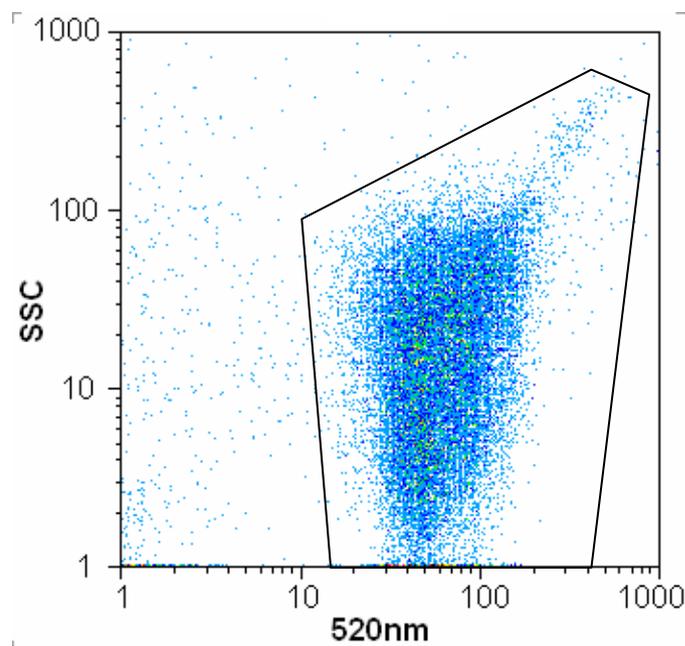
Figure 5.3. AOC bacterial community (Hammes and Egli, 2005) enriched from Chriesbach stream (Dübendorf, Switzerland) was used as HNA enrichment in the current study.

Microbial community analysis

Genomic DNA was extracted from the culture samples by using the UltraClean Water DNA Isolation Kit (Mo Bio, Germany). Total bacterial community analysis was performed by PCR-DGGE, using general bacterial primers (Boon et al., 2002). The processing of the DGGE gels was done with the Bionumerics software 2.0 (Applied Maths, Kortrijk, Belgium). The

calculation of the dendrogram is based on the Pearson (product-moment) correlation coefficient and the clustering algorithm of Ward.

The bands from the DGGE gel for the LNA enrichments were cut out of the DGGE gel with a clean scalpel and added in 50 µl of PCR water. After 12 hours of incubation at 4°C, 1 µl of the PCR water was reamplified with primer set P338F and P518r (Boon et al., 2002). Five µl of the PCR product was loaded on a DGGE gel (see above) and if the DGGE pattern only showed one band, it was sent out for sequencing. In case the DGGE band was not pure enough for sequencing, PCR fragments were cloned by using the TOPO TA cloning kit (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. DNA sequencing of the ca. 180 bp fragments was carried out by ITT Biotech-Bioservice (Bielefeld, Germany).

Genomic DNA of pure isolates was also extracted with UltraClean Water DNA Isolation Kit (Mo Bio, Germany). The eluted DNA was subsequently amplified using universal 16S rRNA primers EUB_9/27f (5'-GCA GAG TTT GMT CCT GGC TCA G-3') and EUB_1492/1512 (5'-ACG GYT ACC TTG TTA CGA CTT-3'). The amplification reaction and program was reported in detail previously (Wang et al., 2007). Target bands were excised from agarose gel and DNA was further purified using Geneclean Kit (Q.BIOgene, Irvine, CA, USA). Purified PCR products were sequenced by Microsynth (Balgach, Switzerland). Analysis of DNA sequences and homology searches were completed with standard DNA sequencing programs and the BLAST server of the Seqmatch provided by Michigan State University. A phylogenetic tree was produced using the same sever.

Scanning electronic microscopy

LNA enrichment cultures were cultivated in sterile river water (as described above). Stationary phase cells were concentrated using centrifugal filter device (Amicon Ultra-15, Millipore, USA). A final concentration of 10^8 cells/ml was achieved. The cells were then fixed with 1% glutaraldehyde for 30 minutes and washed with sterile river water (autoclaved and filtered as described above). The samples were sputter-coated with 10 nm gold and examined by scanning electron microscope (Hitachi S-4800 FEG, Japan), operated at 5 kV Accelerating Voltage.

Cryo-transmission electron microscopy (cryo-TEM)

A 4 µl aliquot of concentrated LNA were adsorbed onto holey carbon-coated grid (quantifoil, Germany), blotted with Whatman 4 filter paper and vitrified into liquid ethane at -178 °C. Frozen grids were transferred onto a Philips CM200-FEG electron microscope using a Gatan 626 cryo-holder. Electron micrographs were recorded at an accelerating voltage of 200 kV and a magnification of 50000 x, using a low-dose system ($10e^{-}/Å^{2}$) and keeping the sample at -175°C. Defocus values were -3 µm. Micrographs were recorded on Kodak SO-163 films and then digitized with Heidelberg Primescan 7100 at 4 Å/pixel resolution at the specimen level.

The cell volume of the individual bacterial cell was calculated based on the measurements from cryo-TEM pictures (Equation 5.4) on the principle of a rod-shaped particle with sphere ends.

$$\text{Cell volume } (\mu m^{3}) = \frac{4}{3} \times \pi \times r^{3} + \pi \times r^{2} \times (L - 2r) \qquad \text{Equation 5.4}$$

where r represents the half of the smallest width and L represents the length of the bacterial cell.

Results

Presence of autochthonous LNA bacterial community in different aquatic environments

Typical LNA and HNA communities were observed in all aquatic samples tested, ranging from an extreme oligotrophic environment (alpine stream) to a relative copiotrophic environment (wastewater effluent). The two communities are clearly separated on the FCM dot-plot of green fluorescence against sideward scatter (SSC) (Figure 5.4). The LNA bacteria were numerically dominant (more than 50%) in the planktonic microbial communities from most sampled aquatic environments (Table 5.1).

A simple separation of HNA/LNA bacterial communities was achieved through 0.45 filtration, which retained about 90% of the HNA bacteria, while this allowed most of the LNA bacteria to pass (Figure 5.1 and 5.2). In a first basic experiment to test the viability of the autochthonous LNA bacterial community, we applied the above mentioned filtration step and then inoculated the filtrate into AOC media. All autochthonous LNA bacterial communities were able to grow on natural AOC. Growth of autochthonous LNA bacterial community was tested using continuous cultivation with natural AOC at 12°C, selected because of the low temperature (10 to 12°C) recorded in the source water. The autochthonous LNA bacteria community established in the continuous culture at a "steady state" level of 1.2×10^5 cells/ml over 150 h at a dilution rate of 0.08 h^{-1} (Figure 5.5). If the LNA bacteria were not able to multiply using natural AOC, their cell concentration should have decreased to less than 5% of the initial value after 24 hours and would have followed the theoretical cell number wash-out line (Figure 5.5). During the entire period of continuous cultivation, the autochthonous LNA bacteria community maintained their FCM identity (i.e., low fluorescence intensity and low SSC signal) (Figure 5.5B and 5.5C).

Table 5.1. Percentage of LNA bacteria in total bacterial communities from different aquatic environments. Results are shown in the format of average ± standard deviation calculated from at least three samples for each source location.

Source	DOC (mg/l)	Total cell concentration (10^6 cells/ml)	Percentage of LNA
Chriesbach stream	3.0 ± 0.3	3.21 ± 0.20	68% ± 2%
Alpine stream	0.5 ± 0.1	0.10 ± 0.00	74% ± 5%
Tap water	0.7 ± 0.1	0.15 ± 0.01	53% ± 1%
Groundwater	0.5 ± 0.2	0.31 ± 0.02	75% ± 3%
Lake Greifensee	3.2 ± 0.3	2.41 ± 0.23	23% ± 5%
Wastewater effluent	7.0 ± 0.5	10.6 ± 1.20	58% ± 2%

Growth properties of LNA enrichment cultures

Typical LNA bacteria were enriched and cultivated from natural water samples with a simple batch growth assay using natural assimilable organic carbon (AOC) (Hammes and Egli, 2005; Vital et al., 2007; Wang et al., 2007) in combination of fluorescence-activated cell sorting (FACS) or extinction-dilution (ED) approaches. Using FACS, only 1 out of 24 samples showed positive growth of LNA bacteria after 14 days incubation (at least 10^5 cells/ml). For ED approach, 3 out of 21 samples at a dilution of 10^{-4} (10 cells/ml inoculated), 2 out of 21 samples at a dilution of 10^{-5} (1 cell/ml inoculated) showed positive growth of LNA bacteria. All samples at a dilution of 10^{-3} (100 cells/ml inoculated), however, showed either HNA bacterial growth or a mixture of LNA and HNA bacteria. The positive LNA samples were re-inoculated (10 cells/ml) into sterile natural freshwater and incubated for another 14 days. Such cycles were repeated until a pure LNA bacterial enrichment was achieved. Three enrichment cultures were obtained (Figure 5.4) from different aquatic environments. They are referred to as LNA enrichment culture A (enriched from Chriesbach stream by FACS; Figure 5.4B), LNA enrichment culture B (enriched from Greifensee by ED; Figure 5.4D) and LNA enrichment culture C (enriched from wastewater effluent by ED; Figure 5.4F). Their positions on FCM dot-plots correspond to the original LNA fraction in the water from where they were enriched (Figure 5.4 and Table 5.2).

Following the successful enrichment, the growth properties of the LNA enrichment cultures were further characterized during batch cultivation. All three LNA enrichment cultures were capable of growing in batch culture on natural AOC, at different temperatures thereby maintaining their typical LNA characteristics. Growth of LNA enrichment cultures was observed from 12 to 30°C and a typical example for culture A is shown is Figure 5.6. Generally, the lag phase observed for the growth at 12°C was extended compared to higher temperatures. The maximum specific growth rate (μ_{max}) increased with ascending incubation temperature up to 30°C, while no growth was detected at 37°C. The recorded μ_{max} values were as follows: 0.10 h^{-1} (12°C); 0.23 h^{-1} (20°C); 0.31 h^{-1} (25°C); 0.37 h^{-1} (30°C). When supplied with identical AOC, all LNA enrichment cultures grew up to similar final cell concentrations (Table 5.2).
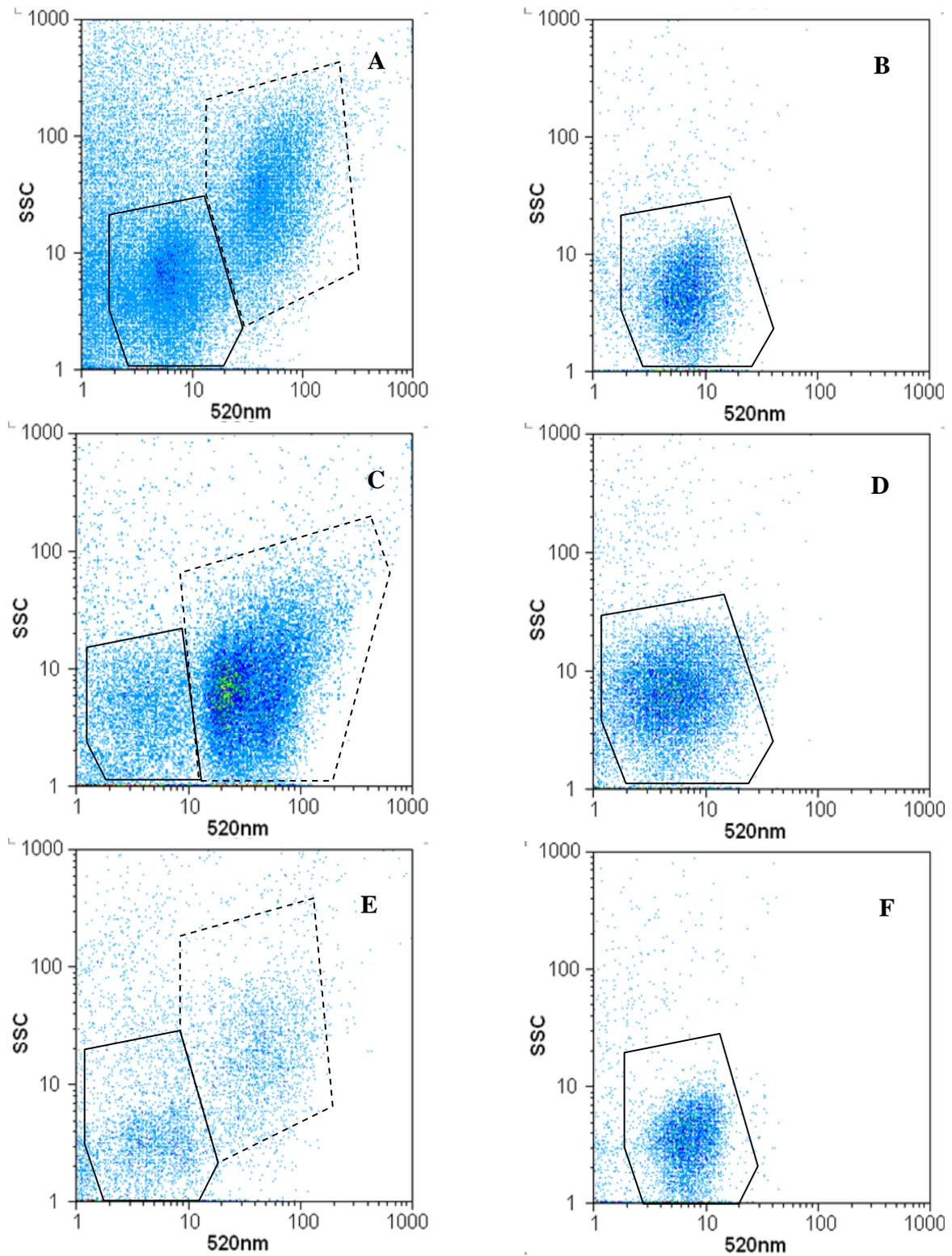
Figure 5.4. FCM plot of CB total bacterial community (A), CB LNA bacterial enrichment (B), Greifensee total bacterial community (C), Greifensee LNA bacterial enrichment (D), wastewater effluent total bacterial community (E), and wastewater effluent LNA bacterial enrichment (F). Solid gated fraction is LNA bacteria and dashed gated fraction is HNA bacteria.

Comparison between LNA and HNA enrichment cultures

During batch growth on natural AOC, LNA and HNA enrichment cultures exhibited distinct characteristics. LNA and HNA enrichment cultures obtained from the same source water (Chriesbach stream) were compared for their grow properties with natural AOC at 20°C. Similar lag phases were observed for both LNA and HNA enrichment cultures (Figure 5.7). The lag phase was followed by a short phase of exponential growth where the cells divided at a maximum rate for 2-3 generations, then the rate gradually decreased until stationary phase was reached (Figure 5.7B). This pattern is typical for growth on complex medium containing carbon/energy sources of different quality, such as LB medium. The maximum specific growth rate (μ_{max}) was higher for the HNA enrichment cultures (0.31 h^{-1}) than that of LNA enrichment cultures (0.23 h^{-1}) (Table 5.2). The HNA enrichment grew from initially 1×10^4 cells/ml to 3×10^6 cells/ml and attained stationary phase after 60 h. In comparison, the LNA enrichment grew from initially 1×10^4 cells/ml to 1.6×10^6 cells/ml and reached stationary phase already after 42 h (Figure 5.7). The final cell concentration of HNA enrichment in stationary phase is about twice that of LNA enrichment (Figure 5.7A).

Cell volume was estimated from the GMean value of sideward scatter (SSC) signal. The GMean value of SSC (recorded as arbitrary units (a.u.)) for the HNA enrichment culture increased from 15 a.u. (inoculum) to 35 a.u. peaked at the early exponential phase, gradually decreased to 10 a.u. in the late exponential phase and finally stabilized. The change in cell size for the LNA enrichment cultures was much less drastic than that of the HNA enrichment, however, a similar trend was observed (Figure 5.7A). The GMean of SSC increased from 4 a.u. to 6 a.u. and stabilized again at 4 a.u; hence, the SSC signal increased only by 50% for the LNA enrichment culture compared to the 133% increase in SSC signal for the HNA enrichment culture. Considering their small cell volume, the LNA enrichment cultures represent only a minor part of the biomass in the total prokaryotic community.

Along with cell growth, the total ATP concentration in the culture of both LNA and HNA increased accordingly (Figure 5.7B). In general, the ATP concentration of LNA enrichment cultures was much lower than that of HNA enrichment cultures (Figure 5.7B). However, their small cell volume explains their low cellular ATP content. If cell size is taken into account, the cytoplastic ATP concentration of LNA cells is comparable to that of HNA cells (Table 5.2).
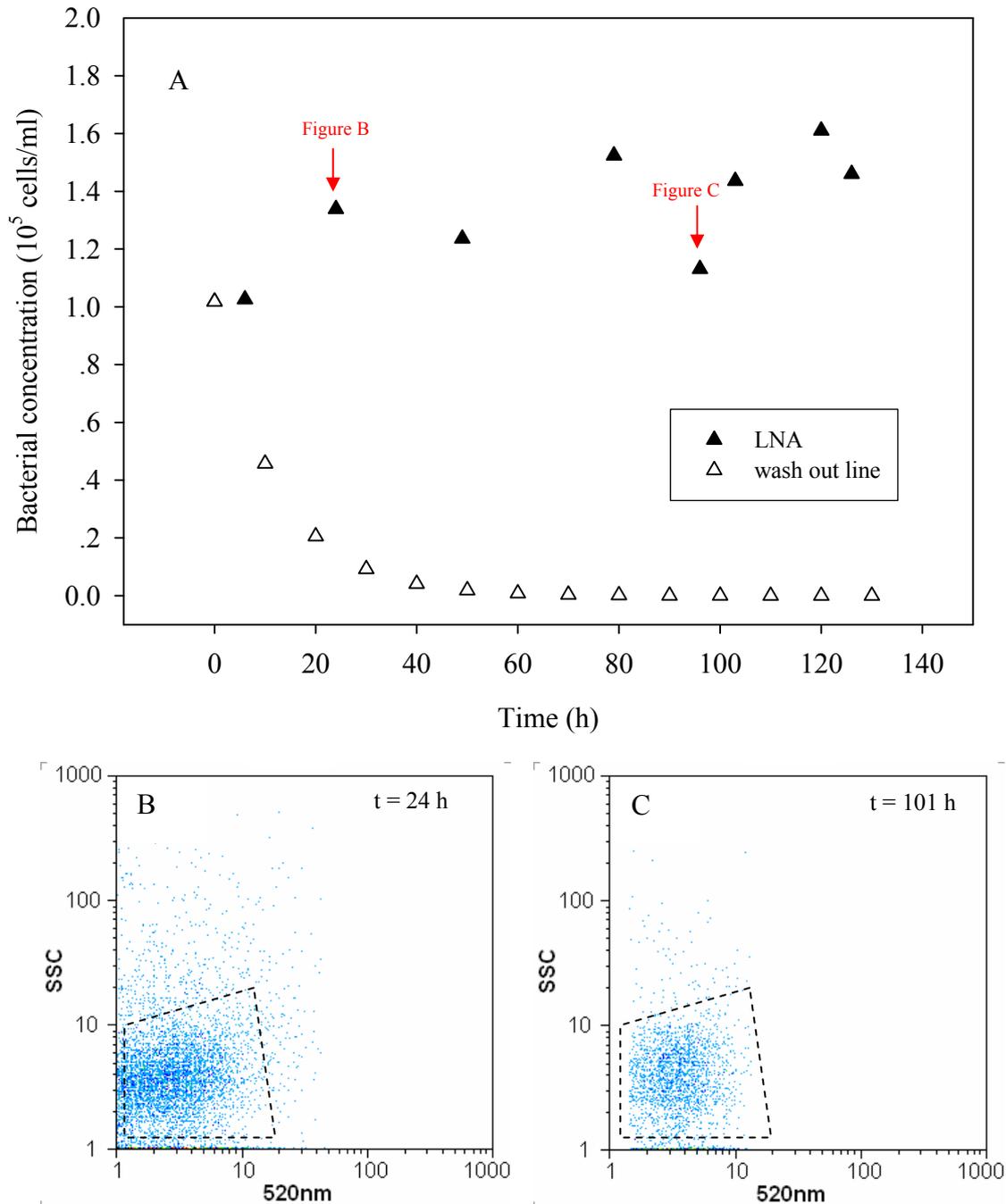
Figure 5.5. Continuous cultivation of autochthonous LNA with sterile river water (D = 0.08 h^{-1}) at 12°C (A). The theoretical wash out line was calculated based on the dilution rate and initial cell concentration. FCM dot-plots at t = 24 h (B) and 101 h (C) were shown below the graph and the LNA bacteria are gated with dashed lines.
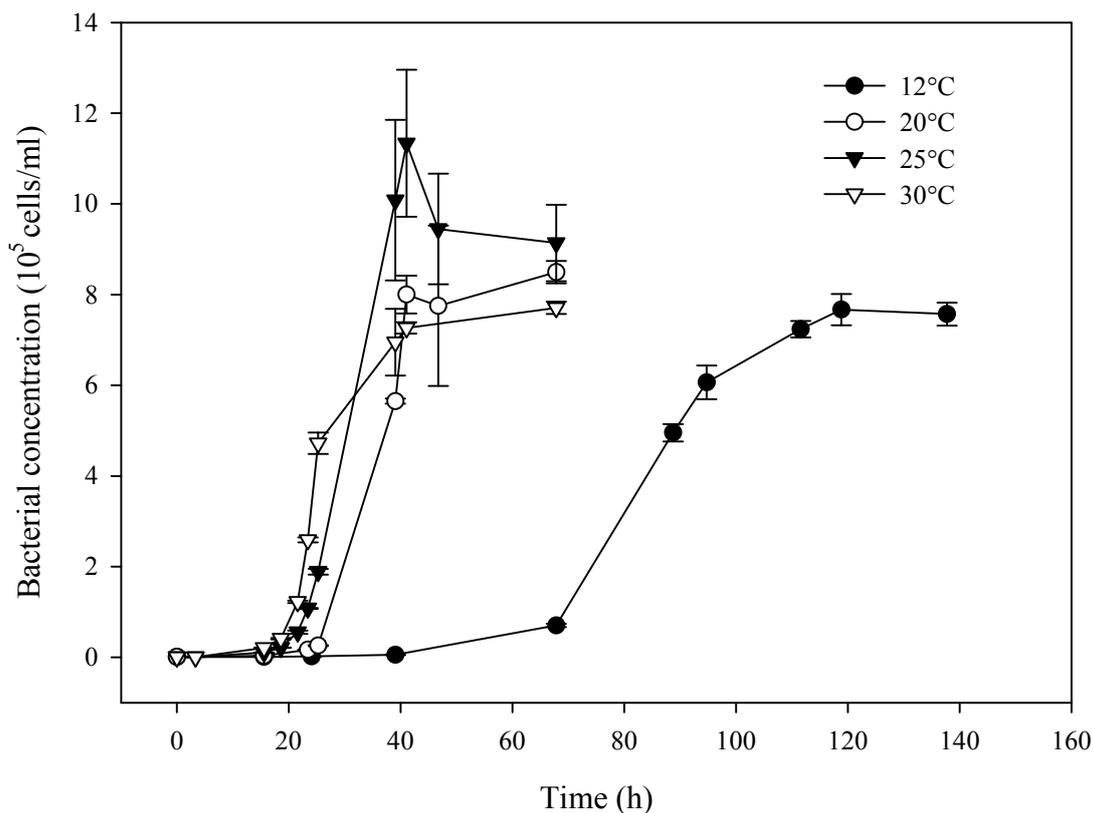
Figure 5.6. Growth of LNA enrichment culture A with natural AOC. (A), growth curve at different temperatures; (B) total ATP concentration measurements during growth at 20°C. Error bars represent the standard deviation for triplicate samples.

Phylogenetic diversity of LNA enrichment cultures and identification of LNA pure cultures

Using a dual approach of FISH and DGGE, we were able to pinpoint the identity of the LNA enrichments. The percentage of DAPI-stained cells that hybridized with EUB338 probe was 89% ± 4% for the LNA enrichments. The CARD FISH revealed that most cells of the LNA enrichments are affiliated to β-proteobacteria cluster (80% for enrichment A, 72% for enrichment B and 88% for enrichment C). No α-*Proteobacteria* were observed in the LNA enrichments, but about 6% of enrichment B belonged to the Gram-positive domain *Actinobacter* and 3% of enrichment C hybridized with *Cytophaga-Flavobacterium* probe. The DGGE analysis showed that one or two bands are dominant in the LNA enrichments we obtained in laboratory (Figure 5.8). Such results make sense in the view of the highly selective procedures we performed to enrich such LNA populations including extinction dilution, single cell sorting and step-wise dilution and re-growth. The predominant bands were further sequenced.
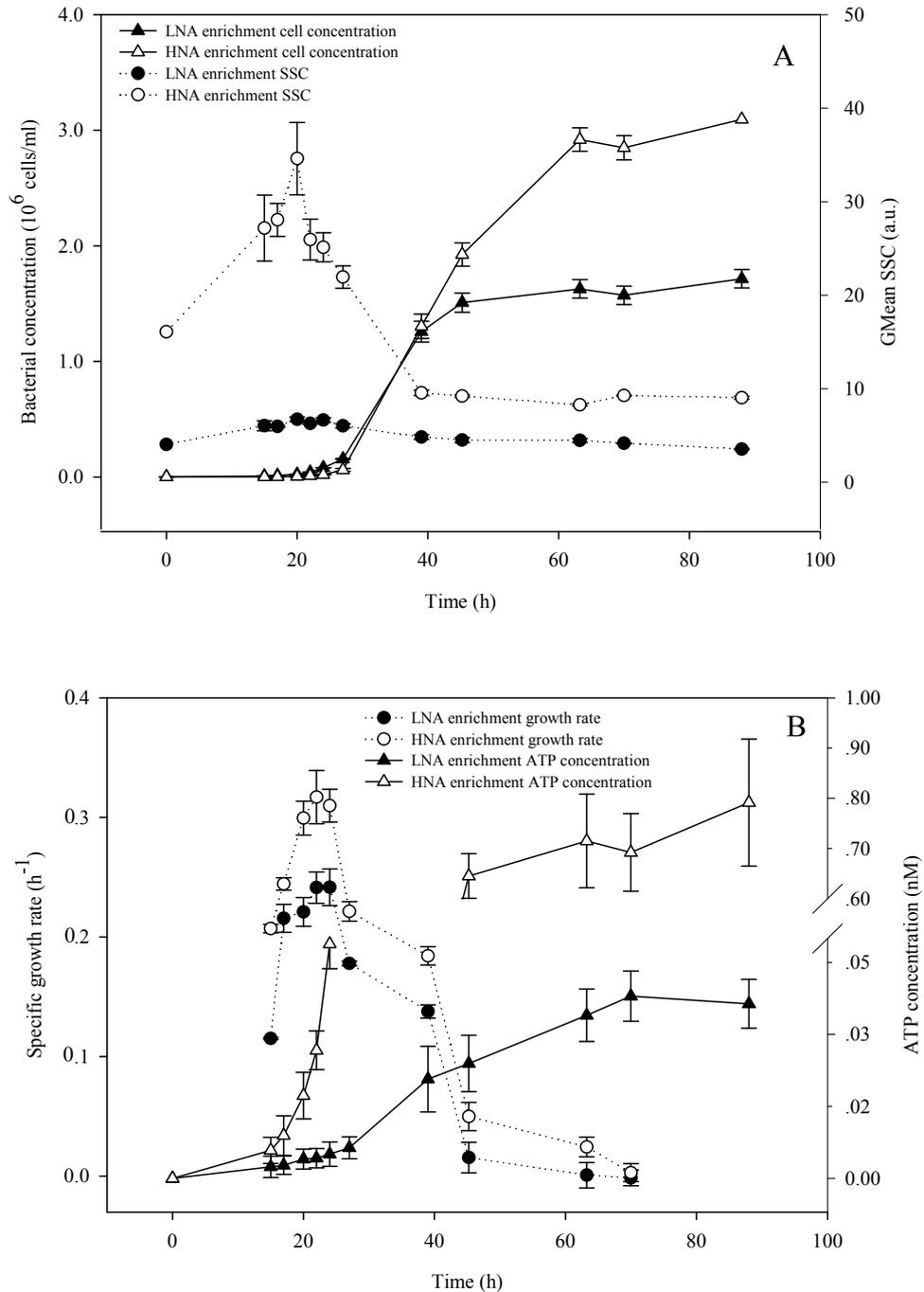
Figure 5.7. Growth comparison between LNA and HNA enrichment cultures. Cell concentration and size changes (A), and specific growth rate and ATP concentration changes (B) during batch cultivation in natural AOC at 20°C. Error bars represent the standard deviation for triplicate samples.

Table 5.2. Growth characterises of LNA enrichment cultures in freshwater medium. GMean values of green fluorescence and SSC were the data of stationary phase cultures. Results are shown in the format of average ± standard deviation calculated from at least three samples from each source location. Cell volume was estimated from the SSC value of the enrichment cultures (Equation 5.2).

	μ_{max} (h^{-1})	Green fluorescence (GMean)	SSC (GMean)	Estimated cell volume (μm^3)	Cellular ATP (10^{-17} gATP/cell)
LNA A	0.23 ± 0.06	7.36 ± 0.56	4.60 ± 0.25	0.01 ± 0.01	1.22 ± 0.26
LNA B	0.19 ± 0.05	5.96 ± 0.63	5.13 ± 0.52	0.02 ± 0.02	3.21 ± 0.36
LNA C	0.24 ± 0.06	8.17 ± 0.59	4.10 ± 0.36	0.01 ± 0.00	1.50 ± 0.31
HNA	0.31 ± 0.08	40.71 ± 1.66	11.56 ± 0.92	0.13 ± 0.02	15.5 ± 2.63

Morphological characterization and identification of LNA pure cultures

In addition, further extinction dilution (1 cell/vial) was carried out on the LNA enrichment cultures to achieve pure culture. Three isolates were obtained by this approach, i.e. isolate CB, isolate GS and isolate WW. The 16s rRNA gene of each isolate was sequenced and deposited in GenBank with accession numbers of EU780139, EU780140, and EU780141. The sequences match 100% with the sequence obtained from the corresponding predominant bands from DGGE analysis (Figure 5.8A), which confirms the purity of the isolates. All three isolates were shown to be closely affiliated to *Polynucleobacter* cluster (Figure 5.8B). Furthermore, repetitive extragenic palindromic-PCR (Rep-PCR) genomic fingerprinting from the isolates indicated that the three isolates were completely different strains (Figure 5.8C).

Pearson correlation [0.0%-100.0%]

DGGE   **DGGE**   **A**

.Chriesbach	LNA enriched
.Wastewater effluent	LNA enriched
.Greifensee	LNA enriched
.Greifensee	Total bacteria
.Wastewater effluent	Total bacteria
.Chriesbach	Total bacteria

B

```
                    ┌ isolate WW
                    │ isolate CB
                    │ Polynucleobacter sp. MWH-T2W17
                    │ Polynucleobacter sp. MWH-MoIso1
                    │ Polynucleobacter sp. MWH-HuW1
                            Polynucleobacter sp. MWH-NZ8W13
                            Polynucleobacter sp. MWH-NZ8W4
                            isolate GS
                    Polynucleobacter necessarius (T)
                    Ralstonia solanacearum (T); ATCC 11696
```

Scale: ├────────┤
          0.01

C

isolate WW

isolate GS

isolate CB

Marker

Figure 5.8. Phylogenetic analysis of LNA bacteria. (A) DGGE and clustering analysis of the water samples. The distance matrix of all the possible gel tracks within the DGGE pattern was calculated by using the Pearson correlation. Based on the values of the resulting matrix, a cluster analysis was performed and visualised as a dendrogram. (B) Phylogenetic tree of three LNA isolates. (C) Rep-PCR of LNA isolates from three different aquatic environments.

The three LNA pure cultures were examined with both SEM and cryo-TEM (Figure 5.9). Different cell shapes were observed from different isolates. Cells of isolate CB and isolate WW have mainly rod shaped structure (Figure 5.9A, C, D and F), while those from isolate GS are in typical curved shape (Figure 5.9B and E). The bacteria possess a cytoplasm full of ribosome particles and contains a diffuse nucleoid. The cell envelope was clearly seen around all cells. The excellent cell preservation of vitrified sample allowed to visualise the lipid bilayer of the plasma membrane (IL) and the outer membrane (OM) (inset in Figure 5.9F) demonstrating that the cell envelope of these LNA isolates is structurally analogous to that of Gram-negative bacteria. The space between the PM and the OM (periplasmic space) is 20 nm thick and contains layered structure similar to the peptidoglycan observed by cryo-sectioning of Gram-negative bacteria (Matias et al, 2003). Furthermore, cells of isolate CB at different cellular cycle stages were imaged by cryo-TEM, documenting a clear cell division process (Figure 5.10).
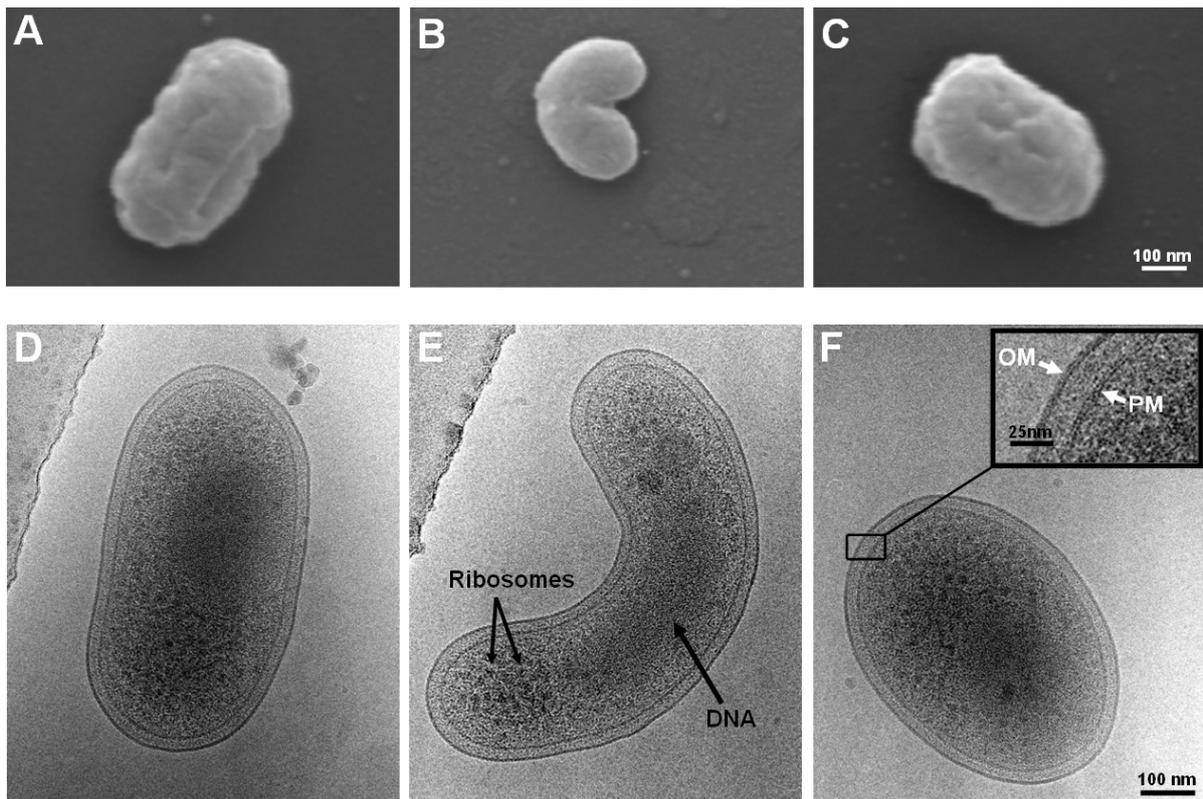
Figure 5.9. Scanning electron micrograph of LNA isolate CB (A), isolate GS (B) and isolate WW (C); and transmission electron micrograph of LNA isolate CB (D), isolate GS (E) and enlarge micrograph to show the membrane structure of isolate WW (F).

During the sample preparation for SEM the bacterial cells probably shrunk due to the dehydration. In contrast, the cells for imaging with cryo-TEM were frozen-hydrated in their native state. Due the well-kept shape and the volume preservation of the frozen-hydrated cells, the volume determination was done from the cryo-TEM micrographs. Cells of all isolates were in the range of 300 to 400 nm in width and 500 to 600 nm in length. The biovolume for the cells was on average below 0.05 μm^3. The periplasmic area is relatively big for the cells. The periplasmic area for isolate CB (Figure 5.9D) accounts for ca. 34% of the total cell volume, ca. 37% for isolate GS (Figure 5.9E) and ca. 23% for isolate WW (Figure 5.9F). The cell periplasmic area is between 3-4 nm thick (inserted in Figure 5.9F). The condensed dark region inside the cell is believed to the chromosome DNA of the cell, which occupies more than 50% of the cytoplasmic area.
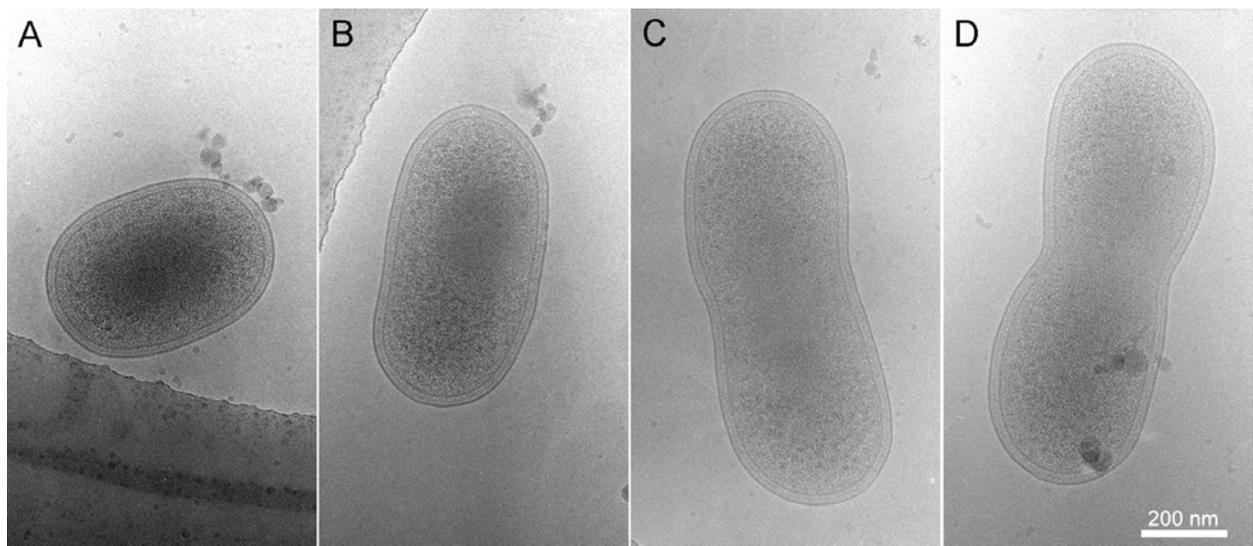
Figure 5.10. Transmission electron micrographs of LNA isolate CB, indicating cell division process (A-D).

Discussion

Presence of LNA bacteria

The separation of natural aquatic microbial flora into LNA and HNA bacterial groups is a typical flow cytometry (FCM) phenomenon based on cellular properties observed by FCM analysis after nucleic acid staining. This observation has been reported in virtually all surface aquatic environments, including sea water (e.g. Li et al., 1995; Gasol and Moran, 1999; Lebaron et al., 2001; Jochem et al., 2004), lakes and rivers (Nishimura et al., 2005; Bouvier et al., 2007), and groundwater, drinking water and wastewater treatment plant effluents (Figure 1, Table 1). Based on cell numbers LNA bacteria had at least an equal share with HNA bacteria in most aquatic microbial communities sampled in the current study (Table 5.1). Therefore, by combining the data from marine environments available in literature (e.g. Li et al., 1995; Marie et al., 1997; Lebaron et al., 2001), we confirmed that the LNA/HNA separation is a general phenomenon of bacterial communities in aquatic ecosystems.

The phenomenon raises the question of microbial activity and diversity. Using FACS in combination with radioactive assays, HNA bacteria have been reported to be the "active" part of the microbial community (e.g., Lebaron et al., 2001; Servais et al., 2003). In contrast, to date, the activities of LNA bacteria has not been clearly demonstrated by the same approach. This cluster has been regarded as ghost or inactive cells by some researchers (Gasol and Moran, 1999; Lebaron et al., 2001; Servais et al., 2003), whereas others reported them to consist of active cells (Zubkov et al., 2001; Jochem et al., 2004; Nishimura et al., 2005). In the current study, after enriching, cultivating and isolating typical LNA bacteria, we were able to address the question of LNA identity more deeply, and our approach gave us the unique chance to investigate their properties in detail.

Cultivation of LNA bacteria

Despite the omnipresence of LNA bacteria in the aquatic environments, there is little information of their growth and characteristics in the literature. To our knowledge, no LNA bacteria have previously been cultivated or isolated. One reason is that the "oligotrophic characteristics" largely handicap investigation of LNA bacteria so far that this is why they

would not be cultivated in conventional rich media, neither complex nor defined. In the current study, such problem is circumvented by applying an unconventional cultivation approach, namely by cultivating the bacteria with natural AOC and monitoring their growth by FCM.

We have observed the growth of the autochthonous LNA bacterial community in continuous culture using sterilized river water as the growth medium (Figure 5.5). If all the cells of LNA bacteria were inactive or dead, they would have washed-out from the reactor under continuous cultivation condition used (Figure 5.5). The ability of LNA bacteria in sustaining at a cell density of 10^5 cells/ml over an extended time proves that the dominant fraction of the autochthonous LNA bacterial community is actively multiplying. Hence, our results demonstrated that the LNA group does not consist of all ghost or inactive cells but that they are able to multiply given the correct cultivation conditions. Consequently, one is now able to investigate whether and how they are different from HNA bacteria. In order to do so, typical LNA enrichment cultures were obtained from different aquatic environments (Figure 5.4).

Growth of autochthonous LNA bacterial communities seemed to occur mainly at lower temperatures (10 - 20°C, data now shown). However, this does not necessarily imply that no LNA bacteria will be able to grow at higher temperatures. The LNA enrichment cultures showed their ability to utilized natural AOC in batch culture at various temperatures (Figure 4A). There is a positive impact of temperature on the specific growth rate of the enriched LNA bacteria, which is consistent with previous report of temperature effect on a natural bacterial community (Vital et al., 2007). The final cell concentrations at different temperatures, however, were similar (Figure 5.6A). Furthermore, the enriched LNA bacterial cells were sensitive to high temperature (37°C). Therefore, the optimal growth temperature likely lies between 20 and 30°C. The growth rates of the LNA enrichments were comparable with natural bacteria communities and other bacteria strains previous studied (Hammes and Egli, 2005; Vital et al., 2007; Wang et al., 2007).

During the batch growth on identical AOC, LNA enrichment cultures displayed growth properties distinctly different from that of the HNA enrichment culture. LNA bacteria showed much less prominent size variation during batch growth on natural AOC than HNA bacteria. Such phenomenon is again explainable by their extreme small cell size. Changes within this size range may not be detectable by FCM. However, a clear cell division and cell size change

were observed by cryo-TEM (Figure 5.10), indicating a normal cell growth cycle. In comparison with HNA enrichment culture, the LNA enrichment cultures had relatively lower specific growth rates and lower production at the same temperature (Table 5.2). In addition, during the enrichemnt process for LNA bacteria, we have observed that the members of HNA bacteria are under most conditions faster growing that the members of LNA bacteria except at low temperature in continuous culture. This is consistent with previous report on LNA versus HNA bacterial production measured by radioactive tracers (Jellett et al., 1996; Zubkov et al., 2001; Nishimura et al., 2005; Tadonleke et al., 2005). However, if the small cell size is taken into consideration, the biomass specific production of LNA bacteria can be similar to that of HNA bacteria (Jochem et al., 2004). Furthermore, the resistance of LNA bacteria to grazing may also partially explained why the LNA bacteria, although having lower growth rates, still persist in high numbers in most natural aquatic environments. It has been reported that grazing pressure is heavier on middle-size class bacteria than those with a small cell volume (Bernard et al., 2000; Grossart et al., 2008). Laboratory studies showed that *Cyclidium* species selectively graze on HNA communities (Tadonleke et al., 2005). Although small bacteria are also vulnerable to grazing, the grazing relating mortality was reported to be much lower (Boenigk et al., 2004).

During the batch cultivation, the total ATP concentration of LNA enrichment cultures increased along with the cell number, which is further evidence of their viability (Figure 5.7B). The small cell volume of LNA bacteria are also closely linked to their low ATP content. The cellular ATP content of a LNA bacterial cell was about 10-times lower than that of an HNA bacterial cell (Table 5.2). The low content of ATP of LNA bacteria may explain why they are always regarded as inactive or dead part of the microbial community. Therefore, the failure to detect activity of LNA bacteria may be due to the sensitivity of the applied methods rather than their actual activity status. Furthermore, the LNA bacteria activity is likely to be masked by that of HNA bacteria and hence ignored by researchers. However, when the data are normalized based on cell volume, the ATP/biovolume for the LNA and HNA communities is quite similar (1.2 vs. 1.5 $\times$ 10^{-15} gATP/μm^3). Therefore, overall low cellular ATP content of LNA bacteria must be attributed to their minute cell volume. Our data suggest that the ATP per microbial biovolume in active cells is a relatively constant value in the range of 1 - 2 $\times$ 10^{-15} gATP/μm^3 for both HNA and LNA bacteria when grown under similar conditions.

Recently, the use of the simplistic dichotomous definition of "active" vs. "inactive" cells in aquatic environments has been criticized (Smith and Giorgio, 2003; Bouvier et al., 2007). Bouvier and co-workers (2007) recently proposed four scenarios for the relationship between LNA and HNA bacteria based on FCM parameters for each group, and suggested each group consisted of cells that are intrinsic to a group, as well as cells that may exchange between groups. The growth data obtained in the current study also showed that the difference between HNA and LNA bacteria is not a simple matter of active or inactive. Our results clearly demonstrate the growth ability of LNA bacteria, and suggest that they are an integral part of the bacterial community. Furthermore, assuming the scenarios proposed by Bouvier and co-workers (2007) are right, we have found and cultivated the LNA bacteria that are intrinsic and do not exchange with HNA bacteria.

Phylogenetic identity

Seeing that LNA enrichment cultures can grow actively with natural AOC and retain their small cell size, the question "what are they?" naturally follows. The phylogenetic identity of LNA enrichment cultures was investigated by CARD-FISH and DGGE. It has been documented that the main bacterial population in freshwater environments are members of the β-proteobacteria group (Glockner et al., 1999; Bruns et al., 2003). Therefore, our finding that the LNA enrichment cells are affiliated mainly with β-proteobacteria was not surprising. The phylogenetic composition of LNA enrichment cultures, however, changes with location (Figure 5.8) and probably also with time. The variation indicates the diversity of LNA bacterial population in different aquatic environments.

Three LNA pure cultures were obtained in the current study. According to their 16S rDNA sequences, the three isolates are completely different strains but are all closely related to the *Polynucleobacter* cluster (Figure 5.8B). Although the original *Polynucleobacter* type strain (*P. necessarius*) is a large and long obligately endosymbiotic bacterium having multiple nucleiods (Heckmann and Schmidt, 1987), several species of this genus were recently reported to be dominant in freshwater habitats (Page et al., 2004; Hahn et al., 2005). Recently, members of *Polynucleobacter* linage were isolated from various sites by gradually enriched cultivation conditions (Hahn, 2003). Although our isolates are phylogenetical closely related to the *Polynucleobacter* spp. isolated by Hahn (2003), they have clearly different growth properties with the former grow restrictly at low nutrient concentration while the latter forms

visible colony on conventional high nutrient agar. The exact systematic position of these isolates still needs further physiological data to confirm.

Morphological characterization

Cultivation and isolation of LNA bacteria not only facilitate investigation of their growth properties, but also give us the chance to obtain their true portrait other than the cluster image on FCM dot-plot. To date, no microscopic image has been published for LNA bacteria. Here, for the first time, we reported morphological characteristics of LNA pure cultures from freshwater (Figure 5.9). Due to their extremely small cell size, there may be limited options of cell shape. Still, the cell shape of the three isolates covers quite diverse shapes, including normal rod (Figure 5.9D), vibrio cell shape (Figure 5.9E) and short-rod (Figure 5.9F). It is believed that surface-to-volume ratio is one of the reasons why most bacteria are rod-, filamentous- or vibrio-shaped, since nonspherical shape increases the ratio for the volume enclosed (Koch, 1996). Cell size estimated by cryo-TEM indicates that the isolates obtained are among the smallest planktonic bacteria known in culture (Schut et al., 1993; Hahn et al., 2003). So far, a marine isolate affiliated with the SAR11 clade has been reported to have the smallest cell size ($0.01~\mu m^3$ estimated with TEM) (Rappé et al., 2002). In comparison, our isolates from freshwater environments have a similar cell size (on average $0.05~\mu m^3$ with the smallest value of $0.01~\mu m^3$ estimated with cryo-TEM). It is known that cells can shrink to certain extend during the SEM sample preparation and therefore are smaller than the actual size. On the other hand, cells may slightly expand when trapped in very thin ice during cryo-TEM examination. Our results showed that the cell size estimated by SEM is up to 5 times smaller than that estimated by cryo-TEM (Figure 5.9). Hence the accurate cell size probably lies between the estimation done by SEM and cryo-TEM. In this study, the cell sizes of LNA isolates were reported according cryo-TEM, since it preserved a much better cell structure than SEM (Figure 5.9). Despite their small cell size, all isolates have a comparable if not larger periplasmic space (20 to 40% of the total cell volume) than that of *E. coli* (20% on average) (Graham et al., 1991). The fact that cells of LNA bacteria have an extremely small volume ($\sim 0.05~\mu m^3$) suggests that their low SSC and low fluorescence intensity is due to their natural size rather than their physiological state.

Cryo-TEM is an optimal method for preserving biological structures (Dubochet at al., 1988). Cryo-TEM of thin vitreous films has become a routine high-resolution technique for the study of isolated small particles such as viruses, liposomes, proteins and others macromolecular

assemblies. The most accurate cryo-technique for viewing prokaryotic ultrastructure involves the use of frozen-hydrated thin sections (Matias et al., 2003); Al Amoudi et al., 2004). However, this method is not suitable for larger objects such as normal bacteria because of their thickness and mass. Due to their extremely small cell size (less than 500 nm diameter), it was possible to obtain cryo-TEM pictures of the frozen LNA bacteria revealing cell envelope structure as well as cytoplasmic content (Figure 5.9). To our knowledge, this is the first study showing the fine structure of frozen-hydrated whole bacteria (without thin sectioning). Our results demonstrate that cryo-TEM is a suitable tool to examine the cell structure of bacteria with minute cell volumes as the "ultramicrobacteria/nanobacteria" group (Janssen at al., 1997; Hahn et al., 2003).

LNA bacteria, "oligotrophs" and "ultramicrobacteria"

Because they cannot be cultivated in conventional high nutrient media and their small biovolume, LNA bacteria may often escape the traditional cultivation attempts and they are therefore still poorly described. Moreover, although LNA bacteria is a typical FCM phenomenon and cannot be reproduced by other techniques, the isolates obtained in the current study are not only representatives of LNA bacteria (i.e., processing typical FCM identity), but also possess the properties of so-called "obligate oligotrophs": they are unable to grow in rich medium, and have properties of "ultramicrobacteria/nanobacteria" (i.e., a cell size < 0.1 μm^3) as well. All these names were created by researchers based on isolated aspects of bacteria (e.g., physiological properties, morphological properties) or different investigation techniques (e.g. flow cytometry, plating). Unfortunately, most of these names are often only vaguely defined and also used differently under various circumstances (Schut et al., 1997). This may lead to confusion of understanding the true nature of the bacteria studied. Having isolates possessing multi-properties of different groups of bacteria indicates the possible overlap of such groups and calls for cautious using of such terms.

In conclusion, we have documented a systematic laboratory investigation, including cultivation, isolation, morphological characterization and phylogenetic analysis, on the LNA bacteria in freshwater environments. Furthermore, the cultivation approach reported here is an important complementary method to the traditional assays applied in microbial ecological studies. We believe that this systematic laboratory study on LNA bacteria would have

significant contribution to a better understanding of aquatic "uncultivated" bacteria, and inspire further investigation on this topic.

Acknowledgement

6. Life under low-nutrient conditions: a batch growth comparison between two very small bacteria

Abstract

Most of the world's natural aquatic environments are low in nutrient concentrations and bacteria living in such environments are typically very small. The growth properties of these small bacteria are poorly understood mainly due to improper cultivation and detection approaches. In the current study, we have used flow cytometry to characterise the batch growth of two very small bacteria (so-called "ultramicrobacteria"), namely isolate CB (a natural stream water bacterial isolate closely affiliated with *Polynucleobacter* cluster based on 16S rDNA sequencing) and *Sphingopyxis alaskensis* (the model "oligotrophic ultramicrobacterium") in low-nutrient environments. Three cultivation media with different carbon quantity and quality were used for the investigation. In general, the two bacteria displayed quite different growth properties. The isolate CB showed higher maximum specific growth rate and achieved higher final cell concentration in natural freshwater than in synthetic laboratory medium at similar carbon concentration. In comparison, *S. alaskensis* exhibited a higher growth rate and final cell concentration in high nutrient concentration synthetic medium. The cell volume of *S. alaskensis* increased, peaked and decreased during the course of a batch cultivation, while such changes were not detectable for the isolate CB. Moreover, it was demonstrated how specific growth rate and final cell number of the two bacteria corresponded to different carbon concentration and incubation temperatures. The difference between growth properties of the environmental isolate and the laboratory model strain challenges the conventional concepts of "oligotroph" and "ultramicrobacteria", and also suggests that strains adapted to laboratory environment may not represent their indigenous behaviour in natural environment anymore. The data presented here provide a further step towards understanding microbial growth in natural environments.

Introduction

Most natural aquatic environments are poor in nutrients, with a dissolved organic carbon (DOC) concentration below 1 mg/l (Morita, 1993). Bacteria living in such environments are mostly small with a biovolume of less than 0.2 μm^3 (Cole et al., 1993; De Duve et al., 1999). These small bacteria (so-called "ultramicrobacteria") constitute a large fraction of the total biomass in aquatic ecosystems and are assumed to play key roles in the biogeochemical cycling of nutrients (Schut et al., 1997). Bacteria living in nutrient poor environments are also often referred to as "oligotrophs", which is a type of microbes that is only vaguely defined (Schut et al., 1997) but in general can grow in low-nutrient media. "Oligotrophs" were further divided into two groups: "obligate oligotrophs" and "facultative oligotrophs" with the former growing only under low-nutrient conditions while the latter can grow in both low- and high-nutrient environments (Morita, 1997). The concept of "oligotrophy" has existed for a long time in microbial ecology, however, with arbitrary and confusing definitions (critical discussed in Schut et al., 1997). Knowledge of the growth properties of this class of bacteria is essential for understanding their impact on ecosystems. However, our current knowledge is largely limited to a few isolates that are able to grow in synthetic media under laboratory conditions. Usually the nutrients are used in high concentrations (g/l range), unlike those typically found in natural aquatic environments where they are present in the μg/l range (Egli, 1995; Morita, 1997).

To date, we still know very little about these ubiquous small and probably oligotrophic bacteria that are abundant in the aquatic environment. The reason probably lies in lack of isolates and proper methods to characterize their growth at low cell density. While the need to comprehensively understand growth in low-nutrient environments has been recognised for many decades (Jannasch, 1969; Morita, 1997), methodological and technological restrictions have resulted in a situation where microbial growth under low-nutrient conditions has not nearly been studied as extensively as pure culture growth in nutrient-rich synthetic media. One of the main reasons for this is that typical analytical tools such as optical density measurements by spectrophotometry, dry weight biomass determination and conventional cultivation on semi-solid growth media are not suitable when studying bacterial growth at low-nutrient concentration as well as low cell densities (Robertson et al., 1998). Flow cytometry has recently become the leading tool for analysing bacterial growth at low-nutrient

concentrations (Hammes and Egli, 2005; Steen, 2000). For instance, Eiler and co-workers (2003) used flow cytometry for analysing growth of aquatic bacteria on different concentrations of natural dissolved organic carbon (DOC). Furthermore, flow cytometry light scatter measurements have been used to estimate bacterial biovolume (Felip et al., 2007) and biomass (Robertson et al., 1998) at low cell densities. Recently in our group, we have also described the potential of flow cytometry for studying microbial growth in natural aquatic environments (Hammes et al., 2005; Vital et al., 2007; Wang et al., 2007).

To our knowledge, the most comprehensive understanding of the growth of oligotrophs comes from a facultative oligotrophic bacterium *Sphingopyxis alaskensis*. Strains of this species are assumed to be very abundant because they were isolated from three geographically separated locations: Resurrection Bay (Alaska), the North Sea, and coastal waters near Japan using the extinction dilution method (Cavicchioli et al., 2003). The bacterium was not able to grow in rich medium after the initial isolation. However, after long time storage at 4°C, an unknown mechanism made it able to grow in high nutrient medium as well (Eugchi et al., 1996). Hence, the strain was later referred as facultative oligotroph. Nevertheless, this bacterium is regarded as the model ultramicrobacteria based on three unique characteristics: (1) a constant ultramicro-size (< 0.1 μm^3) irrespective of whether it is growing or starved, (2) the ability to utilize low concentrations of nutrients, and (3) the ability to simultaneously take up mixed substrates (Schut et al., 1995).

Recently, we have isolated a typical small freshwater bacterium, namely isolate CB, which is closely affiliated with *Polynucleobacter* cluster based on 16S rDNA analysis. The isolate possesses a distinctive small biovolume (<0.05 μm^3) and the ability to grow at low nutrient concentrations but not in conventional rich medium. According to the definitions in literature, the isolate should be considered as a typical "obligate oligotroph" and "ultramicrobacterium" (Cavicchioli et al., 2003; Morita, 1997). The main purpose of this study was to compare the growth properties between this new isolate and the well-known model bacterium *S. alaskensis*, using flow cytometry and low-nutrient cultivation methods. We have investigated their growth in three different media at various carbon concentrations. Furthermore, the temperature and carbon concentration effect on the maximum specific growth rate were studied as well. Here we demonstrated the successful application of an unconventional cultivation approach (namely using natural freshwater cultivation medium and flow cytometry

monitor). The results provide a further step towards a more comprehensive understanding of the growth of the small bacteria in oligotrophic aquatic environments.

Materials and Methods

Cultures and media

Isolate CB from freshwater (Chapter 5) and *Sphingopyxis alaskensis* (RB2256) were used for the current study. The batch growth comparison was carried out in different media. The first was freshwater from a shallow stream (Chriesbach, Duebendorf, Switzerland) containing its natural assimilable organic carbon (AOC) with a DOC concentration of 2.5 - 4.1 mg/l (Vital et al., 2008). This water was sampled in a 1 l Schott bottle, pasteurized at 60°C for 30 min, cooled down to room temperature and then filtered through a 0.1 μm pore size filter (Millipore, USA) before usage. The second medium consisted of different dilutions of Lysogeny Broth (LB) medium. The LB medium was diluted with commercially available bottled water (Evian). The bottled water was poured into a 1 l Schott bottle, pasteurized at 60°C for 30 min, cooled down to room temperature and then full strength LB (10 g/l tryptone, 5 g/l yeast extract and 10 g/l sodium chloride) was added to achieve the correct dilution (i.e., 50,000, 10,000, 5,000, 2500, 1000, 50, and 10-times diluted). The mixture was then filtered through a 0.22 μm pore size filter (Millipore, USA). All filters were pre-washed with nano-pure water (50 ml) to remove contaminating carbon from the filters. All experiments were done in 20 ml carbon-free glassware prepared as described elsewhere (Hammes and Egli, 2005).

Growth characterization

The two bacterial strains were pre-cultivated in 10,000-times diluted LB medium at 30°C for four days. The stationary phase cells were then used as inocula. For each batch cultivation in the media described above, the initial cell concentration was 4000 cells/ml. All growth comparisons were carried out at 30°C. Samples were taken from each vial at regular time intervals till stationary phase was reached. The cell concentrations and cellular biovolume

were analyzed with flow cytometry as described below. The specific growth rate (μ) based on cell number increase in each sample was determined as follows (Equation 6.1):

$$\mu = (Ln(n_t) - Ln(n_0))/\Delta t$$
<div align="right">Equation 6.1</div>

where n_t, n_0 are the cell concentrations measured at two subsequent time points and Δt is the elapsed time interval between these points.

Temperature effect

Isolate CB and *S. alaskensis* were cultivated in pasteurized stream water (as described above) at different temperatures (ranging from 12 to 37°C). The specific growth rate was calculated as described above. All samples were done in triplicate.

Flow cytometry

Flow cytometry was performed using a CyFlow® space flow cytometer (Partec, Hamburg, Germany) equipped with a 200 mW argon ion laser, emitting at a fixed wavelength of 488 nm, and volumetric counting hardware. Bacterial cell concentrations were measured with flow cytometry as described by Hammes and co-workers (2008). Bacterial cells were stained with 10 μl/ml SYBR® Green I (1:100 dilution in DMSO; Molecular Probes), and incubated in the dark for at least 15 minutes before measurement. When necessary, samples were diluted just before measurement in cell-free water, so that the concentration measured in the flow cytometer was always less that 2×10^5 cells/ml. The specific settings were previously described (Chapter 5). The sideward scatter signal (SSC) was recorded as the geometrical mean (Gmean) of all data in arbitrary units (a.u.). The Gmean value was then converted to cellular biovolume using a calibration curve produced based on 5 bacterial strains including *Escherichia coli*, *Pseudomonas aeruginosa*, *Vibrio cholerae*, *S.alaskensis* and isolate CB (Equation 6.2). Bacterial volumes were measured with light microscope and the mean volume was calculated from at least 40 cells. The conversion equation is specific for the flow cytometry used in the current study.

Cellular volume (μm^3) = 0.0006 × (Gmean SSC)$^{2.2}$
<div align="right">Equation 6.2</div>

The carbon biomass was estimated by the calculation proposed by Norland (1993) (Equation 6.3 and 6.4).

Carbon per cell (pg) = 0.12 (pg/ μm^3) × [cellular volume (μm^3)]$^{0.7}$ Equation 6.3

Carbon biomass (pg/l) = cell number (cells/l) × carbon per cell (pg)   Equation 6.4

LC-OCD analysis

The organic matter in 10,000-time diluted LB medium was characterized by liquid chromatography coupled to an organic carbon detector (LC–OCD) using a size exclusion column Toyopearl TSK HW-50S (250 × 20 mm) having a fractionation range of 100–20,000 Da as described by Meylan and co-workers (2007). Cultures of isolate CB, *S. alaskensis*, and an AOC test-community (Hammes and Egli, 2005) were inoculated in the medium, three vials per culture. After 4 days of incubation until stationary phase was reached, samples (10 ml) from each vial were analyzed by LC-OCD. Three blank samples were also analyzed as sterile control. The different fractions, including dissolved organic carbon (DOC), polysaccharides, humics, low molecular weight (LMW) OC, LMW neutrals and hydrophobic OC (as the difference between DOC and the chromatographically detected OC) were quantitatively evaluated by software analysis of the chromatogram (Huber and Frimmel, 1996). The detection limit was 10 µg C/l.

AOC measurement

The AOC concentration in pasteurized stream water and 10,000-times diluted LB medium were measured with a batch growth bioassay with a natural microbial community (Hammes and Egli, 2005; Vital et al., 2007). The final cell concentration is then converted into AOC concentration (µg/l) with a conversion factor of 10^7 cells/(µg AOC) (Vital et al., 2007; Chapter 4).

Results and Discussion

Batch growth in different media

The first of the three media used to cultivate the two strains was natural stream water with an assimilable organic carbon (AOC) concentration of 320 µg/l, as measured by the bio-assay described by Hammes and Egli (2005). The second medium was 10,000-times diluted LB medium, with 700 µg/l of dissolved organic carbon (DOC), within which 309 µg/l was AOC. The third medium was 10-times diluted LB medium, with 700 mg/l DOC. In the natural stream water medium, isolate CB showed similar maximum specific growth rate as *S. alaskensis* (0.37 vs. 0.40 h^{-1}) at 30°C, but with a slightly lower final cell concentration (0.7 vs. 1.0×10^6 cells/ml) (Figure 6.1). When grown in 10,000-times diluted LB medium with a similar AOC content, isolate CB achieved a much lower specific growth rate (0.21 h^{-1}) than did *S. alaskensis* (0.36 h^{-1}). Also the final cell concentration reached by isolate CB was significantly lower than that of *S. alaskensis* (0.26 vs. 8.97×10^6 cells/ml) (Figure 6.2 and Table 6.1). No growth was recorded for the isolate CB in the conventional rich medium (10-times diluted LB medium), while *S. alaskensis* grew normally with an enhanced specific growth rate (0.47 h^{-1}) as well as final cell concentration (5.77×10^8 cells/ml) (Figure 6.3). It is interesting to note that isolate CB coped with the natural carbon source much better than the synthetic carbon source at similar AOC concentrations (Figure 6.1 and 6.2). The opposite was observed for *S. alaskensis*, which grew better in standard complex medium achieving both a higher maximum specific growth rate and a higher final cell concentration (Figure 6.1 and 6.2).

It should be pointed out that isolate CB is a fresh isolate from stream water (Chapter 5) while *S. alaskensis* is a marine isolate that had adapted to laboratory rich medium, though it was also not able to grow in such medium first after isolation (Schut et al., 1997). It was previously argued that the environmental isolates, which had adapted in the laboratory to high nutrient concentrations, may no longer represent its original state in the natural environment (Gottschal, 1992). This may partially explain the different growth properties between isolate CB and *S. alaskensis*.

The study of the growth properties of very small bacteria is seriously hampered by the lack of proper growth media as most of these bacteria cannot grow in laboratory rich media. It was accepted that truly low-nutrient synthetic freshwater media are difficult to prepare. In fact, natural freshwater itself represents the ideal low-nutrient growth environment for most autochthonous bacteria, as suggested from our results and previous reports (Hammes and Egli, 2005; Vital et al., 2007). Here we showed a cultivation strategy that overcomes all those problems, with natural freshwater as a realistic medium and flow cytometry as the appropriate tool to monitor growth. The data presented here showed that one can in fact characterize growth very well with this cultivation strategy.
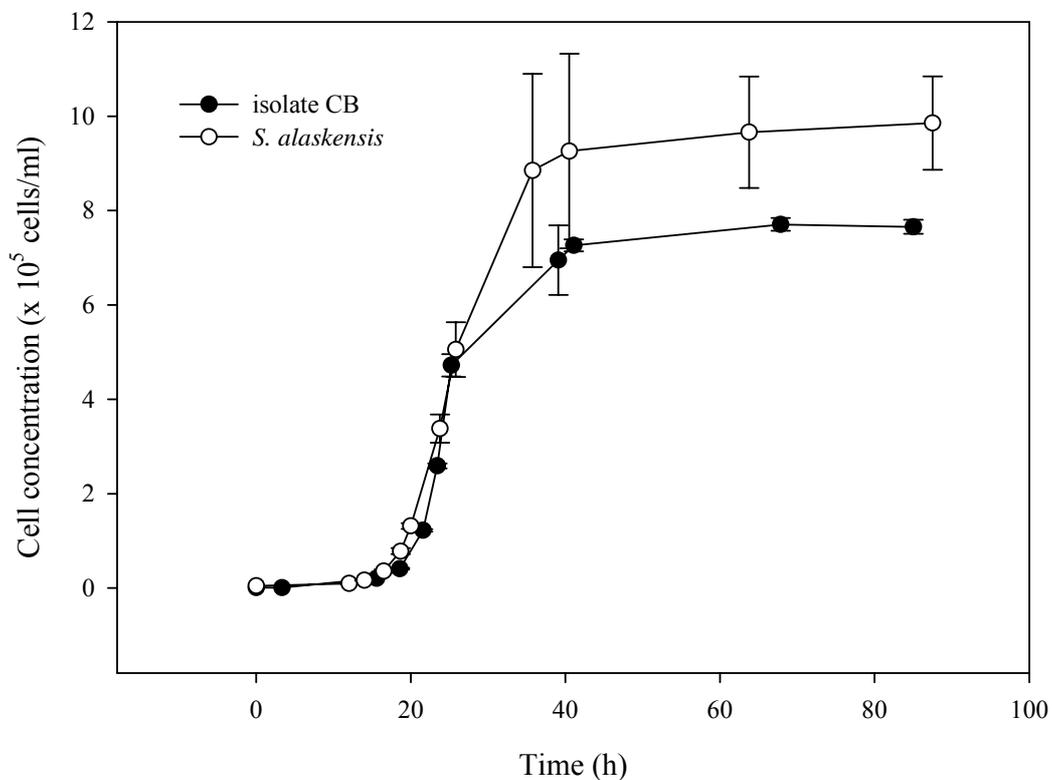
Figure 6.1. Batch growth curves of isolate CB, and *S. alaskensis*, cultivated in natural AOC. Error bars indicate standard deviation on triplicate samples.
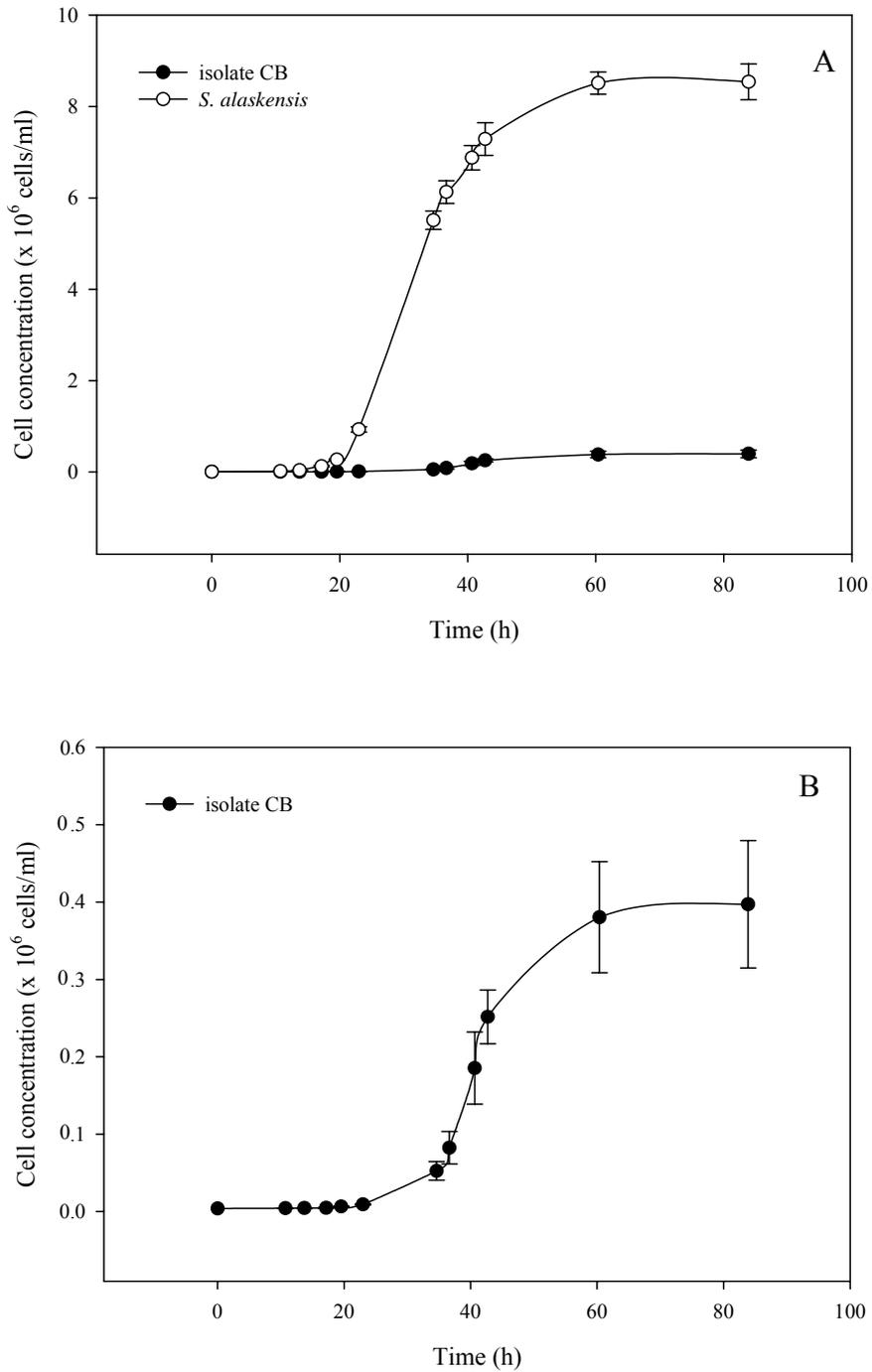
Figure 6.2. Growth curves of isolate CB and *S. alaskensis* cultivated in 10, 000-times diluted LB (A) with the curve for isolate CB was plot separated to show the cell concentration changes clearly (B). Error bars indicate standard deviation on triplicate samples.
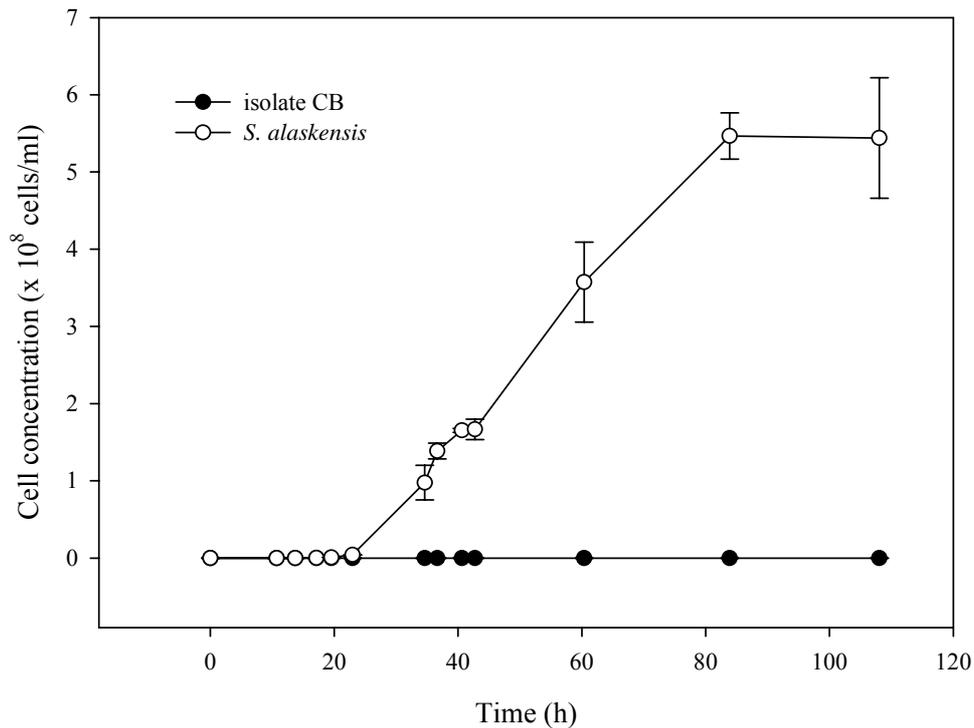
Figure 6.3. Growth curves of isolate CB and *S. alaskensis* cultivated in 10-times diluted LB. Error bars indicate standard deviation on triplicate samples.

Growth rate, cellular volume of batch-grown cells

Cell volume changed considerably during batch growth of *S. alaskensis*. The cellular volume increased, peaked at early exponential phase (0.23 μm^3), decreased and stabilized at stationary phase (0.04 μm^3) (Figure 6.4A and 6.5). The specific growth rate during batch cultivation also follows a similar pattern (Figure 6.4B), indicating there is a positive correlation between cell volume and specific growth rate. It has been reported that there is a strong correlation between the specific growth rate and the cell volume (Marr, 1991; Nystroem and Kjelleberg, 1989; Schaechter et al., 1958; Tempest et al., 1967). Schaechter and co-workers (1958) illustrated the cell volume changes of *E. coli* cells growing in continuous culture at different dilution rates. Similarly, it was shown that *Vibrio* sp. ANT 300 increase in cell volume from 0.48 to 1.16 μm^3 at dilution rates of 0.015 to 0.17 h^{-1} respectively (Morita, 1997). Furthermore, such correlation between growth rate and cell volume was also documented for batch cultivation (reviewed by Wanner and Egli, 1990). Unlike *S. alaskensis*, no significant change in cell volume was observed for the isolate CB. Since cell volume change is intimately

linked to growth, we believe this is caused by the fact that the cell volume of isolate CB is extremely small (< 0.05 μm^3, about half of that for *S. alaskensis*) and changes in this magnitude (e.g. from 0.02 to 0.04 μm^3) is not detectable by the method applied (Figure 6.5).
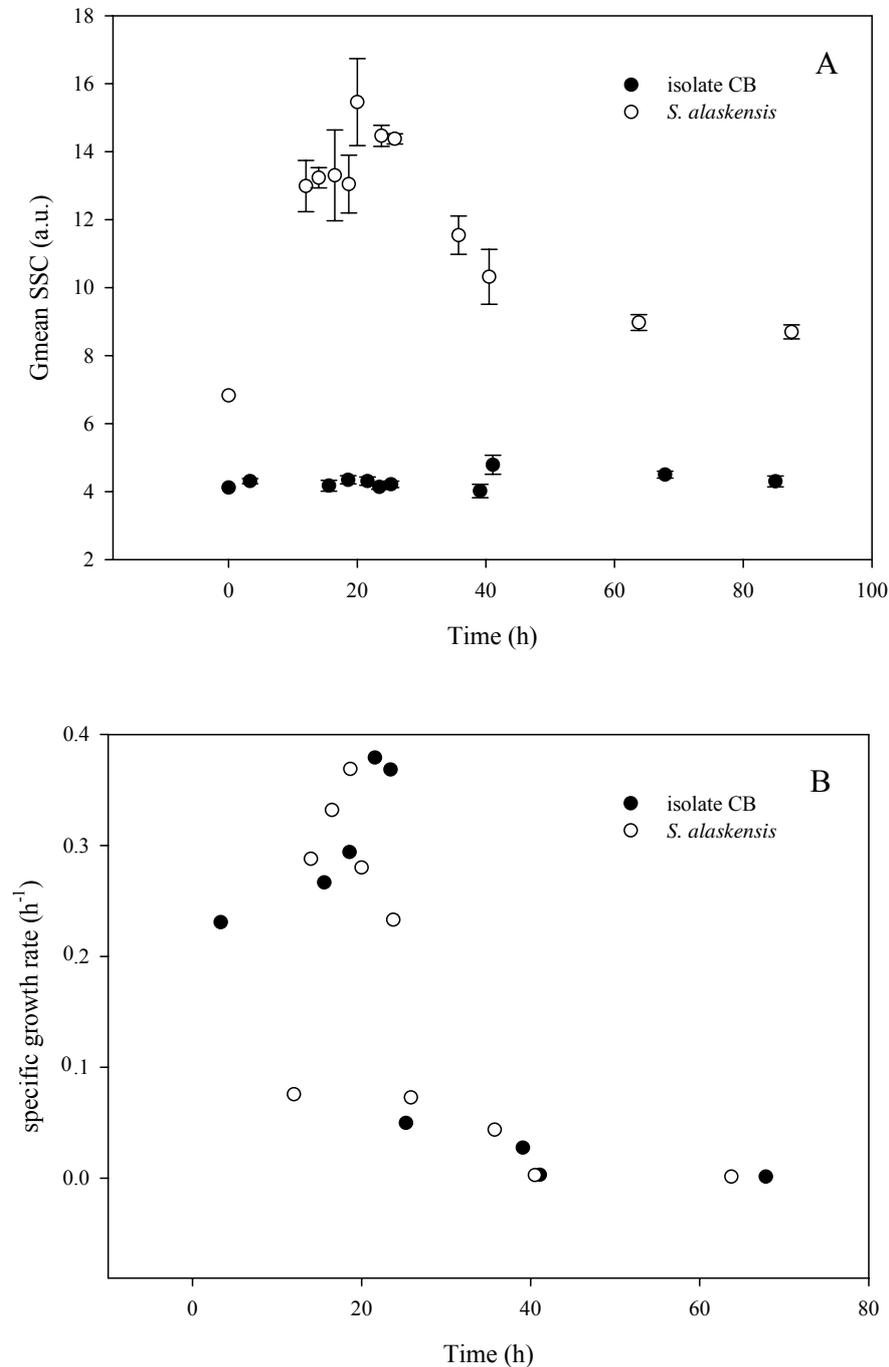
Figure 6.4. Cell volume (A) and specific growth rate (B) changes during batch growth of isolate CB, and *S. alaskensis*, cultivated in natural AOC. Error bars indicate standard deviation on triplicate samples.
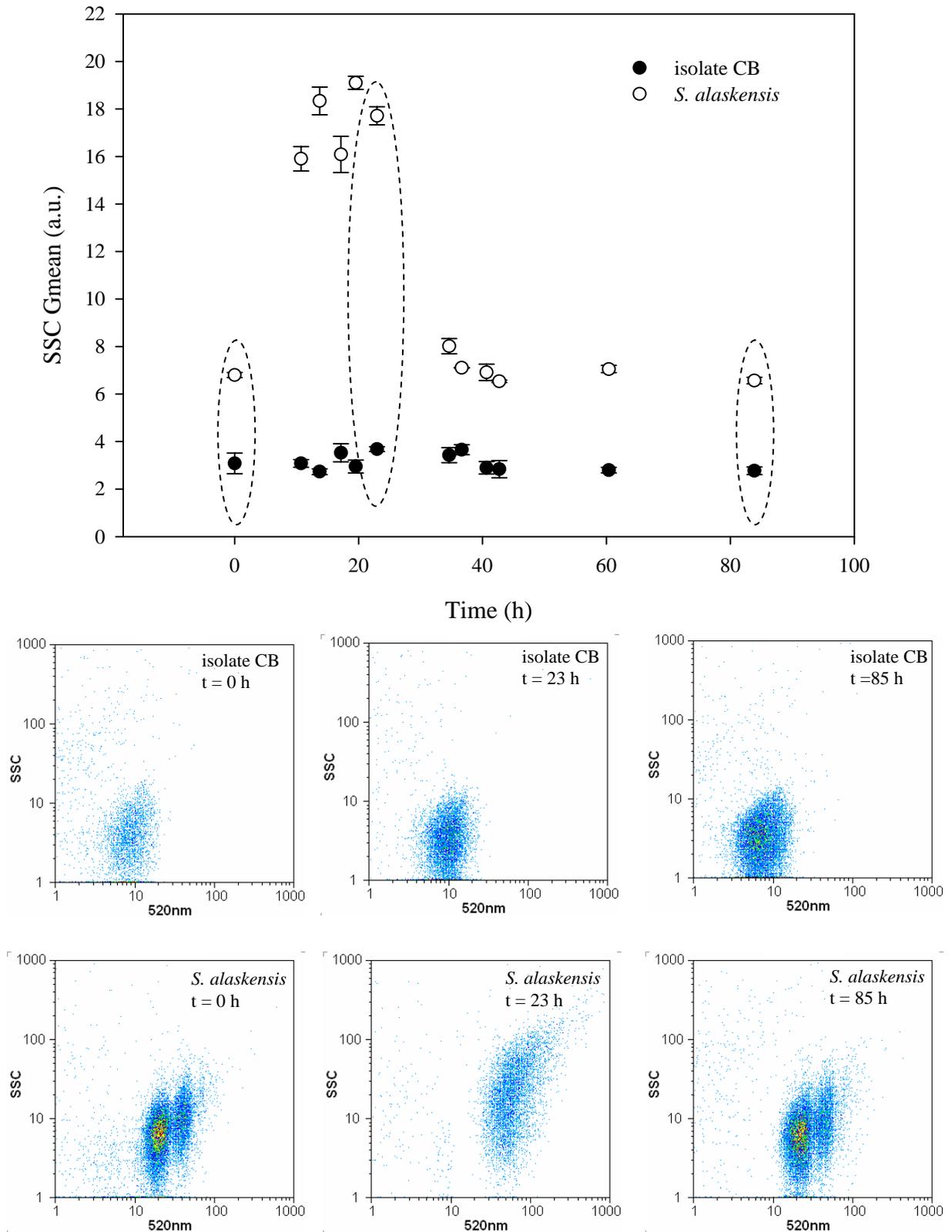
Figure 6.5. Cell volume changes and representative FCM graphs (green fluorescence vs. sideward signal) of isolate CB, and *S. alaskensis* during batch cultivation in natural AOC. Error bars indicate standard deviation on triplicate samples.

It was previously reported that cell volume of "obligate oligotrophs" did not change over a wide range of nutrient concentrations (Ishida and Kadota, 1981). This was also claimed for *S. alaskensis*; it was reported that this model facultative oligotroph maintain constant cell volume irrespective whether it is growing or starved, even when cultivated on nutrient-rich media (Cavicchioli et al., 2003; Fegatelia and Cavicchioli, 2000). However, since growth by binary fission is intimately linked to an increase in cell volume, this claim is probably due to the lack of sensitive methods for detection the volume changes. Indeed, such behaviour of *S. alaskensis* was not observed, and significant cell volume changes were recorded in the present study (Figure 6.4 and 6.5). This may attribute to the fact in previous studies cell volume was measured with microscopy (Christian et al., 1982; Eguchi et al., 1996; Eiler et al., 2003), which may not be sensitive and fast enough to detect the cell volume changes during batch growth. In this study we have used flow-cytometric measurements of the amount of scattered light by each individual particle as indicative of particle size (Felip et al., 2007), and used the geometrical mean value to characterise the average size in the population. The ease and speed of flow cytometric measurements make it possible to analyze significantly larger batches of samples frequently.

The difference in cell volume and its changes between isolate CB and *S. alaskensis* also challenge the concept *S. alaskensis* as a "model ultramicrobacterium". The average cellular volume changed relatively dramatically (from 0.04 to 0.23 μm^3) for *S. alaskensis*, while the average cellular volume was always in the range of 0.05 μm^3 for isolate CB. According to the definition of "ultramicrobacteria", isolate CB would therefore fit this description considerably better than the *S. alaskensis*. Thus, this indicates that one should be careful of extrapolating and generalize the knowledge we gain from a few isolates that we are presently able to handle in the laboratory.

Temperature effect on growth rate

Temperature is always a potential growth limiting factor (Pomeroy and Wiebe, 2001). It was suggested by many previous investigations that temperature has a critical role in regulating bacterial activity (e.g., Kirchman and Rich, 1997; Morita, 1997; White et al., 1991). The impact of temperature on growth of isolate CB and *S. alaskensis* in natural stream water was also investigated and the results are summarized in Figure 6.6. The maximum specific growth rate for *S. alaskensis* increased with increasing temperature in the range tested in the current

study. The result is consistent with previously reported temperature profile of *S. alaskensis* grown in defined glucose-mineral medium, where the optimum growth temperature appeared to be between 30 and 40°C (Eguchi et al., 1996). In comparison, for isolate CB the proportional increase of growth rate to temperature only holds true below 30°C (Figure 6.6). Furthermore, no growth was recorded at 37°C, which indicates that isolate CB is sensitive to elevated temperatures. A similar temperature profile was reported recently for *E. coli* O157 grown in natural river water (Vital et al., 2008). It is interesting to notice that at low temperatures (below 30°C), isolate CB exhibited much higher specific growth rates than *S. alaskensis* (Figure 6.6). This indicates that isolate CB copes with low temperature better than *S. alaskensis*.
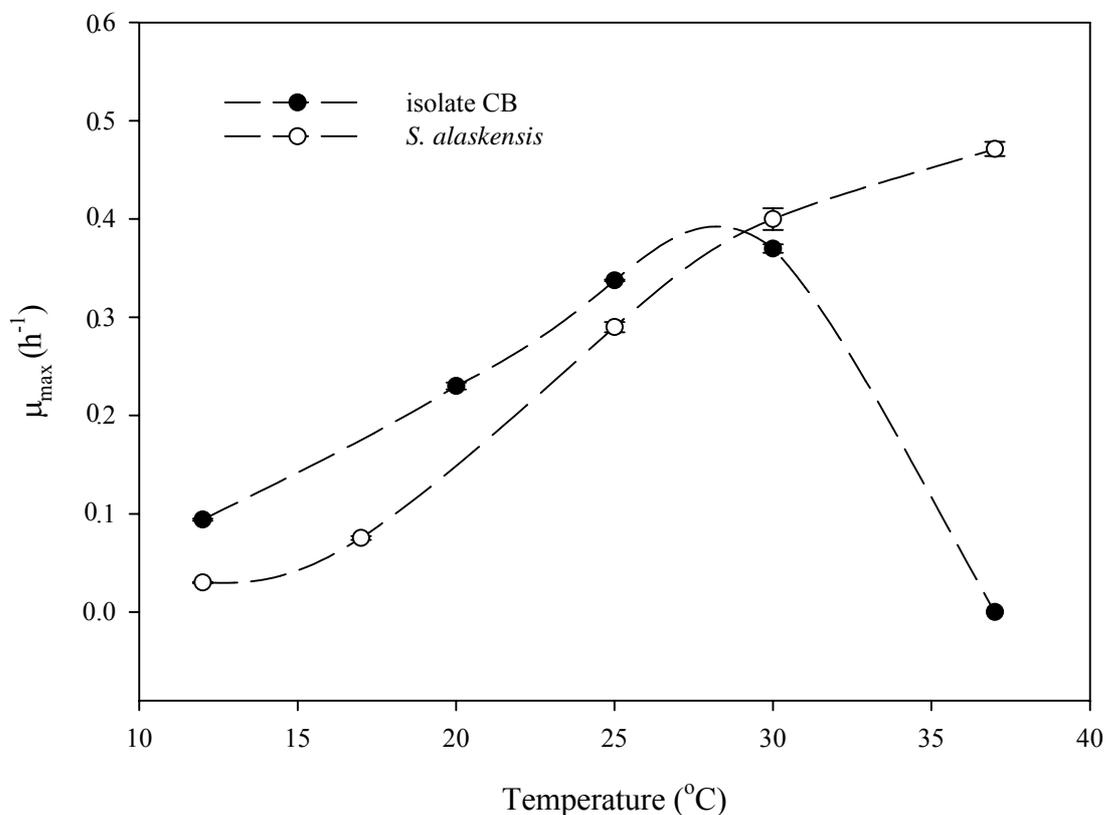
Figure 6.6. Temperature effect on growth rates of isolate CB and *S. alaskensis* cultivated with natural AOC. Error bars indicate standard deviation on triplicate samples.

Dissolved organic carbon (DOC) concentration effect

Besides temperature, DOC appears to be another main factor affecting bacterial growth rate and production. Isolate CB and *S. alaskensis* were grown in batch cultures with diluted LB to determine the effect of DOC concentration on specific growth rate and final cell concentration. For *S. alaskensis*, the specific growth rate increased with the increasing the concentration of DOC in the complex medium (Figure 6.7). Meanwhile, the final cell concentration also changes proportionally to DOC concentration up to 14 mg/l (Figure 6.8). Two linear correlations ($r^2 = 0.99$) were recognized between final cell concentration of *S. alaskensis* and DOC concentration within this range (Figure 6.8B). The slope of the linear regression reduced as the DOC concentration decreased (Figure 6.8B). In contrast, minor changes in the maximum specific growth rate and only a slight increase in the final cell concentration were observed during batch-growth of isolate CB with LB in response to increasing concentrations of DOC up to 1.4 mg C/l (Figure 6.7 and 6.8). A further increase in the initial DOC concentration resulted in reduction of the maximum specific growth rate and final cell concentration of isolate CB and growth ceased when the DOC concentration reach 100 mg C/l (Figure 6.8). This is in consistent with a previous report that the maximum specific growth rate of aquatic bacteria was constrained by the substrate concentration in oligotrophic environments (Eiler et al., 2003).

The response of isolate CB to high nutrient concentrations is that considered to be typical for an "obligate oligotroph" (Morita, 1997). Despite the fact that the term "obligate oligotrophy" is not clearly defined it is normally used for bacteria that can only grow at low carbon concentrations (mg C/l). The line was drawn arbitrarily and the ranges reported by some researchers, e.g., 1-15 mg C/l (Kuznetsov et al., 1979), 5.5 mg C/l (Hood and MacDonell, 1987), 1-6 mg C/l (Fry, 1990) correspond nicely with our observations made for isolate CB. A number of explanations have been put forward for explaining why microbial cells fail to grow at elevated nutrient concentrations. For example, Postgate and Hunter (1964) postulated that the phenomenon might be caused by substrate-accelerated death when the uncontrolled influx of excess nutrients leads to intoxication. Others have proposed for example that cells may be killed by an increased rate of free radical formation at high nutrient concentration (Walker, 1996). Koch (2001) summarized some possible reasons for the inhibition or killing by high nutrient concentration, however, up to date, the reasons are still vague. Definitely, the

concentrations of nutrients in rich media are often unrealistically high and hence improper for study of growth properties for most indigenous aquatic bacteria.
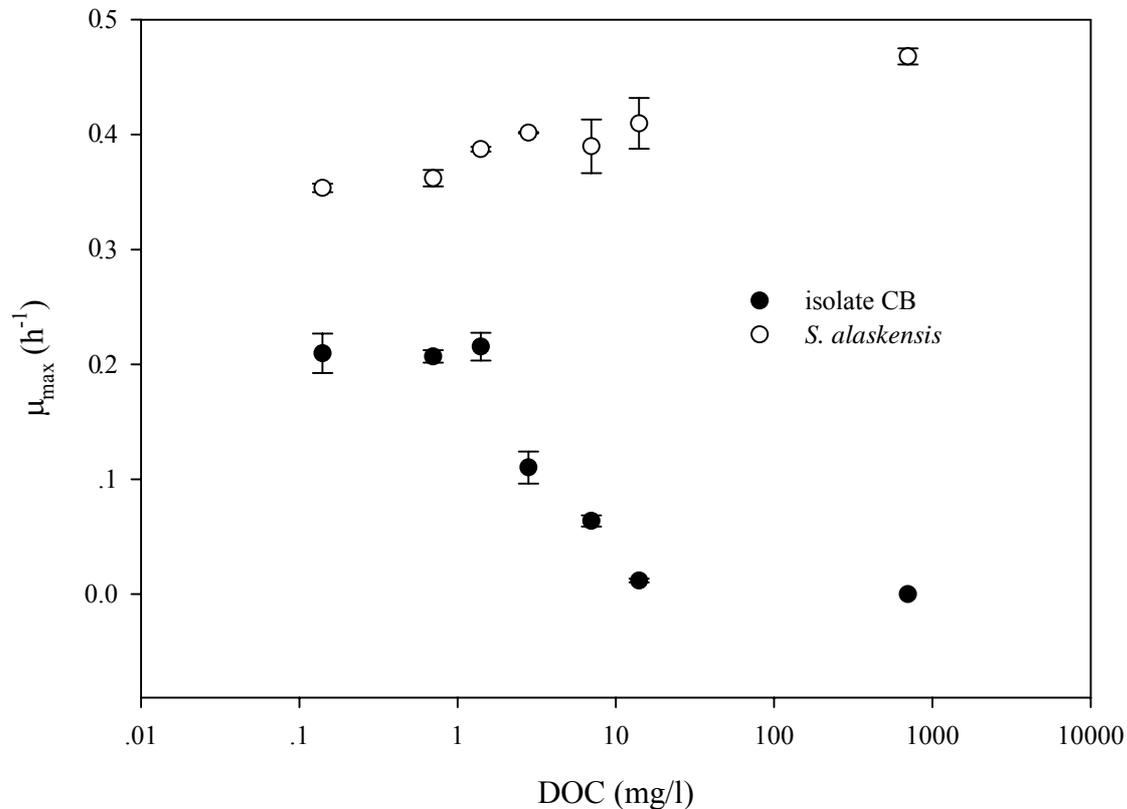
Figure 6.7. Effect of carbon concentration on maximum specific growth rates during batch cultivation of isolate CB and *S. alaskensis* in different dilutions of LB medium at 30°C. Error bars indicate standard deviation on triplicate samples.

The inability of isolate CB to grow in high nutrient medium does not necessary mean that it can never adapt to such an environment, since this is what appears to have happened to *S. alaskensis* after long term incubation at low temperature (Eguchi et al., 1996). Based on this it has been postulated that "obligate oligotrophs" can adapted to high nutrient media and thus become "facultative oligotrophs" (Button et al., 1998; Eguchi et al., 1996) and sometimes even lose their ability to grow on low-nutrient media and become "copiotrophs" (Akagi et al., 1980). Hence, the terms "obligate oligotroph", "facultative oligotroph" and "copiotroph" are therefore not on very solid foundation. In our opinion, one should rather focus on the true nature of bacterial growth than on terms utilized to describe them until a more extensive data base is available. It lacks a logic sense to wait until an environmental isolate adapted to a high

nutrient medium and then its their growth and physiology since it is not likely that such adapted isolates still possess their indigenous properties. The cultivation approach presented in this study, (namely using natural surface water as the medium in combination with monitoring cell growth by flow cytometry) made it possible to investigate the growth and some physiological properties of very small bacteria under conditions that come close to those cells encounter in nature.
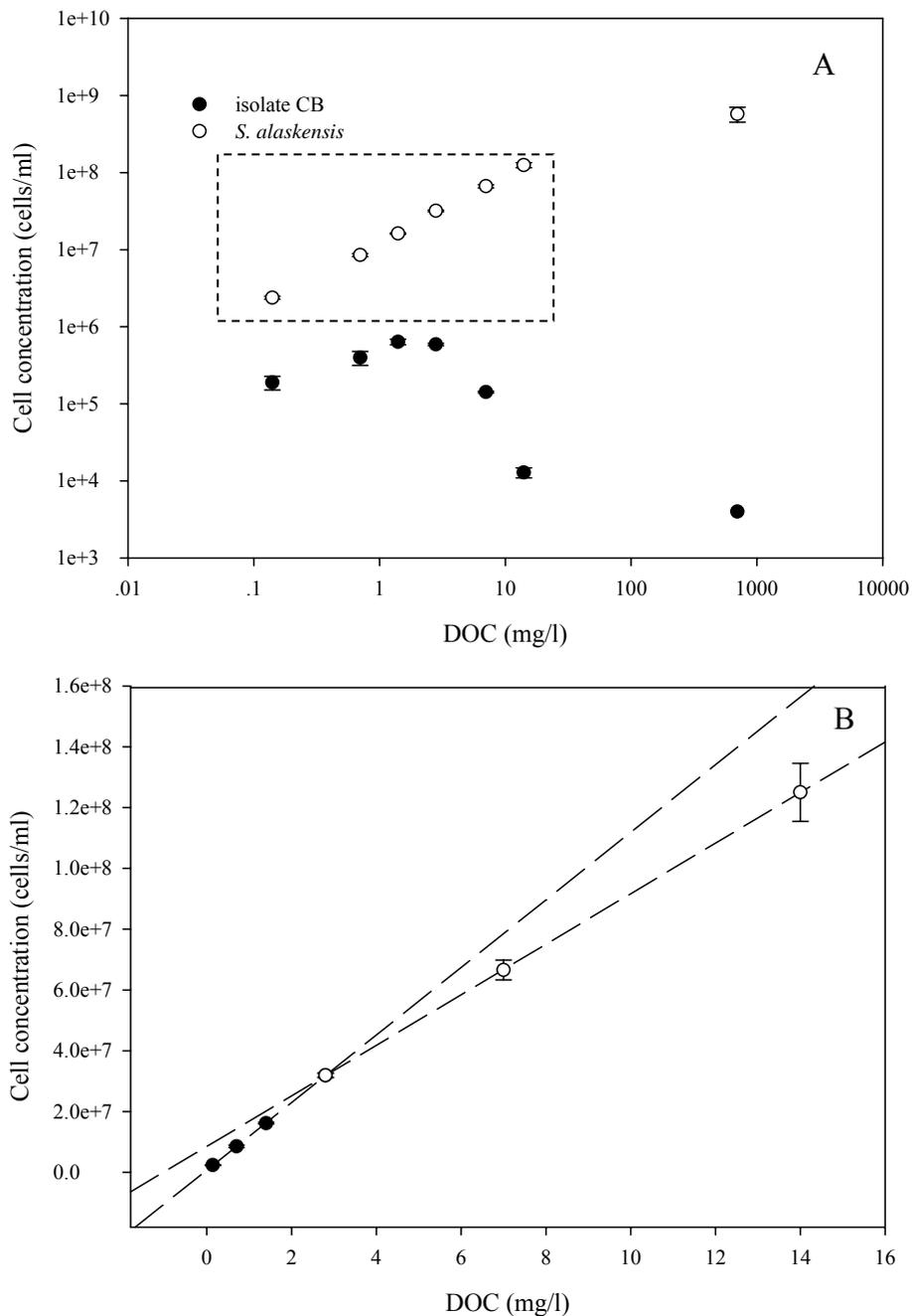
Figure 6.8. Effect of carbon concentration on final cell concentrations at stationary phase during batch cultivation of isolate CB and *S. alaskensis* in different dilutions of LB medium at

30°C (A), and linear regression between DOC concentration and final cell concentrations for *S. alaskensis* (B) within the range of 0.7 to 14 mg C/l (gated with dashed line in (A)). Error bars indicate standard deviation on triplicate samples.

Table 6.1. Comparison between *S. alaskensis* and isolate CB grown in 10,000-time diluted LB medium with a DOC concentration of 700 µg/l. Results were shown as average ± standard deviation from triplicate samples.

Culture	μ_{max} (h^{-1})	TCC ($\times 10^6$ cells/ml)	DOC utilized (µg/l)	Yield (g C biomass/g DOC)
Isolate CB	0.20 ± 0.01	0.26 ± 0.01	76	0.02
S. alaskensis	0.36 ± 0.01	8.97 ± 0.21	291	0.55

DOC utilization and yield

Bacterial production was investigated in some more detail in 10,000-times diluted LB medium. *S. alaskensis* utilized a much higher fraction of the DOC than isolate CB (Table 6.1). The two bacteria also utilized different fractions of the DOC as visualized in the by LC-OCD chromatograms (Figure 6.9). In comparison to the AOC bacterial community (Hammes and Egli, 2005), which by definition removes all AOC from the medium (Vital et al., 2008) isolate CB mainly utilized the low molecular weight acids and humics together with some neutrals. The same fractions were also utilized by *S. alaskensis*, but this bacterium was also able to utilize high molecular weight humics as well as some building blocks (Figure 6.9). This indicates that isolate CB prefers relatively easy carbon sources while *S. alaskensis* may have a broader substrate spectrum. It was reported by Amon and Benner (1994) that high molecular weight DOC compounds were utilized to a higher degree and more rapidly than low molecular weight compounds in sea water by bacterial communities. However such results

should be interpreted carefully, especially since the seawater was amended with inorganic nitrogen and phosphate (Amon and Benner, 1994). Furthermore, isolate CB had a much lower yield with respect to cell number in such synthetic LB medium than that of *S. alaskensis* (Table 6.1). The results also indicate that LB is not the perfect food for isolate CB, which is in consistent with the results from the previous section on media comparison.
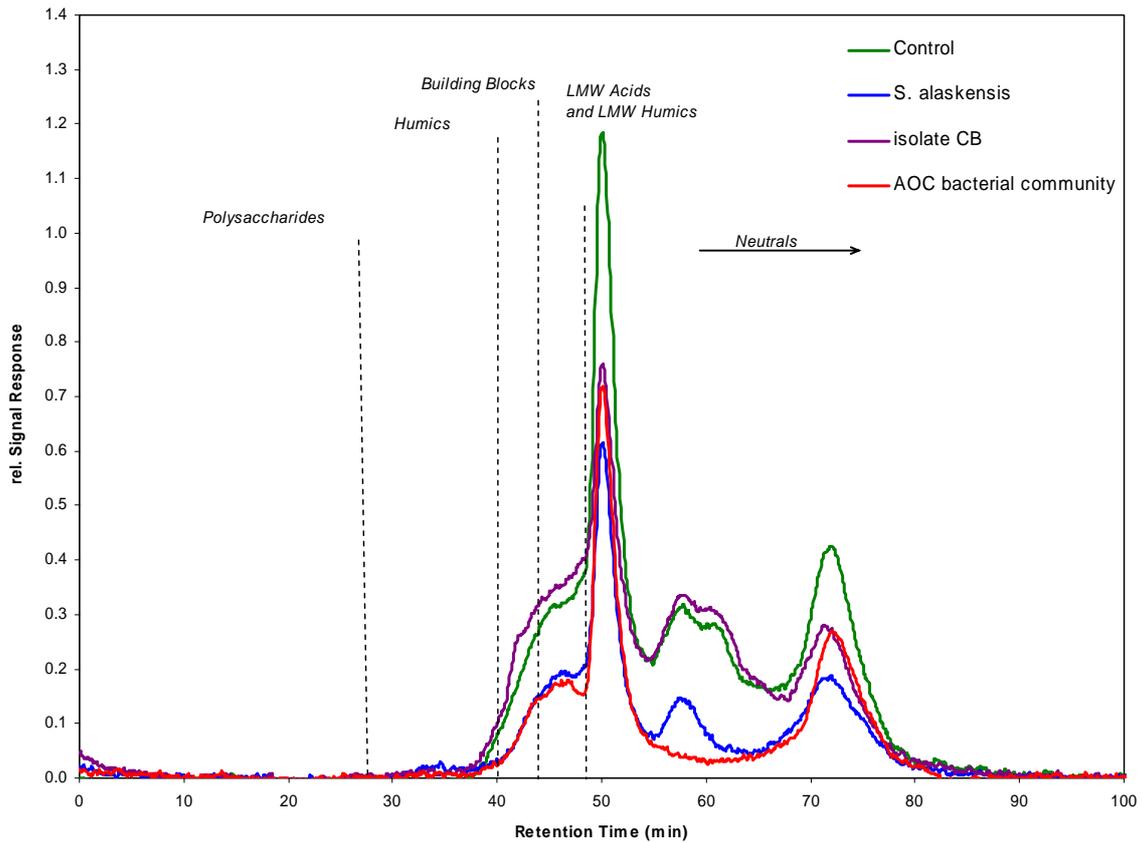
Figure 6.9. Size exclusion chromatograms of DOC utilization in 10,000-time diluted LB medium by *S. alaskensis*, isolate CB and AOC bacterial community, respectively.

Conclusion

It was reported that the very small bacteria are representatives of the autochthonous bacterial communities in aquatic environments (Cavicchioli and Ostrowski, 2003). However, they are undoubtedly the portion that cannot be recovered by the presently used cultivation approaches. The data presented here have demonstrated the value of flow cytometry for following

microbial growth at low cell concentrations. Unlike microscopy or plating, the speed of flow cytometric measurements allows generation of a large amount of data easily, including both cell number measurements as well as biomass estimation.

Under the experimental conditions tested in this study, we observed distinctly different batch growth characteristics for our freshwater isolate CB and the model "ultramicrobacterium" *S. alaskensis*. Because very small bacterial cells dominate in number the aquatic environments and very few of them have been isolated, investigators have to use culture data obtained for growth and metabolism to interpret their observations. The fact that such data are available only from a few model bacteria (i.e., *S. alaskensis*) leads to potential problems. Furthermore, since the mechanism of *S. alaskensis* adaptation to laboratory rich media is still unknown, extrapolation of its physiological properties to indigenous strains in natural environment may cause problems. The cultivation of isolate CB provides strong evidence that those cells may be readily cultivable when appropriate cultivation conditions are used. The study of bacterial growth is the basic method to understand the physiology of microorganisms (Monod, 1949). Knowing that and how they can be cultivated we may be able to extend our studies to the physiological level. This will also help in our search for more isolates. Our results demonstrated the great potential of the cultivation strategy in growth characterization under low-nutrient conditions and brought us closer to a better understanding of very small bacterial growth and their true nature.

Acknowledgement

7.   General conclusions and remarks

Cultivating the unseen majority

The cultured bacterial strains available in the literature only represent less than 1% of the microbial communities in natural environments. Genetic analysis based on rRNA has also shown that the majority of the microbes present in nature have no counterpart among previously cultured isolates we have studied so for in the laboratory (Rappé and Giovannoni, 2003; Tyson and Banfield, 2005). For a comprehensive understanding of the properties and abilities of these organisms, and of the complex environmental processes in which they engage, we are largely dependent on their cultivation. Therefore, there is an urgent need to find ways to cultivate those microbes presently thought to be "uncultivable" – the so-called "unseen majority". To achieve this goal, one needs a suitable cultivation approach, which includes the proper media and detection methods.

The term "cultivability" is often used to describe bacterial ability to grow in conventional rich medium to a detectable level (i.e., visible optical density and visible colony forming units). Hence the cultivability of the bacteria is quite misleading. The inability of bacterial cells to grow in a provided medium does not correspond with their inability to grow in general. However, all those efforts trying to cultivate "natural" bacteria ended with the cultivation of isolates in nutrient-rich media. Many reports successfully proved that most of the aquatic bacteria are able to multiply under low nutrient concentration conditions, but still kept to the idea of reaching high cell concentration as a goal (e.g., Button et al., 1993; Schut et al., 1997). The mechanisms of environmental isolates changing from no growth to growth in rich medium shall be investigated in much detail before we take it for granted that the laboratory isolate obtained by this way is what we actually looking for.

Recently, there have been advances in single cell manipulation techniques such as optical tweezers and laser micro-dissection (reviewed by Brehm-Stecher and Johnson, 2004; Fröhlich and König, 2000), fluorescence-activated cell sorting (Davey and Kell, 1996) and gel micro-droplets (Zengler et al., 2002). Some of the technical problems regarding the separation of target cells have been solved. The major remaining problem is choosing a suitable medium to cultivate these bacteria. Considerable effort has been made with respect to the improvement

of cultivation. For example, researchers modified the traditional cultivation approaches by increasing incubation times (Jassen et al., 2002), adjusting oxygen concentrations and nutrient levels (Stevenson et al., 2004) and adding signal compounds (Bruns et al., 2003). However, despite those few successes, most of the effort using traditional rich medium failed to isolate more bacteria. At the same time, researchers tried to employ cultivation media with a composition more related to their natural environments (Button et al., 1993; Connon and Giovannoni, 2002; Kaeberlein et al., 2002; Rappé et al., 2002).

Most of the approaches taken are based on the preliminary that for successful isolation a cell has to for a colony. However, I feel that a paradigm shift away from the concept of conventional cultivation is needed – i.e., cells do not need to make colonies on plates to grow. In fact, surface water itself represents the ideal low-nutrient growth environment for planktonic bacteria originated from aquatic environments. For instance, Zengler and co-workers (2002) applied media like filter-sterilized sea water amended with different concentrations of nutrients. However, one drawback of such media is that such filter-sterilized sea water cannot be regarded as bacteria-free water, since we have demonstrated that a significant part of the microbial community in aquatic environments are able to pass through sterilizing filters and subsequently grow on the available nutrients (Chapter 2; Wang et al., 2007). Still, the application of natural water as medium, in my opinion, is the right direction. To avoid the effect of filterable bacteria, I recommend using natural surface water sterilized by heat or by ultrafiltration. This has been successfully applied in the enrichment and isolation of previously uncultivable cells (Chapter 5), and also for the investigation of bacterial growth at low nutrient concentrations (Chapter 2 and 6, Vital et al., 2007; 2008; Wang et al., 2007).

The successful application of using natural surface water as a growth medium depends on coupling it to a suitable detection method. The cell concentrations that can be reached in such media is typically below the detection limit of most conventional techniques used to quantify growth such as optical density. In such cases, microscopy and flow cytometry are most suitable quantitative techniques, with the latter being faster and more accurate. Flow cytometry has been applied in medical research for more than 50 years and it has become one of the key techniques in this field. It has also been applied in microbiological research mainly for single cell analysis. However, one of the powerful characteristic of flow cytometry - fast and accurate counting - is not always fully appreciated. We have demonstrated here in this thesis the application of flow cytometry for the detection and quantification of filterable

bacteria (Chapter 2), and for monitoring and characterizing the growth of targeted bacteria (Chapters 5 and 6). Together with recent reports from our group (Hammes et al., 2008; Vital et al., 2007; 2008), we have shown the tremendous advantage and potential of flow cytometry in the research of bacterial growth in low nutrient environments.

With the proper medium and a suitable detection method, we proposed a new cultivation approach, namely to cultivate bacteria in natural surface water medium and monitor the growth with flow cytometer. In general, our approach not only gives us the opportunity to isolate strains that one could not possible to isolate previously, but also to investigate their growth properties under low nutrient conditions that are closely related to the natural environment in which they live. The cultivation approach used in this thesis has overcome most problems that hampered the cultivation of the microbial "unseen majority": 1) improper medium with too high concentrations of nutrients; 2) chemical component not suitable for growth; 3) inappropriate method to quantify cells at low densities; 4) lack of fast methods to detect cellular changes at low cell density. Hence, the cultivation approach developed in the thesis provided us with the unique chance to explore the unseen part of the microbial world. The approach not only allows characterizing the growth of bacteria, it also further opens the door for investigating a number of bacterial physiological properties at low cell density. For example, the spectrum of substrates utilized by isolates could be tested by supplying 80-100 µg C/l of these substrates to a virtually carbon-free but growth supporting water (such as filtered bottled water). Up to now, such studies have largely relied on high cell density. How to adapt and modify the cultivation approach to meet the requirements of physiological study still needs further investigation. Furthermore, the recently advances in molecular technologies makes it possible to characterize genetic properties without the necessity of high cell density. Such methods will help us to reveal the true physiological properties of such "uncultivable" bacteria and will help us to understand their contribution to the various biogeochemical cycles. In this way, the presented cultivation approach will help us to achieve a new level of microbial research.

Very small bacteria

Bacteria with a very small biovolume have been one of the major research targets for researchers due to their ubiquitous presence and possible import role in biogeochemical processes. With the report of "nanobacteria", even more attention was drawn to this area (e.g., Çiftçioglu et al., 2006; Kajander and Çiftçioglu, 1998), even though this was later suggested to be erroneous (Hamilton; 2000; Martel and Young, 2008). Filterable bacteria have been to certain extent a synonym for of bacteria with small size and, therefore, filtration has been one of the main methods to search for so-called "ultramicrobacteria" and "nanobacteria". It has been a general believe that the smaller the cell volume the easier it is for bacterial cells to pass through micro-pore size filters. Mostly, filters with 0.22 μm pore size have been used to search for very small bacteria. We have observed that filtration is – no doubt – a quick and simple way to separate bacteria with different sizes (Chapter 5). However, as the pore size decreases, there is an increased selection for cell shape but not cell size (Chapter 3; Wang et al., 2008). Therefore, filtration through a 0.22 μm pore-size membrane is likely to give a selection of the bacterial community with a certain shape but not necessarily with a very small cell size (Chapter 2). Hence, filtration with proper pore-size is critical in the search and isolation of very small bacteria. In my opinion, the traditional assumption that the smaller the pore size is, the better the chance of enriching very small bacteria is highly questionable and should be substantiated with solid experimental data.

In addition, filterable bacteria on its own have been a research topic since 1930s due to its possible medical implications. Here in this thesis, the omnipresence of filterable bacteria in natural freshwater and drinking water was clearly demonstrated and their ability to pass through 0.1 μm pore size filters was specifically highlighted (Chapter 2). Furthermore, such laboratory results were validated in an industrial-scale filtration device (Chapter 4). In this respect the results of filterable bacteria presented in this thesis challenge the current "sterilization-by-filtration-practice" and the established filter validation processes, and we expect that this report will have profound implications for preparation, production and handling of all liquid solutions processed by filtration. Besides, the selection of spirillum shaped bacterial cells (Chapter 2 and 3) suggested that special care must be taken when dealing with samples containing pathogenic bacteria with similar shape, especially in the medical field.

Proliferation of terms

"The significant problems we face cannot be solved at the same level of thinking we were at when we created them." — **Albert Einstein**

Various terms have been created by researchers in the hope of better describing and characterizing target bacterial groups that are present in ecosystems, but apparently differ distinctly in their properties. Hence, terms like "oligotrophs", "copiotrophs", "dormant cells", "viable but non-cultivable (VBNC) cells", "not immediately cultivable (NIC) cells", "ultramicrobacteria (UMB)", "nanobacteria (NB)", "dwarf cells", "high nucleic acid content (HNA) bacteria", "low nucleic acid content (LNA) bacteria" and many more have been invented by different researchers based on various criteria (Table 7.1). The most used terms, part of their origin and possible interrelationships are visualized in Figure 7.1. Here I focused on free-living heterotrophic bacteria. Many of the terms are poorly defined and used arbitrarily in individual reports from different fields.

Table 7.1 Different criteria used to describing heterotrophic bacteria by various terminologies.

Criteria	Terms	References
physiological properties	oligotroph; copiotroph; VBNC etc.	Amann et al., 1995; Whitman et al., 1998
activity	dormant cells; resting cells etc.	Morita, 1997; Kell et al., 1998
cell volume	ultramicrobacteria, nanobacteria; dwarf cells etc.	Morita, 1993; Kajander and Ciftcioglu, 1998
detection methods	HNA; LNA etc.	Gasol et al., 1999; Lebaron et al., 2001
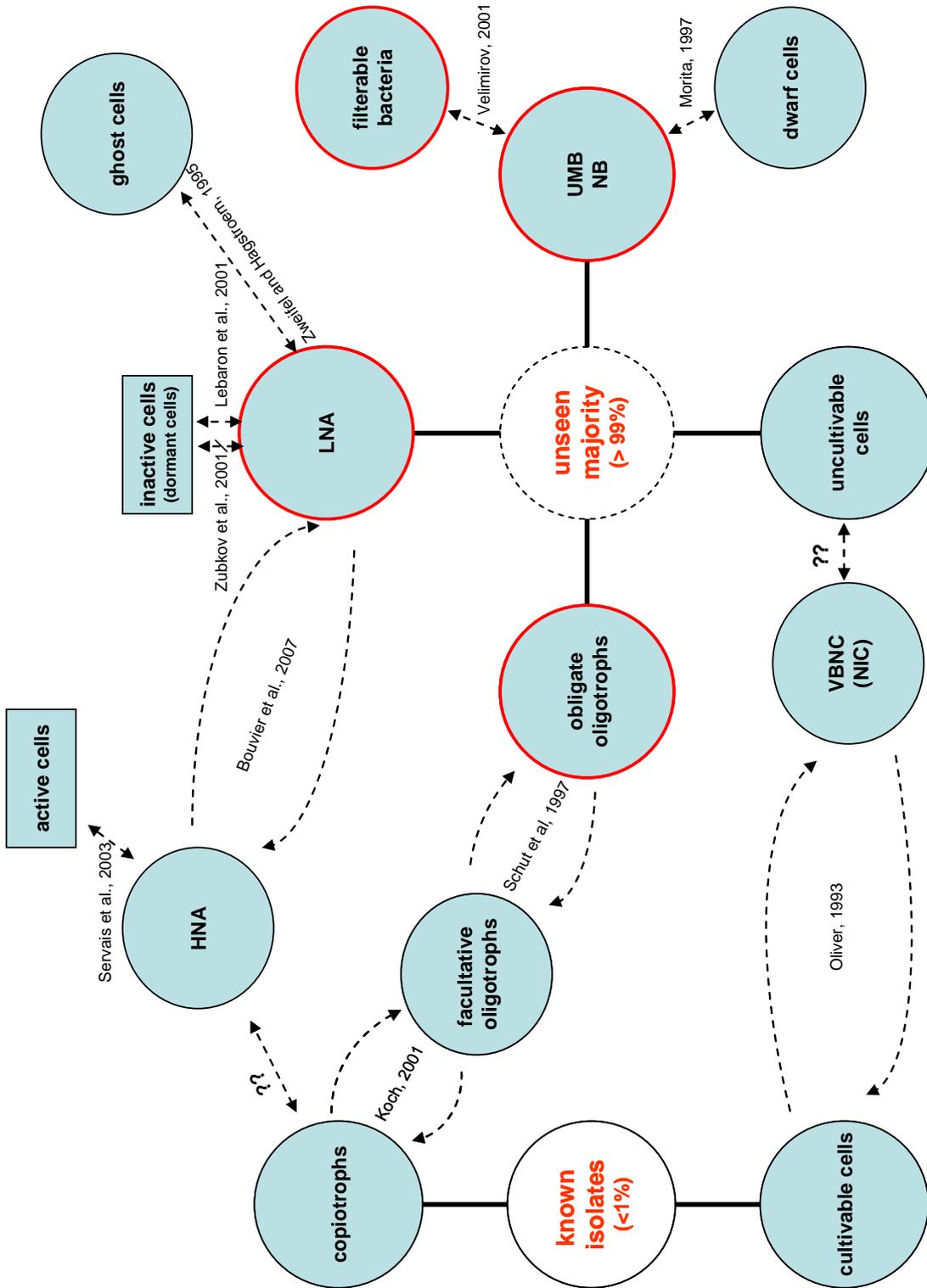
Figure 7.1. Sketch indicating the major terminologies applied to describe microorganisms in the literature and their possible interrelationships. For some cases, selected references are given where the interrelationship was proposed. The red circles indicated the fields I focused on in this thesis.

Recognizing that the major terms are unable to describe some of the members within these groups, one started to introduce new sub-groups or proposed new classes. For example, within "oligotroph" there are "obligate oligotroph" and "facultative oligotroph" (reviewed by Morita, 1997); within "HNA" there are subgroups named "very high nucleic acid content bacteria (VHNA)" (Nishimura et al., 2005), "high nucleic acid content and high light scatter bacteria (HNA-hs)" and "high nucleic acid content and low light scatter bacteria (HNA-ls)" (Zubkov et al., 2001; Tadonléké et al., 2005). As a result, more and more terms appeared in the literature without accurate definition and clear relationships with each other. Even apparently clearly named groups are at a closer look only vaguely defined. An excellent example is oligotrophic microbes for which at least 10 different definitions exist (see review by Schut et al., 1997).

Here I tried to sort out some of the possible interrelationships among different terms (Figure 7.1) based on available research articles and reviews (and common sense). Clearly the less we know about a bacterial group, the more terms there are to describe them. So there have been much more terms created to describe the unseen majority (more than 99% of the natural bacterial community) than for the known isolates. It is frequently difficult to link all the terms due to their poor definition and various meanings reported by different researchers. For example, the term "VBNC" was originally used to describe cells of known isolates entering a stage that cannot be cultivated anymore (reviewed by Oliver, 1993). However, it was used later by some researchers for bacteria from natural environments that cannot be cultivated in rich media but display metabolic activity (Defives et al., 1999). In my opinion, the term "uncultivability" is also particularly dangerous to use. In general, this term is used in literature synonymous to "uncultivability on agar media". Such inability of bacteria to grow (or more precisely to form a visible colony) cannot be taken as a proof for being uncultivable. It can at most be regard as researchers' inability to detect growth with a certain established method. So the use of such terms should be coupled with a very clear definition to avoid ambiguity.

The term that is used is largely dependent on the researchers' preferences and sometimes their educational background. The label given is often misleading and masks the true nature of the target microorganisms. Although the different groups of bacteria have been labelled with distinctly different names, they may not be that far apart. For example, "ultramicrobacteria" were defined as "cells which are less than 0.3 μm in diameter, which are not, or hardly, capable of growing in nutrient-rich media and which do not increase significantly in size in

such media" (MacDonell and Hood, 1982; Torrella and Morita, 1981). Such a definition suggests that "ultramicrobacteria" possess the important traits of so-called "oligotrophic" bacteria. Are they actually the same class of organisms? Another typical example for this is the research on so-called LNA and HNA bacteria. Such differentiation of bacterial communities is based on the FCM technology, which cannot be re-produced on any other instruments. The division of LNA and HNA is created by microbial ecologists and then become an ongoing debate on their activity, taxonomic relationships (discussed in Chapter 5). The relationship between LNA and HNA is more than just active cells vs. inactive cells, which was reported shortly after the names were proposed (Lebaron et al., 2002).

The interrelationships between the artificially defined groups of bacteria are still far from clear and Figure 7.1 is only a first attempt to understand and sort-out the presently existing concepts. The results presented in this thesis shed light on parts of these complications. The isolates obtained from freshwater environments (Chapter 5) have been demonstrated to be 1) representatives of LNA bacteria, 2) to possessing typical "oligotrophic" growth characteristics, and 3) to be very small bacteria with a cell size that falls into the category of "ultramicrobacteria/nanobacteria". Hence, I think that although researchers tried to solve the puzzle of the unseen majority by different approaches based on their speciality, it is highly likely that one is dealing with a similar group of microorganisms. Unfortunately, the various terminologies applied in different fields hampered the collaboration, interpretation and exchange of knowledge between different groups (i.e. ecologists, physiologists, geochemists, etc.). I suggest that when possible, investigators should avoid using terms like "oligotroph" and "copiotroph" unless clear operational definitions are provided. In fact, it is generally not necessary to use such terms, and more precise terms which indicate the methods applied, such as heterotrophic plate count, total cell count, specific dye-positive cells, etc. are more accessible and less prone for misinterpretation.

Finally, the comparative study of growth reported in Chapter 6 highlights the clear difference between the environmental isolate (Chapter 5) and the so-called "model" organism *S. alaskensis*. The mechanisms for *S. alaskensis* to adapt to conventional rich medium are still unclear. Researchers proposed it is either adaptation or mutation. How much such strains after all these processes still exhibit the indigenous bacterial properties are highly questionable. Therefore, it would be more sensible to study the environmental isolates as much as possible when they are newly isolated or clearly document its evolution under cultivation conditions in

the laboratory. Despite the invaluable information obtained from the adapted isolates, the extrapolation from laboratory adapted strains to environmental performance has to be judged cautiously.

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