Association of hygienically relevant microorganisms with freshwater plankton

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Miriam Tewes

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Biofilm Centre, Aquatische Mikrobiologe

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Gutachter: Prof. Dr. Hans-Curt Flemming

Prof. Dr. Bernd Sures

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Für meine Eltern

“The role of the infinitely small in nature is infinitely great.”

— *Louis Pasteur*

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"Was aus Liebe getan wird, geschieht immer jenseits von Gut und Böse!"
Friedrich Nietzsche (*Jenseits von Gut und Böse*, Aph. 153)

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Glossary

CFDA	carboxyfluorescein diacetate
cfu	colony forming units
CTC	5-cyano-2,3-ditolyl tetrazolium chloride
DAPI	4’,6-diamidino-2-phenylindole dihydrochloride
DNA	deoxyribonucleic acid
DVC	direct viable count
EDTA	ethylene diamine tetra-acetic acid
EPS	extracellular polymeric substances
FDA	fluorescein diacetate
FISH	fluorescence in situ hybridization
HPC	heterotrophic plate count
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PI	propidium iodide
PMA	propidium monoazide
PNA	peptide nucleic acid
rRNA	ribosomal ribonucleic acid
VBNC	viable but non-culturable

Abstract

In aqueous environments bacteria can occur planktonically in the water phase, or associated in biofilms attached to solid surfaces or other phase boundaries. Plankton organisms in surface waters provide external surfaces which can be colonized by biofilms. Plankton surface can comprise areas up to thousands of km^2 in a lake. Possible associations of potentially pathogenic bacteria with phyto- and zooplankton were observed in a field study in a freshwater environment (Lake Baldeney, Essen, Germany) and in laboratory experiments.

Hygienically relevant microorganisms considered were, bacteria with faecal origin (*Escherichia coli*, coliforms, intestinal enterococci, *Clostridium perfringens*), an obligate human pathogen of faecal origin (*Campylobacter* spp.), and environmental opportunistic bacteria (some coliforms, *Pseudomonas aeruginosa*, *Aeromonas* spp., *Legionella* spp.). For all of the investigated hygienically relevant bacteria an association with freshwater plankton could clearly be demonstrated, except for *Campylobacter* spp. which was only found in water and *Legionella* spp. which was not detected by culture. The bacterial abundance in all samples was found to be higher with plankton than compared to the free water. With the culture-independent methods, fluorescence in situ hybridization (FISH) and quantitative polymerase chain reaction (qPCR), the organisms *P. aeruginosa* and *Legionella* spp. were found in significant higher concentrations in water and plankton than with cultural methods. This observation indicates that both may occur in a viable but nonculturable (VBNC) state.

In laboratory experiments hygienically relevant bacteria were co-cultivated with *Daphnia magna* in microcosms. To study cladoceran-bacteria associations in detail, it was discriminated between carapace-associated and ingested bacteria.

The organisms *P. aeruginosa* and *A. hydrophila* were found more frequently located on the carapace of *D. magna*, whereas most of *E. faecalis* was located in the gut. FISH analysis indicated the possibility of VBNC cells for these organisms in association with *D. magna*.

The field study as well as laboratory microcosms indicate that there are associations and accumulations of pathogenic bacteria, with different characteristics and origins, with plankton. Plankton can act as a reservoir and a vector for potentially pathogens and may spatially enhance bacterial concentrations up to infectious doses. In case of VBNC bacteria, it is possible that the VBNC cells resuscitate and regain their

virulence. Plankton-pathogen associations are of relevance considering human health in drinking water production and recreational use of the surface water.

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1 Introduction

1.1 Background of this study

Plankton organisms in surface waters provide large surfaces which can be colonized by bacteria, including hygienically relevant organisms. Plankton can act as a hide, a nutrient source or as a vector for these pathogens. This has been shown for *Vibrio* spp. and a few other pathogens, but mostly in marine environments. Furthermore, the microorganisms can enter the viable but nonculturable (VBNC) state and will not be detected with conventional culture methods. The transition of potentially pathogenic bacteria into the VBNC state when living in association with freshwater plankton has not been considered yet, except for *E. faecalis*.

In the present study the associations of pathogenic microorganisms with freshwater plankton and with the macrophyte *Elodea nuttallii* are investigated. Two main objectives are taken in consideration, (i) a field study, the examination in a natural surface water, Lake Baldeney in Essen/Germany, (ii) the simulation of associations in microcosms with selected pathogens and *Daphnia magna* as a zooplankton model organism.

Hygienically relevant microorganisms considered in this study are ubiquitous in surface waters. They belong to the categories of faecal indicator bacteria (*Escherichia coli*, coliforms, intestinal enterococci, *Clostridium perfringens*), an obligate human pathogen of faecal origin (*Campylobacter* spp.), and environmental opportunistic bacteria (e.g. some coliforms, *Pseudomonas aeruginosa*, *Aeromonas* spp., *Legionella* spp.). These organisms can originate from urban and agricultural run-off, sewage overflow, or dropping of birds. In the picture (Figure 1.1) the objects of interest that can be colonized by bacteria, such as phyto- and zooplankton, as well as macrophytes, and possibly pathways for hygienically relevant bacteria into the lake are illustrated.

The novelty of the project lies in the fact that the available information about the interaction between plankton and hygienically relevant microorganisms in freshwater is very scarce. Possible correlations between certain plankton species and the target organisms were not investigated, or less is reported unitl now.

This research will provide in the first place fundamental knowledge about the association and interaction of the target organisms with plankton. It offers knowledge relevant for public health in terms of a deeper understanding and, eventually, control

of these organisms in terms of hygienic safety of recreational waters, aquacultures and quality of raw water for drinking water production.

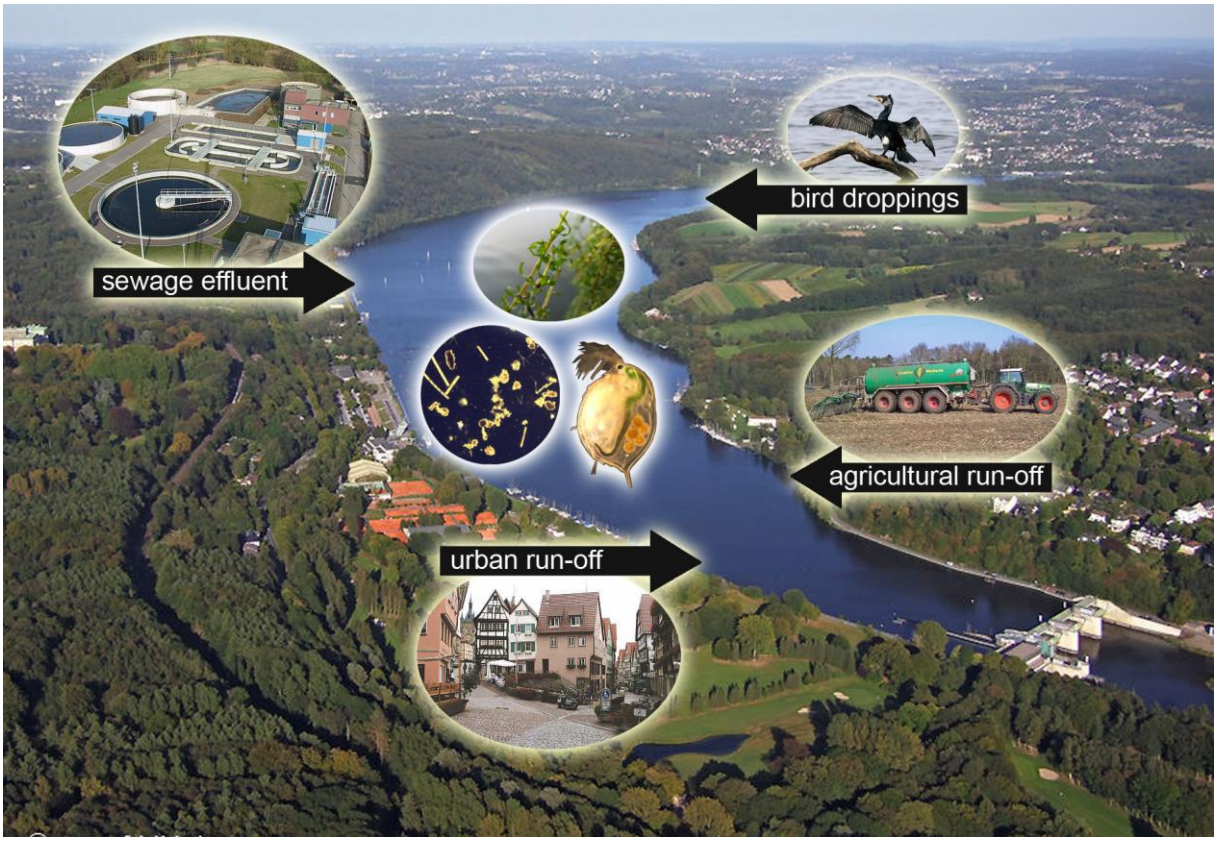


Figure 1.1 Lake Baldeney with possibly pathways of contamination by bacteria with hygienical relevance. The objects of interest are phyto-, zooplankton and *Elodea nuttallii*, which can be colonized and associated with pathogens from the surrounding water. (Sources of pictures: see appendix)

1.2 Plankton organisms as a habitat for hygienically relevant microorganisms

In aqueous environments, bacteria generally occur in two distinct states: (i) free-living in the water phase (planktonic state) or, more frequently, (ii) in a biofilm that is associated with solid surfaces and other phase boundaries. Biofilms are microbial conglomerations which are attached to a surface. The biofilm cells are embedded in a matrix of self-produced extracellular polymeric substances (EPS) (Donlan, 2002; Flemming & Wingender, 2010; Watnick & Kolter, 2000). Microorganisms integrated in a biofilm share features of ecological benefits like horizontal gene transfer and intercellular communication facilitated by the EPS matrix (Wingender & Flemming, 2011). Aquatic biofilms can host human pathogens. Pathogenic bacteria are capable of initiating biofilm formation (primary colonizers) or becoming incorporated in pre-established biofilms (secondary colonizers) (Costerton et al., 1987; Declerk, 2010; Wingender, 2011). Since biofilm cells are regularly dispersed into the water phase (Watnick & Kolter, 2000), the prevalence of hygienically relevant bacteria within biofilm communities is an aspect important to consider when assessing water-associated health risks.

Bacteria in aquatic environments have expanded their habitats by exploitation of organic matter like particles or aggregates such as phyto- and zooplankton. Plankton in surface waters provide large solid-liquid interfaces which can be colonized by biofilm-forming bacteria (Bidle & Fletcher, 1995). Plankton organisms can basically be subdivided into bacterioplankton (mainly heterotrophic prokaryotes), phytoplankton (cyanobacteria and eukaryotes) and zooplankton (eukaryotic unicellular and pluricellular organisms) (Dussart, 1965).

As an example, the volume of the zooplankton organism *Daphnia magna* is 5.6×10^7 μm^3 (surface area: 2.1×10^9 μm^2) and for the diatom species *Fragilaria capucina* it accounts 84.3 μm^3 (surface area: 161 μm^2).

To understand physiological adaptations and population dynamics of aquatic bacteria the key is to consider their alternate lifestyle between free-living and surface-associated (Figure 1.2). It became obvious that aquatic bacteria are often motile and concentrate at nutrient hotspots and interactions between bacteria and higher organisms show that they use bacterial motility and are controlled by chemotaxis (Grossart et al., 2001; Grossart, 2010; Kiørboe et al., 2002; Stocker et al., 2008).

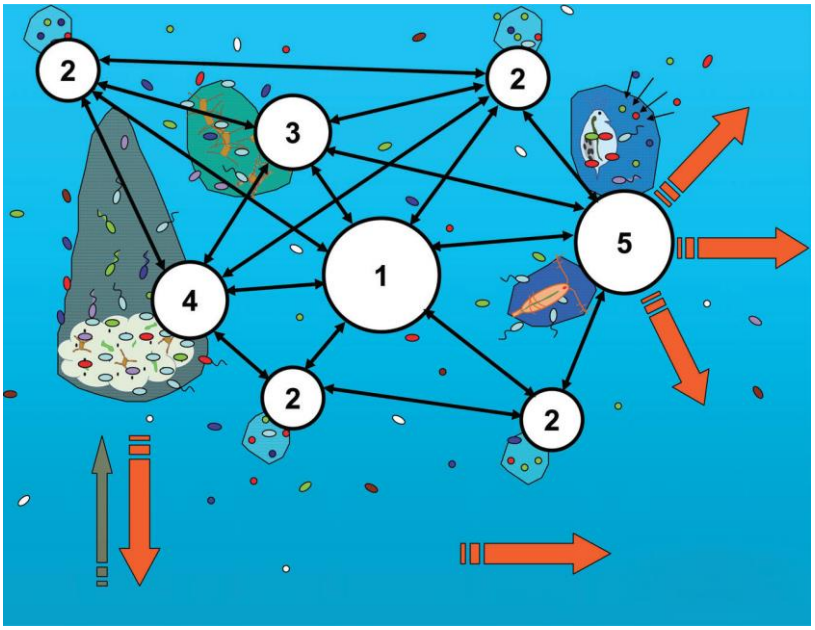


Figure 1.2 Conceptional view on aquatic bacteria and their network. (1) Free-living stage; (2) Bacteria associated with microparticles; (3) Bacteria clustering around photosynthetic organisms; (4) Chemotactic bacteria surrounding an aggregate; (5) Bacteria associated with motile organisms (Grossart, 2010).

Bacterium-plankton associations are considered a widespread phenomenon and have been studied for a long time (Carli et al., 1993; Huq et al., 1983; Huq et al., 1984; Maugeri et al., 2004). Previous studies mainly focusing on marine environments have revealed interactions of bacteria with phytoplankton such as diatoms (Plough & Grossart, 2000) and zooplankton such as small crustaceans and copepods (Huq et al., 1983, Huq et al., 1984, Lipp et al., 2002). Epibiotic bacteria can survive longer than free forms, the biotic surfaces represent nutrient sources for certain microorganisms favouring their attachment and biofilm development (Maugeri et al., 2004; Watnick & Kolter, 2000). Plankton-colonizing biofilms can harbor pathogens. First investigations have been carried out in marine environments with *Vibrio* species. Huq et al. (2005) found greater numbers of vibrios associated with zooplankton than in the water column. Chitinolytic bacteria such as *Vibrio* spp., i.e. *V. parahaemolyticus* or *V. cholerae*, which produce an active chitinase utilize the chitineous exoskeleton of copepods as source of both carbon and nitrogen (Kaneko & Colwell, 1975, Yu et al., 1991). The association of *V. cholera* with zooplankton in aquatic environments of Bangladesh was found to be a particular factor of human epidemic cholera outbreaks (Islam et al. 2007, Rawlings et al. 2007). In the presence of copepods, these bacteria have a competitive advantage when other sources of nutrients are scarce (Heidelberg et al., 2002). *Vibrio cholerae* possesses multiple

strategies for colonization of both abiotic and biotic surfaces (Mueller et al., 2007) and even associations with cyanobacteria enhanced their survival (Islam et al., 1990, 2004).

Emblazing the question about the distribution of potentially pathogenic bacteria as free living or plankton associated, Møller et al. (2007) found elevated abundance and growth rates for bacteria living associated with copepods compared to free living bacteria. It may be advantageous for bacteria to stay close or attached to the copepods, because copepods produce dissolved organic matter. Exterior bacteria can be found near the mouth, between segments or close to the anus (Carman & Dobbs, 1997). Electron micrographs of the zooplankton organism *Daphnia magna* during this study revealed large numbers of bacteria located on the surface of the carapace embedded in matrix, as illustrated in Figure 1.3.

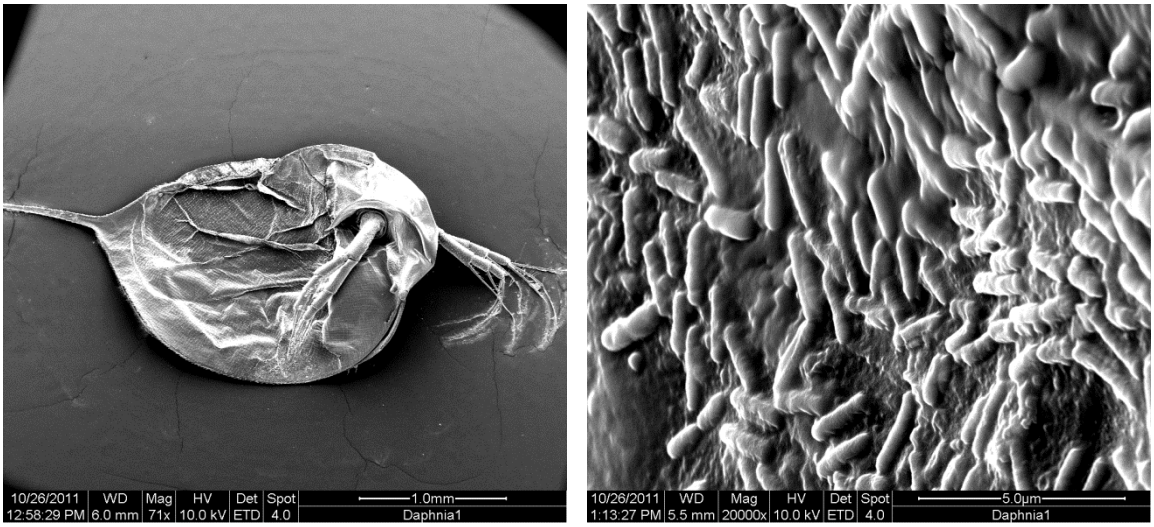


Figure 1.3 Scanning electron micrograph (SEM) of *D. magna.*
Left: overview of *D. magna*. Right: Part of the abdomen of *D. magna* covered with bacteria. (Source: Miriam Tewes, Biofilm Centre, University of Duisburg-Essen)

The association of hygienically relevant bacteria to marine plankton has been acknowledged for various pathogenic species such as *Vibrio* spp. (e.g. Carli et al., 1993; Heidelberg et al., 2002; Huq et al., 1983; Maugeri et al., 2004), *Campylobacter* spp. (Maugeri et al., 2004), and *Helicobacter pylori* (Carbone et al., 2005), and for the faecal indicators *Escherichia coli* (Maugeri et al., 2004) and *Enterococcus* spp. (Maugeri et al., 2004; Signoretto et al., 2004; Signoretto et al., 2005). Amongst environmental opportunistic pathogens, *Aeromonas* spp. has been linked to plankton colonization (Maugeri et al., 2004), but no information is available so far about the connection of other representatives of this category such as *Pseudomonas aeruginosa* or *Legionella* spp. to plankton.

In previous studies it was shown that wind, birds, and land animals can help carry aquatic bacteria from lake to lake, and that bacteria can spread quickly through a particular water mass simply by their extremely rapid growth rates (Hervàs et al., 2009; van der Gucht et al., 2007). Earlier studies also show that bacteria can move downward with the larger, heavier particles of organic detritus that constantly rain upon the seabed (Simon et al., 2002; Turley & Mackie, 1995). If bacteria travel with zooplankton species that migrate from the depths to the surface, they can overcome huge distances, either vertically or horizontally, expand their habitat and are transported to favourable feeding areas within their journey. Their idea requires that bacteria not only attach to the upwardly migrating zooplankton, but that they later detach into the free water again. This is of high ecological importance in habitats where spatio-temporal changes may occur rapidly (Grossart 2010). Free-living and particle-associated bacterial communities should not be perceived as separate entities, but rather as interacting assemblages. There is an active exchange of bacteria between plankton organisms and the surrounding water (Hansen & Bech, 1996; Riemann & Winding, 2005), the bacteria actively attach to the phyto- or zooplankton surface (Simon et al., 2002), they may enter their gut via ingestion by the zooplankton and are released together with gut flora by defecation or are egested unharmed (Tang 2005). Since copepods are the main dietary constitutens of many marine carnivores, including fish, bacterial attachment to the copepod integument can contribute to the transfer of pathogens through the food chain (Dumontet et al., 1996).

Algal exudates are important nutrient sources for heterotrophic bacteria in aquatic environments. Their cellular products can promote growth of indicator organisms, and there is potential for pathogenic bacteria to persist and grow on these algae (Byappanahalli et al. 2003; Kaplan & Bott, 1989). Reche et al. (1997) found out that algae and bacteria can balance grazing losses by compensatory growth. High masses of zooplankton stimulated bacterial growth, whereas release of organic carbon by phytoplankton declined. The algal decrease of organic carbon supply for bacteria could affect the balance and lead to a change from competition to commensalism.

Growth of bacteria on algal aggregates enhanced bacterial abundance and activities and even changed the composition of the free-living communities in the surrounding water (Tang et al. 2006). The colonization of plankton by bacteria can spatially

enhance bacterial concentration, increase the possibility for humans to be exposed to infectious doses and therefore pose a health concern (Omar et al. 2002).

1.2.1 Biology and ecology of *Daphnia magna*

In this study the zooplankton organism *Daphnia magna* was used to investigate associations with hygienically relevant mircroorganisms. It is a well-established model species and has been used in biological research for ecotoxicology, ecology and evolution studies since the 18th century (Ebert, 2008; Lampert, 2011; Routtu et al., 2010; Schaffer 1755). *D. magna* is a planktonic freshwater crustacean, a member of the Phyllopoda (Branchiopoda) and within the branchiopods they belong to the Cladocera. It is an ecologically important species in freshwater environments as a key grazer of algae and also being preferred as prey for fish (Lampert 2006). *D. magna* is a relatively large species (up to 5 mm) it is widespread in the northern hemisphere and easy to maintain in laboratories. *Daphnia* populations in their natural environment can be found, in lakes or ponds and are often one of the dominant zooplankton organisms. The density of the populations vary strongly throughout the seasons. Density peaks can be observed two or three times per year and especially in the cold or dry season it is possible that they disappear entirely. In the early season there is rapid population increase by recruitment from resting eggs and/or surviving females. A peak in *Daphnia* density occurs often after a peak in algae density and results in the clear-water phase in which *Daphnia* have removed most of the phytoplankton out of the water (Ebert 2005).

Daphnia are key herbivors in many freshwater ecosystems and efficiently consume heterotrophic bacteria (Brendelberger at al., 1991; De Mott 1986; Gophen & Geller, 1984). Porter et al. (1983) calculated filtering rates of *D. magna* with approximately 2.77 mL per individual and hour. They found that filtering rates of cladocerans increase with increasing body size. Also the filter efficiency is enhanced by the presence of larger particles, bacteria can be associated to larger particles and therefore be more easily collected by filtering appendages (Porter et al., 1983).

The body is an uncalcified shell called carapace, which mainly consists of chitin (Ebert 2005).

The English name for *Daphnia*, waterflea comes from the jumping-like behavior. This behavior originates from the beating of their antennae, which they use for swimming. *Daphnia* are filter feeders, feeding on small suspended particles in the water. With their flattened leaf-like legs, the phylopds, they produce a water current for the

filtering apparatus. Usually the food is made up of planktonic algae, but also bacteria can be collected. Green algae are the best food e.g. *Scenedesmus* or *Chlamydomonas* and therefore mainly used in laboratory experiments. (Ebert 2005).
The life cycle of *Daphnia* is characterized by an asexual mode (apomixes) whereas a female produces a clutch of parthenogenetic (amictic) eggs. These eggs are placed in the brood chamber (Figure 1.4).

Figure 1.4 Lightmicroscopic picture of *D. magna.* The gut filled with algae and the eggs located in the brood chamber. (Source: Miriam Tewes, Biofilm Centre, University of Duisburg-Essen)

The embryos hatch from eggs within 1 day but remain in the brood chamber for further development for almost 3 days until they are released from the mother by ventral flexion of the post-abdomen. The juvenile *Daphnia* look more or less than the adult, but have not yet a developed brood chamber. Before a juvenile becomes primipare, i.e. produces eggs for the first time it takes around 5 – 10 days (at 20°C). Until their death an adult female *Daphnia* produces eggs every 3 – 4 days. Under laboratory conditions may live for more than 2 months (Ebert 2005).

1.2.2 Appearance and abundance of the macrophyte *Elodea nuttallii*

The investigation of macrophytes was initially not intended within this study but due to the massive growth of the waterweed *Elodea nuttallii* in the year 2009, it provided another objective for the investigation of associations with hygienically relevant microorganisms. In literature there is less reported about macrophyte-pathogen associations in freshwaters yet.

Macrophytes are ubiquitous in freshwater environments. They excrete metabolites which can act as nutrient source for epiphytic bacteria. These bacteria can form biofilms on the plants´surface and use it as a habitat or a hide. In freshwater and marine habitats there are often associations of bacteria of the Cytophaga-Flavobacteria-Bacteriodetes group and Alpha- and Betaproteobacteria described (Eiler et al., 2004; Riemann et al., 2000; Sapp et al., 2007). There are investigations about heterotrophic biofilms on plants in freshwater (Hempel et al., 2008) but there is no information about the association of macrophyta with hygienically relevant bacteria.

In the catchment area of the Ruhr, especially in the reservoirs like Lake Baldeney, an invasive proliferation and massive growth of the neophyte *Elodea nuttallii* could be observed within the last years. Disregarding from the fact that the massive growth poses a problem for recreational use of the surface waters, e.g. for sailing or motor boats, the plant surface presents an attachment side for pathogens.

The macrophyte *Elodea nuttallii* is a species of waterweed which is a perennial aquatic plant with a thin branching stem and narrow recurved leaves. Stalks of the water plant are disseminated by abscising and floating until they anchor to the ground. The species can grow in oligotrophic as well as polytrophic surface waters and has a slender demand of light (Vöge 1995). *Elodea nuttallii* is native in North America and was found in Europe for the first time in 1939. Nowadays the waterweed is widespread and can be found in all dams of the catchment area of the river Ruhr, e.g. Lake Hengstey, Lake Harkort and Lake Baldeney. In the year 2000 the first comprehensive massive proliferation of the neophyte was noticed in Lake Harkort (Figure 1.5), where the plant was growing from ground up to the surface of the lake.

Figure 1.5 Massive growth of *Elodea nuttallii.*
Massive growth at Lake Harkort in 2004 (left) (Ruhrverband 2009); *Elodea nuttallii* (right) (J. Wingender, Biofilm Centre, University Duisburg-Essen)

The change of a phytoplankton-dominated to a macrophyte-dominated surface water is the result of the reduction of ortho-phosphate concentrations by extension of sewage water plants (Scheffer et al., 1993; Scheffer 1989; Scheffer 1998). In contrast to other macrophytes *Elodea nuttallii* has physiological advantages, they start already to be in bud at 4 °C and can take up nutrients, e. g. phosphate either with their leaves as well as their roots. Furthermore they can exist with low concentrations of phosphorus (0.2 % of the dry weight) (Garbey et al., 2004; Simpson 1990). In view of the water ecology the macrophytes have a positive effect for zooplankton organisms, macrozoobenthos and fish populations (Ruhrverband 2009).

1.3 Bacteria in the viable but non-culturable state

Biofilms in water systems act as a reservoir for hygienically relevant microorganisms and serve as a source for contamination of water by the release of pathogens. Significant numbers of pathogens can be found in environmental reservoirs, therefore it is important to assess their viability status to determine whether they pose a risk to public health. A review article of Keer & Birch (2003) provides a suitable overview of molecular methods for the assessment of bacterial viability.

Bacteria in the viable but non-culturable (VBNC) state fail to grow on routine culture media on which they would normally grow, although they are still alive and show metabolic activity. This phenomenon is described for numerous microorganisms (Mc Kay, 1992; Oliver 2005, 2009, 2010) including pathogens considered in this project, e.g. *A. hydrophila* (Mary et al., 2002), *P. aeruginosa* (Kimata et al., 2004), *L. pneumophila* (Steinert et al., 1997), *E. coli*, *E. faecalis* (Signoretto et al., 2004, 2005), *C. jejuni*, *C. coli* (Rollins and Colwell, 1986). Cells enter the VBNC state as a response to a variety of environmental stresses like starvation, osmotic stress, temperature variations, shifts in oxygen concentration or exposure to biocides or toxic metal ions (Oliver, 2005). Therefore the transformation into the VBNC state is considered to be a survival strategy and it has to include the capability to increase metabolic activity and to regain culturability (Whiteside and Oliver, 1997).

Pathogens, e.g. *V. cholera* and *Aeromonas* spp., were found attached to plankton in the viable but nonculturable state in a freshwater environment in India (Shukla et al., 1995). VBNC *V. cholerae* O1 were also found in marine environments of Argentina, where they were associated with phyto- and zooplankton (Binzstein et al., 2004). In the harbor of Naples in Italy Dumontet et al. (2000) found *Vibrio* spp. and *A. caviae* in the VBNC state adhering to copepods. The detection of *E. faecalis* in both lake water (Lake Garda) and sea water (Adriatic Sea) showed the organism mostly bound to plankton and that adhesion to copepods accelerated the entry into the VBNC state (Signoretto et al., 2005).

In this study it has to be investigated whether bacteria, particularly opportunistic pathogens, pass into the VBNC state, when they are associated with plankton in a freshwater environment. This phenomenon should furthermore be proved in batch cultures where pathogens are co-cultivated with plankton organisms. There is the aim to find out, if the VBNC state is induced when bacteria live attached to the outer surface of the zooplankton organism, or when they were ingested and are released after gut passage.

If human pathogens like *A. hydrophila*, or *E. faecalis* enter into a state in which they are no longer detectable with cultural methods they present a public health concern, because it has been demonstrated that these bacteria, remain viable, conserve their pathogenic characteristics and are able to resume growth again (Kell et al., 1998; Oliver 2000; Pruzzo et al., 2002). Thus determination of pathogens on the basis of culture methods alone are expected to be unreliable in order to establish the load of hygienically relevant bacteria in environmental waters.

The transition from a culturable into a non-culturable state can provoke morphological and physiological changes in the bacteria, like a reduced cell size, modified cell membrane compositions or reduced respiration rates. But generally VBNC bacteria maintain some viability markers, e.g. integrity of cytoplasmic membrane, respiratory activity or the presence of ribosomes (Lleó et al. 2000; Oliver, 2009). The detection of bacteria in the VBNC state can be improved if the standard culture-based methods are combined with molecular methods (Grobe et al. 2010; Keer & Birch, 2003).

Table 1.1 Overview of approaches and methods used for the assessment of bacterial viability (adapted from Keer & Birch, 2003)

	Methods		**Literature**
Presence of nucleic acids	DNA	(real-time) PCR	Behets et al., 2007
	mRNA	RT-PCR NASBA	Birch et al., 2001 Chan & Fox, 1999 Maher et al., 2001
	rRNA	FISH, PNA-FISH	Bottari et al., 2006 Moter & Göbel, 2000
Metabolic activity	Respiratory activity	CTC assay	Nwoguh et al., 1995 Rodriguez et al., 1992
	Enzymatic activity	FDA, CFDA	Ziglio et al., 2002
	Cell elongation	Direct viable count (DVC)	Kalmbach et al., 1997 Kogure te al., 1979
Cellular integrity	Membrane integrity	PI, PMA SYTO 9, GFP persistence	Banning et al., 2002 Nocker et al., 2007
	Respiratory activtiy	Rhodamine 123; $DIBAC_4$ (3)	Banning et al., 2002 Lowder et al., 2000

To assess metabolic activity of a bacterial cell, preferential in mixed bacterial populations, respiration activity can be determined by intracellular hydrolysis of 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) and detection of CTC-formazan crystals with fluorescence microscopy (Rodriguez et al., 1992). To determine enzyme activity, fluorogenic esters, like fluorescein diacetate (FDA) and carboxyfluorescein diacetate (CFDA) can be used. The analysis of membrane integrity is based on the ability of bacterial cells to exclude fluorescent dyes, e.g. propidium iodide (PI) or propidium monoazide (PMA) (Nocker et al., 2007).

For specific detection and quantification of hygienically relevant bacteria such as *P. aeruginosa* or *Legionella* spp. the molecular methods quantitative real-time PCR (qPCR) or fluorescence in situ hybridization (FISH) are preferential and were used in this study. Quantitative PCR is a technique based on the exponential in vitro amplification of a double-stranded DNA (dsDNA) target sequence from a DNA template by a heat-stable polymerase enzyme. The resulting amplification product is referred to as the “amplicon”. In contrast to conventional PCR, qPCR employs fluorogenic molecules which allow the detection and quantification of the amplicon in real-time (Mackay et al., 2007; Shipley 2006). There are primer and probe systems available for *Legionella* spp., *L. pneumophila* and *P. aeruginosa,* but PCR methods do not give information about the viablility of bacteria (Declerck et al., 2007; Wellinghausen et al., 2001; Qin et al., 2003). Due to its high degree of sensitivity, qPCR is prone to errors caused by matrix effects. Numerous contaminants may interfere and eventually causing partial or total PCR inhibition by matrix compounds and may lead to false-negative results. Often, inhibiting substances originate from the sample matrix itself as humic acids, polysaccharides (Wilson, 1997), or iron and manganese (Wullings & van der Kooij, 2006). This phenomenon was observed in analyzing cooling water samples, presumably due to their relatively high matrix complexity (Yaradou et al. 2007). Furthermore extracellular DNA or DNA from dead cells can lead to an overestimation of VBNC cells, due to false-positive results (Lehtola et al., 2007; Wellinghausen et al., 2001).

The principle of FISH involves the staining of ribosomal RNA by fluorescent dye-labelled nucleic acid probes. Probes may be general, binding to ribosomes of every cell regardless of the domain, or specifically designed to fit to certain species. By treating fixed cells with appropriate chemicals, they become permeable and allow intrusion of the probe solution. In a subsequent hybridization step, the probe binds to

the 16S rRNA of the cell's ribosomes. FISH allows detection and quantification of culturable and non-culturable cells (Madigan et al., 2006; Moter & Göbel, 2000). This method has limitations since it is known that the physiological state of bacteria can affect the amount of rRNA. A positive FISH signal might not be a direct indicator of metabolic activity, because stresses, like starvation or disinfection can degrade rRNA over time and lead to a weak or absent fluorescent signal, although the cells might be still alive (Bjergbæk & Roslev, 2005; Lehtola et al., 2007). This might lead to a false-negative result. On the osther side, rRNA is remaining stable for a long time, although cells are already dead. Since this would indicate false-positive results, the method should not be used to assess the viability of individual cells (Tolker-Nielsen et al., 1997, Prescott et al., 1999).

Is FISH coupled with the method of direct viable count (DVC) it is possible to detect the capacity of bacteria to metabolize nutrients. Therefore gyrase inhibitors, like pipemidic acid or nalidixic acid, are used to inhibit cell division and provoke cell elongation of dividing cells with accumulation of ribosomes.

Afterwards the FISH method is applied and viable, culturable as well as VBNC cells are detected (Regnault et al., 2000; Armisen & Servais, 2004; Piqueres et al., 2006).

As a recent development, FISH methods using probes made up from peptide nucleic acid (PNA) instead of DNA are available. PNAs are uncharged and analogous to DNAs but possess 2-aminoethyl-glycinelinkages instead of a phosphodiester backbone. This structural change provides resistance to enzymatic cleavage, better salt tolerance, and higher thermal stability for the molecule, but nevertheless allows it to bind to complementary nucleic acid sequences (Bottari et al., 2006).

Also, PNA probes can be designed with a shorter length, allowing them to pass the cell membrane more easily and the binding to nucleic acids is stronger than that of oligonucleotide probes. Comparisons of DNA and PNA probe efficiency for the same target site in *Legionella* have shown that PNA probes surpass comparable DNA probes in terms of target specificity and signal intensity, and proved their superiority for the detection of legionellae in environmental samples (Wilks & Keevil, 2006).

Some studies reported long-term persistence of DNA and RNA (Lázaro et al., 1999, Lleó et al., 2000), while others have shown evidence for nucleis acid contents to gradually decrease in VBNC cells (Weichart et al., 1997).

To estimate the viability of bacteria it is useful to determine their viability markers, e.g. membrane integrity with propidium iodide (PI) or propidium monoazide (PMA)

(Nocker et al., 2007). Respiration can be detected by intracellular hydrolysis of 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) (Rodriguez et al. 1992).

Resuscitation after non-culturability is possible when the environmental conditions change or the stress factors are reduced (Kell et al., 1998). The process of resuscitation can be enhanced by co-cultivation with protozoa for example *L. pneumophila* regained culturability in association with *Acanthamoeba castellani* (Steinert et al. 1997), or by inoculation of *C. jejuni* into the yolk sacs of embryonated eggs (Cappelier et al., 1999). Signoretto et al. (2004, 2005) found that the persistence in aquatic environments for *E. faecalis* in the VBNC state is warranted by the adhesion to copepods. *P. aeruginosa* which was transformed into the VBNC state by copper stress, was resuscitated and regained infectivity to human lung cells by use of a copper chelator (Dwidjosiswojo et al., 2011). In the monitoring of viability with the current available methods it has to be considered that none of the methods is universally appropriate, rather several methods should be used simultaneously or combined to increase accuracy of the result (Lisle et al., 2001).

1.4 Distribution of hygienically relevant organisms in surface water

A well documented summary about hygienically relevant organisms in biofilms of man made water systems is reported by Dr. Jost Wingender in Biofilm Highlights (Flemming et al., 2011).

The following organisms with hygienical relevance were selected to represent a variety of members of three different groups. They are all ubiqutious bacteria in aquatic environments with pathogenic properties and originate, for instance, from sewage effluents, urban or agricultural run-off and birds. The environmental opportunistic pathogens, such as *P. aeruginosa*, *Aeromonas* spp. and *Legionella* spp., are of particular relvance in drinking water distribution system, since they are known to form biofilms or integrate into pre-existing biofilms. But they are also of significance in waters used for recreational purposes. The bacteria of faecal origin (coliforms, *Escherichia coli*, intestinal enterococci and *Clostridium perfringens*) are indicator for a faecal pollution water. Some are inhabtitants of the intestinal tract of humans and warm-blooded animals, but they can also be found in natural environments, such as soil or surface waters. Their incidence is also considered in the EU bathing water directive. *Campylobacter* spp. represent common human enteric obligate pathogens with faecal origin, some species can cause severe gastrointestinal diseases.

1.4.1 Environmental pathogens

1.4.1.1 *Pseudomonas aeruginosa*

The Gram-negative, aerobic, non-spore-forming rod-shaped bacterium *Pseudomonas aeruginosa* (Madigan et al., 2006; Stover et al., 2000) is an ubiquitous environmental organism, occurring in sediments and soil, in freshwater and marine surface waters, on plant and animal tissue, as well as in man-made environments such as drinking water distribution and plumbing systems, swimming pool environments, hospitals, and wastewater effluents (Hardalo & Edberg, 1997; Khan et al., 2007; Pellett et al., 1983; Schwartz et al., 2006; Valentino & Torregrossa, 1995). Elevated levels of *P. aeruginosa* in surface waters are often related to anthropogenic alterations of the water body (Pirnay et al., 2005). Kimata et al. (2004) and Khan et al. (2007) isolated *P. aeruginosa* from the open ocean. Its natural occurrence, however, rather might be associated with sediments and submerged surfaces, suggesting that surveillance of *P. aeruginosa* should not be restricted to water alone

(Pellet et al., 1983). *Pseudomonas* spp. were found in association with marine phytoplankton (Berland et al., 1976) and recently with *Daphnia* (Qi et al., 2009).

Infections of humans with *P. aeruginosa* can arise in water bodies that are used for recreation purposes (e.g. swimming). The main route is contact of skin or mucous tissue with contaminated water, ingestion of water rarely leads to infections. Transmitted through wounds that are exposed to contaminated water, *P. aeruginosa* causes bathing-water-associated ear, eye, and skin infections (Grobe et al., 1994; Wingender et al., 2009). In technical water systems, *P. aeruginosa* is capable of integrating into pre-existing biofilms or, acting as a primary coloniser, to develop biofilms, thus promoting the subsequent incorporation of other species into the biofilm (Declerck, 2010; Moritz et al., 2010; Wingender et al., 2009). *P. aeruginosa* is able to form multispecies biofilms together with other microorgansims (Al-Bakri et al., 2004; Stewart et al., 1997), often being one of the dominant organisms in this habitat (Andersson et al. 2008; Shin et al. 2007)

P. aeruginosa is an opportunistic human pathogen of high clinical relevance (Anaissie et al., 2002; Exner et al., 2007; Reuter et al., 2002; Szewzyk et al., 2000; Trautmann et al., 2009), causing a multitude of infections in immuno-compromised hosts, such as cancer patients, aids and transplant patients, young children and elderly persons, but rarely in healthy people. In hospitals and other medical environments, *P. aeruginosa* accounts for a significant fraction of nosocomial infections of burn wounds, the urinary tract, the respiratory system, and the bloodstream (Anaissie et al., 2002; Trautmann et al., 2009). In cystic fibrosis patients suffering from chronic lung infections, it is a frequent cause of morbidity and mortality (Trautmann et al., 2005).

1.4.1.2 *Aeromonas* spp.

Aeromonas spp. are widespread in natural habitats and have been isolated from soil, fresh and brakish water, sewage and waste water, drinking water and a variety of foods (Buchanan & Palumbo et al. 1985; Araujo et al., 1991). *Aeromonas hydrophila* is a Gram-negative, motile, oxidase positive, non-sporeforming facultative anaerob bacterium (Joseph & Carnahan, 1994). It is ubiquitous in the water environment, but is normally not an inhabitant of the gastrointestinal tract in humans (Messi et al., 2002). *A. hydrophila* has been reported to cause in humans both intestinal and extraintestinal infections (Galindo et al., 2006; Janda 2001; Vila et al., 2003) including gastroenteritis, hepatobiliary tract infections, urinary tract and wound infections,

septicemia, cellulitis, soft tissue infections, broncho-pulmonary infections and occasionally meningitis and peritonitis (Galindo et al., 2006; Hornemann et al., 2007; Janda et al., 1988). The most frequent route of infection is direct contact with contaminated water (Janda et al., 1991).

A. hydrophila was found in adhesion to zooplankton, especially in association with both dead and live copepods, in case of alive copepods this could allow transport of bacteria to other locations (Alfredsson et al., 1995; Dumontet et al., 2000; Krovacek et al., 1994; Maugeri et al., 2004). Hazen & Esch (1983) found a positive correlation between *A. hydrophila* and algal blooms in an estuarine habitat influenced by fertilizer effluents. Accumulation of bacteria on plankton surfaces can lead to consumption of infectious doses by humans (Dumontet et al. 1996; Omar et al., 2002). Some strains of *A. hydrophila* produce virulence factors and are capable of causing septicemia in fish and amphibians (Krovacek et al., 1993), whereas *A. salmonicida* is a well-known fish pathogen (Austin & Austin 1993). *A. hydrophila* is known to produce a number of potential virulence factors including cytotoxin, haemolysin and enterotoxins (Houston et al., 1991; Krovacek et al. 1991; Kühn et al. 1997; Wadstrom et al., 1976). The wide distribution of the bacterium in different aquatic habitats underlines its ability to adapt to environments that differ in nutrient supply and presence of other aquatic microorganisms (Messi et al., 2002).

1.4.1.3 *Legionella* spp.

Legionellae are Gram-negative, aerobic bacteria which occur rod-shaped or filamentous (Declerck, 2010). Over 50 different *Legionella* species including numerous serogroups have been described so far, about half of which are considered to be opportunistic human pathogens, causing legionellosis, an infection of the respiratory tract that is acquired by inhalation or micro-aspiration of *Legionella*-carrying aerosols from contaminated sources, e.g. taps, shower heads, whirlpools, air conditioning systems, or fountains. *Legionella pneumophila* with the predominant serogroup 1 is regarded to be the medically most important species amongst *Legionella* (Yu et al., 2002).

Legionellae ubiquitously inhabit both natural and anthropogenic freshwater environments (Fliermans et al., 1981). Natural environments are rarely associated with outbreaks of legionellosis, because the lower water temperatures do not support significant proliferation of legionellae (Declerck, 2010; Fields et al., 2002).

Tison et al. (1980) found *L. pneumophila* serogroup 1 in association with cyanobacteria. The bacterium is provided by algal extracellular products which can be used as energy source and this maintains the distribution in natural habitats.

L. pneumophila is also capable to consume and grow on dead bacteria such as *Pseudomonas putida* and *Escherichia coli* (Temmermann et al., 2006). Moreover, they are able to replicate as parasites in biofilm-grazing protozoans such as amoebae (Fields et al., 2010; Kuiper et al., 2004; Kuiper et al., 2006; Greub & Raoult, 2004). Association of *L. pneumophila* with biofilm communities and amoebae provides the bacterium with necessary nutrients and thus, despite its fastidious growth requirements, allows it to thrive in oligotrophic environments (Lau & Ashbolt, 2009; Declerck, 2010; Fields et al., 2010).

Although legionellae do not produce monospecies biofilms under environmental conditions (Declerck, 2010), they readily incorporate into existing biofilms as a secondary coloniser where they persist or even multiply (Moritz et al., 2010; Rogers et al., 1994). Survival and growth of *L. pneumophila* depends on their cohabitants, several Gram-negative bacteria e.g. *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, as well as *Aeromonas hydrophila* were found to inhibit *L. pneumophila* growth and biofilm formation (Toze et al., 1990; Guerrieri et al., 2008).

1.4.2 Bacteria of faecal origin

1.4.2.1 Coliform bacteria

To the group of coliform bacteria belong a wide range of aerobic and facultatively anaerobic, Gram-negative, non-spore-forming, rod shaped bacteria (Rompré et al., 2002). Coliforms such as *Escherichia*, *Citrobacter* and *Enterobacter* are common inhabitants of the intestinal tract of humans and of warm-blooded animals, but they are also heterotrophic and can be found in natural environments such as soil or surface waters where they are able to survive or multiply in biofilms even under low-nutrient and low-temperature conditions (Le Chevallier et al. 1987; Camper et al. 1996; Leclerc et al., 2001). Coliforms from environmental sources which are adapted to oligotrophic conditions are more likely to colonize surfaces and form biofilms in water systems than bacteria which are adapted to elevated nutrient levels. Are there no autochthonous sessile microorganisms present coliforms are also able to act as primary colonizer and form biofilms (Szabo et al., 2006). Coliforms have been used as indicator organisms for faecal pollution of drinking water. Some are facultative pathogens and can cause e.g. wound infections, pneumonia and septicemia (Feuerpfeil et al. 2009). There is no recent knowledge about associations of bacteria of the coliform group with plankton organisms in aquatic habitats, except for *E. coli*.

1.4.2.2 *Enterococcus* spp.

Enterococci are Gram-positive, ovoid, non-spore-forming, facultative anaerobic bacteria (Fisher & Philips 2009). Their origins can vary from environmental sources as well as from humans or animals, as they are part of the microflora in warm-blooded animals and humans. They have been found associated with soil, plants, zooplankton and algae. *E. faecalis* was found in association with zooplankton organisms and is able to persist in the environment for long periods (Signoretto et al. 2004; Signoretto et al. 2005). *E. faecalis* can enter the viable but non-culturable (VBNC) state as a survival strategy when they are released in fresh or marine water environments (Lleò et al., 1998). Mote et al. (2012) found that both *E. faecalis* and *E. casseliflavus* were able to survive and multiply in plankton suspensions for longer times than just in artificial seawater. This implies that *Enterococcus* spp. may be highly associated with plankton or other particles and furthermore they are able to survive longer in marine environments than fecal coliforms (Cabelli et al. 1983). The EU bathing water quality directive defines a bathing water in good quality with a

concentration of intestinal enterococci of 200 cfu/100 mL (Directive 2006/7/EC). Enterococci were found in high densities attached to the green alga *Cladophora* (Byappanahalli et al., 2003) in shoreline waters of Lake Michigan. Plankton may serve as a reservoir for enterococci which enhances growth due to nutrient supply and persistence of this fecal indicator e.g. by protection from sunlight (Byappanahalli et al., 2003; Signoretto et al. 2004; Signoretto et al. 2005). The U.S. Environmental Protection Agency (EPA) recommended to use enterococci instead of fecal coliforms as indicator organism for fecal pollution (U.S. Environmental Protection Agency, 1986). And in addition the findings by Mote et al. (2012) imply the effectiveness of enterococci as an indicator of water quality, especially in particle-rich aquatic habitats.*E. faecalis* and *E. faecium* are the most common ones in the human gastrointestinal tract (Klein, 2003). They can cause urinary tract infections, wound infections endocarditis (Guzmán et al., 1991).

1.4.2.3 *Escherichia coli*

E. coli are straight cylindrical rods which are Gram-negative, motile, aerobic and facultatively anaerobic (Scheutz & Strockbine, 2005). The organism is commonly found in the human intestine but there are also obligate pathogens existing like enterotoxigenic and enterohaemorrhagic variants (Nwachuku & Gerba, 2008). Association and persistence of *E. coli* with the green alga *Cladophora* at Lake Michigan was investigated by Whitman et al. (2003). *Cladophora* harbour high densities of *E. coli* and provides a habitat and nutrients for survival and growth of this organism (Byappanahalli et al. 2003; Whitman et al. 2003; Olapade et al., 2006). In the presence of protozoa Sibille et al. (1998) observed enhanced elimination of *E. coli* by grazing activity in an experimental distribution system. Mc Mahon & Rigler (1965) found that *Daphnia* are efficiently grazers on *E. coli*.

E. coli is able to become incorporated into pre-existing drinking water biofilms, but also to form monospecies biofilms (Jones & Bradshaw, 1996; Bridier et al., 2010). It is a widely used indicator of fecal contamination but this is contended because of its persistence and common occurrence in natural habitats (Whitman et al. 2006). *E. coli* can occur in habitats that are not influenced by sewage inputs. They can survive in soil or sand for months and be washed into the water body due to rainfall. Once the cells are adsorbed to particulate matter in moist areas they are protected from environmental stresses, like desiccation and UV light (Whitman et al., 2006). The

current European guideline for bathing waters uses *E. coli* enumeration for determination of fecal contamination instead of total or fecal coliforms. The concentration for a good bathing water quality is 500 cfu *E. coli* /100 mL (Directive 2006/7/EC).

1.4.2.4 *Clostridium perfringens*

The organism *Clostridium perfringens* is Gram-positive, anaerobic and is able to form endospores. This species produces different soluble substances that cause a variety of toxic effects (Rainey et al., 2005). *C. perfringens* is widespread in the environment and found in soil as well as aquatic habitats. The organism can be found in the intestine of animals or humans where it can be pathogenic, in humans it is able to cause gangrene and gastrointestinal diseases. *C. perfringens* produces various toxins and enzymes which results in lesions of healthy cells and leads to the specific symptoms. For epidemiological studies it is important to identify the *C. perfringens* pathovars because each type of toxin induces a certain syndrome (Petit et al., 1999).
Clostridia play an important role in anoxic organic-rich environments, because they degrade via fermentation complex organic materials into acid, alcohols, hydrogen and carbon dioxide. Besides fecal coliforms and fecal streptococci the most frequently used indicator for fecal pollution in water is *C. perfringens.* This species is consistently present in wastewater at concentrations of 10^3 to 10^4 cells per 100 mL. Because of its resistance to chlorination and diverse environmental factors, its indicator value resembles that of enteric viruses (Fujioka and Shizumura, 1985). *C. perfringens* and its spores had been specified as an indicator parameter of quality of water for human consumption in the Directive of the European Community (EC, 1998). The presence of spores and the ratio between vegetative cells and spores can be a potential sensitive indicator for recent fecal pollution by agricultural sources or microorganisms entering streams with municipal wastewater (Bisson and Cabelli, 1979; Lango, 1999; Sørensen et al., 1989). *C. perfringens* spores can persist in water for a long time and are considered as much longer than the persistence of most enteropathogenic bacteria (Cabelli et al., 1982). Both vegetative cells and spores are used to monitor water treatment process efficiency (Ryzinska-Paier et al., 2011).
There is no recent knowledge about associations of *C. perfringens* with plankton organisms neither in freshwater nor in marine environments.

1.4.3 Human pathogen

1.4.3.1 *Campylobacter* spp.

Campylobacter spp. are fastidious Gram-negative, non-spore-forming, motile, microaerophilic, spiral-shaped bacteria (Vandamme & De Ley, 1991; Rowe et al., 2000). They are of clinical and epidemiological relevance because of enteric food- and waterborne disease causing gastrointestinal illness. Point source outbreaks involving contaminated raw milk, poultry as well as water are well described, in most cases these events are sporadic with no clear source (St. Pierre et al. 2009). Campylobacters can form monospecies biofilms (Joshua et al. 2006) and integrate into pre-existing mixed-population biofilms, e.g. isolates of *C. jejuni* and *C. coli* were found to survive in such biofilms (Buswell et al., 1998). There are different possibilities for *Campylobacter* spp. to contaminate environmental water, e.g. streams, rivers or lakes because the organism colonizes a variety of hosts like domestic animals or wild birds and thus the organism is excreted via the faeces, agricultural run-off, or it is discharged from wastewater treatment plants (Obiri-Danso & Jones, 1999; St. Pierre et al. 2009). Campylobacters that are excreted into aquatic environments or upon starvation undergo a physiological change and can enter the viable but non-culturable state (VBNC) (Rollins & Colwell, 1986; Tholozan et al. 1999; Chaveerach et al. 2003). They can persist in water and survive for months under various conditions (Cools et al. 2003). The survival of campylobacters is contributed by the ingestion of planktonic grazers. Ishii et al. (2006) observed *Campylobacter* associated with *Cladophora*. The green algae serves as a protective and nutritive reservoir for pathogenic bacteria. It was shown that *Daphnia* were highly efficient grazers of aquatic bacteria and affect the biomass, productivity, size structure and species composition of aquatic bacterial biofilms (Jürgens 1994; Kamjunke et al. 1999). Schallenberg et al. (2005) found *Daphnia carinata* grazing on *C. jejuni* and to reduce the survival of the pathogen.

Campylobacters in natural aquatic habitats were found to survive in cold water at temperatures below 10°C in the winter period much longer than they survive in water at higher temperatures in Finland and Norway (Korhonen & Martikainen, 1991; Brennhovd et al., 1992)

Campylobacter spp. in particular *C. jejuni* is one of the most important causes of food-borne gastrointestinal disease (Koenraad et al. 1997). Foods of animal origin and water are regarded as the main transmission routes due to the presence of these organisms in the intestine of many animals (Pearson et al., 1996). *C. jejuni* and *C.*

coli are the most common human enteric pathogens among the thermotolerant campylobacters and cause acute bacterial diarrhea (Frost 2001).

1.5 Aims of the study

- Overall goal is to assess the role of plankton as a site for accumulation, transport and possible proliferation of hygienically relevant microorganisms

    - Determination of the association in a freshwater environment, in Lake Baldeney/Essen/Germany
    - Investigation of pathogens existing in the VBNC state
        - Use of cultural and culture-independent methods
            - Quantitative polymerase chain reaction (qPCR)
            - Fluorescence in-situ hybridization (FISH)

    - Simulation of the plankton-bacteria association in batch cultures with the model organism *Daphnia magna* and selected pathogens (*Pseudomonas aeruginosa*, *Aeromonas hydrophila* and *Enterococcus faecalis*)
        - Balance studies between attached pathogens on the carapace of *D. magna* and the amount ingested, thus located in the gut (with cultural methods and FISH)

- Evaluation of the results in concern of relevance for public health considering drinking water production and recreational water quality

2 Material and methods

2.1 Organisms

2.1.1 Bacterial test strains

The organisms used in this study listed in the table below were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Germany) and kept in cryogenic vials (Mast Diagnostica) at -70°C.

Additionally used organisms were *P. aeruginosa* PAO1 (Nottingham wild type from Holloway collection) and *A. hydrophila* AH-1N (wild type, Swift et al. 1999).

Table 2.1 Microorganisms used as a reference

Organism	**DSMZ No.**	**ATCC No.**
Aeromonas hydrophila	30187	7966
Clostridium perfringens	756	13375
Enterococcus faecalis	20478	19433
Escherichia coli	30083	11775
Legionella pneumophila	7513	33152
Pseudomonas aeruginosa	50071	10145

2.1.2 *Daphnia*

The strain of *Daphnia magna* K34Q used in this study was originally isolated by Prof. Dr. Winfried Lampert from „Großer Binnensee" in Schleswig-Holstein/Germany (Lampert, 1991). The Daphnids were obtained from Dr. D. Martin-Creuzburg, Limnological institute, University of Konstanz and Prof. Dr. R. Tollrian, Animal ecology, evolution and biodiversity, University of Bochum.

2.1.3 Algae

The unicellular green algae *Scenedesmus obliquus* (SAG 276-3a) was used for feeding of *Daphnia magna*. The algae was obtained from Dr. D. Martin-Creuzburg, Limnological institute, University of Konstanz.

2.2 Growth media

Preparation of media:

Media were prepared using deionized water and autoclaved at 121 °C for 20 min. If not mentioned otherwise, the preparation was done according to the manufacturers' instructions. All solid growth media were poured in portions of 25 mL into sterile Petri dishes.

Acetamide nutrient broth (Fluka Analytical)

Composition in g/L: acetamide 2.0; magnesium sulfate 0.158; sodium chloride 0.2; sodium molybdate 0.005; ferrous sulfate 0.0005; dipotassium hydrogen phosphate 0.2; pH 7.0 ± 0.2 at 37°C.

Aachener *Daphnia* medium (ADaM) for cultivation of *Daphnia magna*

For cultivation of *Daphnia magna* the artificial medium Aachener Daphnien medium (ADaM; pH 7.1) was used. The original recipe for the medium was developed by Klüttgen et al. (1994), but in this study a modified version used.

Table 2.2 Stock solutions for ADaM

Stock solution	Chemicals	Concentration [g/L]
A	$CaCl_2$	117.6
B	$NaHCO_3$	25.2
C	SeO_2	0.07

Table 2.3 Preparation of 10 L ADaM

Deionised Water [L]	Sea salts (Sigma) [g]	Stock solution A [mL]	Stock solution B [mL]	Stock solution C [mL]
10	3.33	23	22	1

Ampicillin-dextrin agar

According to Havelaar et al. 1987

Composition in g/L: Bacto Agar (Beckton Dickinson) 15.0; dextrin from potato starch (Fluka analytical) 10.0; Bacto Tryptose (Beckton Dickinson) 5.0; sodium chloride (VWR) 3,0; yeast extract granulated (Merck) 2.0; potassium chloride (Merck) 2.0; magnesium sulfate heptahydrate (Fluka analytical) 0.2; iron(III)chloride hexahydrate (Merck) 0.1.

8 mL of bromothymol blue solution (Merck) was added (10 mg/ml) and pH was adjusted with NaOH (1 M) to 8.0 ± 0.2 at 25°C.

The medium was autoclaved for 20 min at 121°C, cooled down to 55°C and freshly prepared, filter sterilized (pore size 0.2 µm) 10 mL of ampicillin sodium salt (AppliChem) (final conc. 1 mg/ml) and 10 mL of deoxycholic acid sodium salt (Fluka Analytical) (final conc. 10 mg/ml) were added.

Bile aesculine azide agar (Merck)

Composition in g/L: peptone from casein 17.0; yeast extract 5.0; peptone 3.0; ox bile dried 10.0; sodium chloride 5.0; aesculine 1.0; ammonium iron(III)citrate 0.5; sodium azide 0.15; agar-agar 13.0. pH 7.1 ± 0.2 at 25°C.

BCYEα agar (OXOID)

Commercial agar plates, ready-to-use.

Composition in g/L (ISO 11731:1998): yeast extract 10.0, agar 12.0, activated charcoal 2.0, alpha-ketoglutarate, monopotassium salt 1.0, ACES buffer (N-2-actetamido-2-aminoethanesulfonic acid) 10.0, potassium hydroxide 2.8, L-cysteine hydrochloride monohydrate 0.4, iron(III)pyrophosphate 0.25, deionized water ad 1000 mL.

mCCDA Preston (Campylobacter blood free selective agar base) (OXOID)

Composition in g/L: Nutrient broth No. 2 25.0; bactericological charcoal 4.0; casein hydrolysate 3.0; sodium deoxycholate 1.0; ferrous sulphate 0.25; sodium pyruvate 0.25; agar 12.0; pH 7.4 ± 0.2 at 25°C

22.75 g of the commercially available granulate of mCCDA agar base were suspended in 500 mL of deionized water and autoclaved (121 °C, 20 min). The content of one vial of CCDA Selective Supplement (cefaperzone 16.0 mg; amphotericin B 5.0 mg; Oxoid) was dissolved in 2 mL of sterile deionized water was and added to 500 mL of autoclaved agar base cooled to 50 °C.

Cetrimide agar (Merck)

Composition in g/L: peptone from gelatin 20.0; magnesium chloride 1.4; potassium sulfate 10.0; N-cetyl-N,N,N-trimethylammonium bromide 0.3; agar-agar 13.6. pH 7.2 ± 0.2 at 25°C.

Chromocult® Enterococci Agar (Merck)

Composition in g/L: Peptones 10.0; sodium chloride 5.0; sodium azide 0.2; dipotassium hydrogenphosphate 3.4; potassium dihydrogenphosphate 1.6; ox bile 0.5; Tween ® 80 1.0; chromogenic-mixture 0.25; Agar-agar 11.0. pH 7.0 ± 0.2 at 25°C.

33.0 g of the commercially available granulate were suspended in 1 L of deionized water by heating in a boiling water bath until complete dissolution while stirring. After cooling down to 45 – 50 °C the agar was poured into plates.

GVPC agar (OXOID)

Commercial agar plates, ready-to-use.

Composition in g/L (ISO 11731:1998) yeast extract 10.0, agar 12.0, activated charcoal 2.0, alpha-ketoglutarate, monopotassium salt 1.0, ACES buffer (N-2-actetamido-2-aminoethanesulfonic acid) 10.0, potassium hydroxide 2.8, L-cysteine hydrochloride monohydrate 0.4, iron(III)pyrophosphate 0.25, ammonium-free glycine 3.0, polymyxin B sulfate 80.000 iu, vancomycin hydrochloride 0.001, cycloheximide 0.08, deionized water ad to 1000 mL.

LB medium (Lenox Broth)

Composition in g/L: tryptone 10.0, sodium chloride 5.0, yeast extract 5.0 / pH 7.0 ± 0.2 at 25 °C. The compounds were dissolved in deionized water, pH was adjusted to 7.0 ± 0.2 and deionized water was added up to 1 L.

Malachite-green broth (base) (Merck)

Composition in g/L: peptone from meat 5.0; meat extract 3.0; di-potassium hydrogen phosphate 0.37. pH 7.3 ± 0.2 at 25 °C.

8.4 g (single concentrated broth) and 16.8 g (double concentrated broth) were dissolved in 1 L deionized water and the solutions were autoclaved (121 °C, 20 min).

0.15 g malachite-green oxalate (Merck) were dissolved in 90 ml, filter sterilized and 12 ml were added to the double concentrated broth base or 6 ml to the single concentrated broth which was before cooled to 50°C.

m-CP agar (OXOID)

Commercial agar plates, ready to use.

Composition in g/L: tryptose 30.0; yeast extract 20.0; saccharose 5.0; L-Cysteine hydrochloride 1.0; magnesium sulphate x 7 H_2O; bromocresol purple 0.04; agar-agar 15.0; polymyxin B sulphate 0.025; D-Cycloserine 0.4. pH 7.6 ± 0.2 at 25 °C.

Nutrient agar (NA, Merck)

Composition in g/L: peptone from meat 5.0, meat extract 3.0, agar 12.0 / pH 7.0 ± 0.2 at 25 °C.

Preston broth (OXOID)

Composition in g/L: ‘Lab-Lemco’ Powder 10.0; peptone 10.0; sodium chloride 5.0; pH 7.5 ± 0.2 at 25 °C.

12.5 g of the commercially available granulate of Nutrient broth No. 2 (agar base) were suspended in 500 mL of deionized water and autoclaved (121 °C, 20 min). The content of one vial of modified Preston Campylobacter Selective Supplement (polymyxin B 2500/U; trimethoprim 5.0 mg; rifamipicin 5.0 mg; amphotericin B 5.0 mg; Oxoid) was dissolved in 2 mL of sterile deionized water and was added to 500 mL of autoclaved agar base cooled to 50 °C.

Pseudomonas Agar F (base) (Merck)

Composition in g/L: peptone from casein 10.0; peptone from meat 10.0; magnesium sulfate 1.5; di-potassium hydrogen phosphate 1.5, agar-agar 12.0. pH 7.2 ± 0.2 at 25 °C.

Pseudomonas Agar P (base) (Merck)

Composition in g/L: peptone 20.0; magnesium chloride 1.4; potassium sulfate 10.0; agar-agar 12.6. pH 7.2 ± 0.2 at 25 °C.

Pseudomonas selective agar (CN agar, Oxoid)

Composition in g/L: gelatin peptone 16.0, casein hydrolysate 10.0, potassium sulfate 10.0, magnesium chloride 1.4, agar 11.0 / pH 7.1 ± 0.2 at 25 °C.

24.2 g of the commercially available granulate of Pseudomonas agar base were suspended in 500 mL of deionized water and 5 mL of glycerol were added before autoclaving (121 °C, 20 min). The content of one vial of Pseudomonas CN supplement (100 mg cetrimide, 7.5 mg sodium nalidixate; Oxoid) was dissolved in 2 mL of a 1:1 (vol/vol) mixture of ethanol and sterile deionized water and the solution was added to 500 mL of autoclaved agar base cooled to 50 °C.

R2A medium (Difco)

Composition in g/L: yeast extract 0.5, Difco proteose peptone no. 3 0.5, casamino acids 0.5, glucose 0.5, soluble starch 0.5, sodium pyruvate 0.3, potassium hydrogen phosphate 0.3, magnesium sulfate heptahydrate 0.05, agar 15.0 / pH 7.2 ± 0.2 at 25 °C.

18.2 g of the commercially available granulate were dissolved in 1 L of deionized water.

Slanetz & Bartley Medium (OXOID)

Composition in g/l: tryptose 20.0; yeast extract 5.0; glucose 2.0; potassium hydrogen phosphate dihydrate 4.0; sodium azide 0.4; tetrazolium chloride 0.1; agar 10.0; pH 7.2 ± 0.2 at 25°C.

WC_g medium for cultivation of *Scenedesmus obliquus*

For cultivation of the algae *Scenedesmus obliquus* the WC_g medium (pH 8) was used (Guillard, 1975). It is a general purpose medium for freshwater algae that prefer slight alkaline milieu.

Table 2.4 Preparation of WC_g medium:

Salt solution	
Component	Molar concentration in final medium [M]
K_2HPO_4	1.25×10^{-5}
NH_4Cl	5.01×10^{-5}
$CaCl_2 \times 2\ H_2O$	2.50×10^{-5}
$MgSO_4 \times 7\ H_2O$	4.14×10^{-5}
$NaNO_3$	2.50×10^{-5}
$NaHCO_3$	3.75×10^{-5}
H_3BO_3	2.43×10^{-5}
$Na_4EDTA \times 2\ H_2O$	2.50×10^{-5}
Metal salt solution	
$FeCl_3 \times 6H_2O$	1.17×10^{-5}
$Na_2EDTA \times 2H_2O$	1.17×10^{-5}
$CuSO_4 \times 5\ H_2O$	3.93×10^{-5}
$Na_2MoO_4 \times 2H_2O$	2.60×10^{-5}
$ZnSO_4 \times 7\ H_2O$	7.65×10^{-5}
$CoCl_2 \times 6\ H_2O$	4.20×10^{-5}
$MnCl_2 \times 4\ H_2O$	9.10×10^{-5}
Vitamin solution	
Vitamin B_1	2.96×10^{-7}
Vitamin H	2.05×10^{-9}
Vitamin B_{12}	3.69×10^{-10}

For the preparation of 1 L medium each 500 µL of the salt solutions, 1 mL each of the metal salt solutions and 250 µL of the vitamin solution were mixed and the pH was adjusted with NaOH (0.1 M) to pH 7.8

2.3 Buffers and other solutions for general use

0.9 % NaCl solution

9.0 g NaCl were dissolved in 1 L deionized water. The solution was autoclaved for 20 min at 121 °C.

Acid buffer (ISO 11731: 1998)

Solution A: 0.2 M HCl

200 mL 1 M HCl in 800 mL of deionized water.

Solution B: 0.2 M KCl

14.9 g KCl were dissolved in 1 L deionized water.

For preparation 3.9 mL of solution A were mixed with 25 mL of solution B. After adjusting the pH to 2.2 ± 0.2 with 1 M KOH, the buffer was filter sterilized (pore size 0.2 µL).

DAPI stock solution (25 µg/ml) in 2 % (v/v) formaldehyde

12.5 mg 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma) were dissolved in 500 mL formaldehyde solution (2 %) and filtered through a cellulose acetate filter (pore size 0.2 µm).

DNA Ladder

MassRuler™ DNA Ladder Mix, ready-to-use, range 80-10000 bp (Fermentas, SM0403).

Loading Dye

6 x TriTrack™ DNA Loading Dye (Fermentas, R1161).

Composition: 10 mM Tris-HCl (pH 7.6), 0.03% bromophenol blue, 0.03% xylene cyanol FF, 0.15% orange G, 60% glycerol, 60 mM EDTA.

Ethidium bromide solution

10 mg/mL (Bio-Rad, 161-0433)

McFarland standard (bioMérieux, 70900)

Particle-free deionised water

Deionized water was filtered through a cellulose acetate filter (pore size 0.2 µm) and then autoclaved (20 min, 121 °C).

2.4 Commercial kits

API®20 NE (bioMérieux, 20 050)

API NaCL 0.85 % Medium (bioMérieux, 20 070)

API 20 NE test stripes

JAMES (bioMérieux, 70 542)

NIT 1 + NIT 2 (bioMérieux, 70 442)

Zn reagent (bioMérieux, 70 380)

Mineral oil (bioMérieux, 70 100)

API®Campy (bioMérieux, 20 800)

API NaCL 0.85 % Medium (bioMérieux, 20 070)

API Campy test stripes

NIT 1 + NIT 2 (bioMérieux, 70 442)

FB (bioMérieux, 70 562)

NIN (bioMérieux, 70 491)

Mineral oil (bioMérieux, 70 100)

API®rapid ID 32 E (bioMérieux, 32 400)

Aquadien™ Kit (BioRad)

iQ-Check™ Legionella Quantification Standards (BioRad)

iQ-Check™ Screen L. pneumophila Kit (BioRad)

iQ-Check™ Screen Legionella spp. Kit (BioRad)

Quant-iT™ PicoGreen® dsDNA Kit (Invitrogen)

DNeasy Kit (Qiagen)

2.5 Chemicals

4’,6-Diamidino-2-phenylindole dihydrochloride (DAPI; Sigma)

50x TAE (TRIS/acetic acid/EDTA) buffer (BioRad)

Agarose (Pulsed Field Certified; Bio-Rad, 162-0137)

Bovine serum albumin (BSA), 100x, 10 mg/mL (New England BioLabs, B9001S)

Calcium chloride dihydrate, ≥ 99 % (Fluka Chemie AG)

di-Sodium tartrate dihydrate, ≥ 99.5 % (Merck)

Ethanol Rotipuran (Roth)

EDTA tetrasodium salt dihydrate, ≥ 99 % (Sigma-Aldrich)

Ethidium bromide (10 mg/ml) (BioRad)

Folin Ciocalteu’s phenol reagent, 2N (Sigma-Aldrich)

Formaldehyde solution, ~ 36 % in H2O (Fluka, 47630)

Formamide deionised, ≥ 99.5%, p.a (Roth, P040.1)

Glycerol (KMF Laborchemie Handels GmbH)

Humic acid (Fluka Chemie AG)

Loading Dye TriTrack (Fermentas)

Magnesium chloride hexahydrate, grade for analysis (Merck)

Paraformaldehyde (Merck, 1.04005)

Rotipuran Water (Roth, p.a., ACS)

Sodium carbonate, anhydrous, ≥ 99.8 % (Roth)

Sodium dodecyl sulfate (SDS), 99 % (Sigma-Aldrich)

Sodium hydroxide pellets, 99 % (KMF Laborchemie)

TRIS PUFFERAN®, ≥ 99.9 % (Roth)

Water for Molecular Biology (Roth, DPEC treated)

2.6 Equipment

Table 2.5 Equipment used in this study

Equipment	Manufacturer
Biofuge fresco	Heraeus Instruments
Cary 50 Bio UV-Visible Spectrophotometer	Varian
Centrifuge 5415 D	Eppendorf
Filtropur S plus 0.2 membrane filters, 0.20 µm porosity	Sarstedt
Electrophoresis chamber HE 33 mini horizontal submarine unit	Amersham Biosciences
iCycler	BioRad
iQ™5 Multicolor Real-Time PCR Detection System	BioRad
LabelGuard™ Microliter Cell	Implen
Leica DMLS	Leica Microsystems Wetzlar GmbH
Microseal® 'B' Film	BioRad
Molecular Imager Gel Doc XR System	BioRad
Omnifix 10 ml syringe	B. Braun Melsungen AG
PCR plates, 96-well	BioRad
Polycarbonate membrane filters, white, 0.40 µm porosity	Millipore
Power Supply Pack P25	Biometra
SFM 25 A fluorescence spectrophotometer	Kontron Instruments
Thermomixer comfort	Eppendorf
Cooling centrifuge, Sorvall® RC26PLUS	Sorvall
Bactident® Oxidase Test Strips	Merck, 1.13300
Black polycarbonate membrane filters, pore size 0.2 µm	Millipore
Desk centrifuge 5415 D	Eppendorf
Disposable Bottle Top Filter OR, non-fiber releasing membrane, surfactant free cellulose acetate pore size 0.20 µm	NALGENE®
Disposable cuvettes PMMA semi-micro, 12.5 × 12.5 × 45 mm	BRAND, 7591 15

Equipment	Manufacturer
Epifluorescence microscope: Leitz Laborlux S	Leitz
Objectives: PL Fluotar 100x / 1.32 oil Eye-piece: 2x Periplan 10x / 18 UV-unit HBO 50	Leitz
Gel documentation system Universal Hood II	Bio-Rad
Hybridisation oven	Thermo electron cooperation
Light microscope; Leica DM LS	Leica Microsystems
Mastercycler ep gradient S	Eppendorf
Membrane filters black, MicroPlus-31 ST, 0.45 µm pore size, ø 50 mm, mixed cellulose ester	Whatman
Membrane filters white, 0.45 µm pore size, ø 47 mm, mixed cellulose ester	Pall
Nikon Microscope: Eclipse Ni H 550 L Objectives: Plan Fluor 4x/0,13 OFN 25 WD 17,2 Plan Apo 100x/1,40 oil OFN 25 Eye-piece: CFI 10x/22 UV-unit Intenislight C-HGFIE Camera: Nikon DS Fi 1 Software: NIS-Elements AR 4.10.01	Nikon
pH meter WTW (ph 549 ELP)	MultiCal®
Phase contrast microscope; Leica DM LS	Leica Microsystems
Quanti-Tray® Sealer Model 2X	IDEXX
Reaction chambers for Fluorescence in situ hybridisation	Vermicon
Six-fold stainless-steel vacuum filtration apparatus	Millipore
Thermo Scientific Diagnostic slides epoxy-coated 8-well 6 mm	Menzel
Thermomixer comfort	Eppendorf
Three-fold stainless-steel membrane filtration apparatus	Sartorius
Tissue Culture Plate 6-Well Flat Bottom with lid (Polystyrene)	Sarstedt

2.7 Software

Table 2.6 Software used in this study.

Software	Manufacturer
Microsoft Office Excel 2003/2010	Microsoft Deutschland GmbH, Unterschleißheim, Germany
iQ™5 Optical System Software 2.0	BioRad, Hercules, CA, USA
Simple Reads 2.0	Varian Australia Pty Ltd., Mulgrave, VIC, Australia
Quantity One 4.6.3	BioRad, Hercules, CA, USA
Win UV Scan	Varian Australia Pty Ltd., Mulgrave, VIC, Australia
Chromas Version 2.01	Technelysium Pty Ltd, Australia
APILAB Plus V 3.3.3	bioMérieux

2.8 Sampling of water and plankton at Lake Baldeney

Water and plankton samples were obtained from Lake Baldeney (Essen, Germany), the lowermost reservoir within the course of the River Ruhr. Between April and September 2010, six successive sampling events were deployed at monthly intervals. At each event, three different transects (T1, T2, T3) across the lake (from the northern to the southern shore) were sampled (Figure 2.2). Sampling included the collection of one water sample (at T1 only) and the collection of zoo- and phytoplankton at all transects. This lead to a total of 42 samples (18 each for zoo- and phytoplankton plus 6 water samples).

Physico-chemical parameters (pH value, air temperature, water temperature, electric conductivity and oxygen concentration) were measured on-site halfway along each transect while water samples were taken at the same spot (T1 only), approximately 30 cm below the water surface using glass bottles. Additionally to surface water samples, samples were also taken at the lake bottom with a ‘Ruttner Schöpfer’ (Figure 2.1) and the depth was measured with an ultrasonic sensor.

Figure 2.1 Ruttner Schöpfer for sampling of water at the lake bottom (Hydro-Bios Apparatebau GmbH, Kiel, Germany)

Sampling of plankton was carried out using different plankton nets for phytoplankton (55 µm) and zooplankton (200 µm) along diagonal hauls from the lake bottom up to the surface. Therefore, the plankton net was first lowered until it almost reached the lake ground (depth measured before using an ultrasonic sensor) and then slowly lifted while the boat slowly moved toward the opposite lake shore. After each haul, the net was emptied and its content transferred into polyethylene containers (200 ml). Altogether four replicate samples (2–4 hauls each) were taken, two of which were further processed for bacterial analysis and the other two for identification. Plankton samples for indentification were fixed and preserved in Lugol´s fluid (5%), while plankton samples for bacteriological analysis were transported alive without preservation (but cooled at 4° C). All sampling and transport was done following DIN EN ISO 19458 (2006) and processed within 6 h after sampling.

Once arrived at the laboratory, plankton samples for bacteriological analysis were filtered through a piece of gauze (Hydro-Bios Germany, mesh size 55 µm) and the wet weight of the biomass was measured. Plankton was then resuspended in defined volumes of sterile and filtered (pore size 0.2 µm) lake water and homogenised by stirring on a magnetic stirrer for 5 min. Water samples and homogenised plankton suspensions were immediately subjected to cultural methods and DNA extraction or stored in the dark at -20 °C until further analysis.

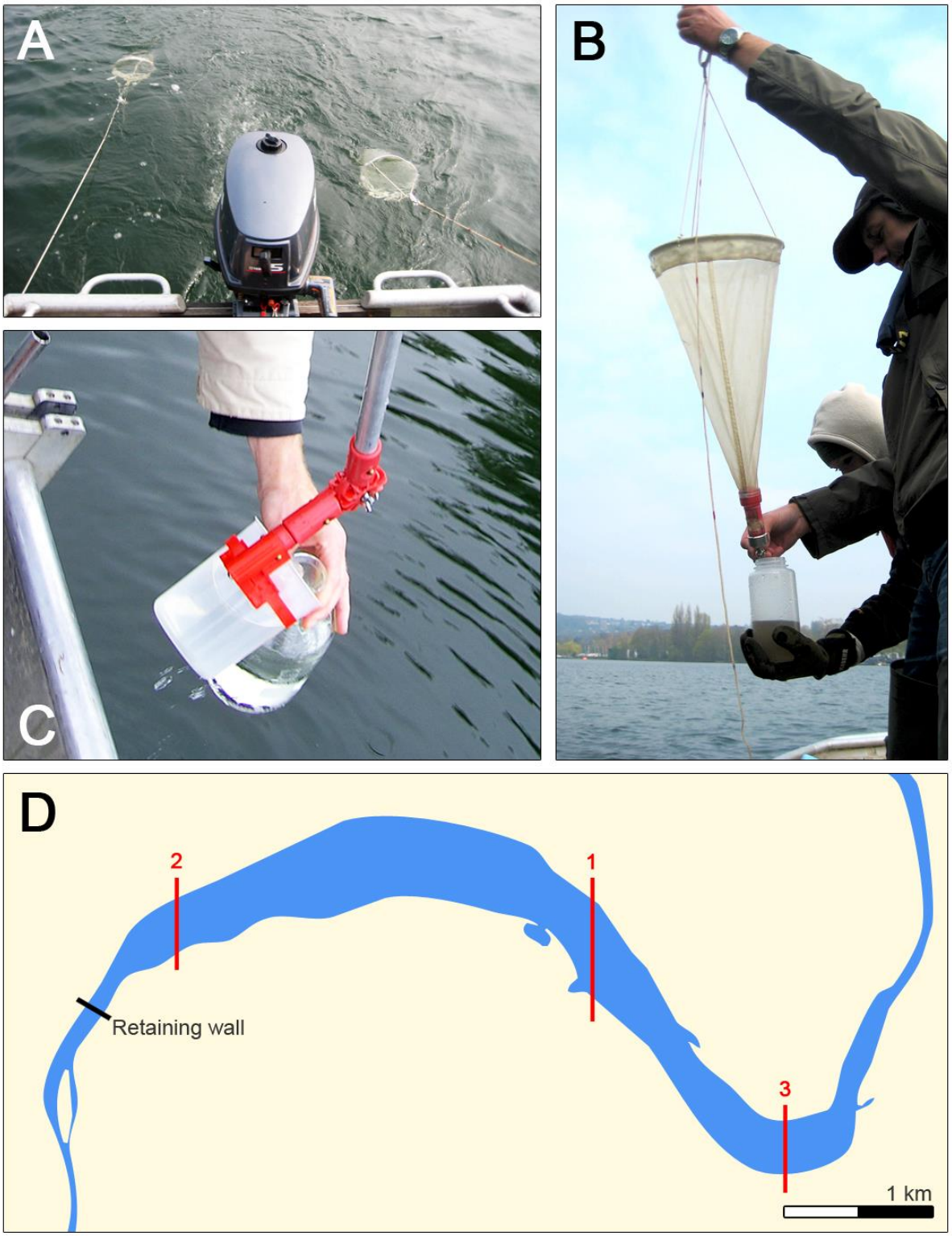


Figure 2.2 Overview of the sampling procedure
(A) Plankton nets are hauled along a transect to harvest phytoplankton and zooplankton, respectively; (B) Plankton is recovered in a sterile polyethylene bottle by opening a valve at the lower end of the plankton net; (C) Collection of water samples in sterile glass bottles; (D) Schematic overview of Lake Baldeney. Red lines indicate transects 1, 2, and 3. The retaining wall impounding the River Ruhr is indicated as a black line. Flow direction is from East (right) to West (left). (Source A – C: J. Wingender, Biofilm Centre, University of Duisburg-Essen)

2.8.1 Identification of plankton organisms

The preserved plankton samples were separated into zooplankton and phytoplankton and identified under a dissecting microscope or binoculars (Olympus SZ 51 and BX 51). Dominant taxa (abundance >5%) were identified to the lowest possible taxonomic level.

Phytoplankton density was determined by averaging the counts of five replicate subsamples (10 µL each). The subsamples were fully processed under the microscope. Zooplankton samples were fully processed and counted (without sub-sampling). Identification of phytoplankton followed recommendations of Mischke and Behrendt (2007), which is the most recent standard for phytoplankton identification in Central Europe and DGL (2007). Zooplankton species (Rotatoria, Copepoda, Cladocera) were identified using Kiefer & Fryer (1978).

2.8.2 Determination of plankton mass and sample preparation

For the determination of the wet weight of the obtained plankton masses, both plankton samples were each filtered through a net (mesh size 55 µm) and weighed with an analytical balance. Furthermore the plankton samples were washed one time in filter-sterilized lake water (0.2 µm) and resuspended in 600 mL of filter-sterilized lake water and stirred on a magnetic stirrer for 5 min. Additionally a ‘plankton-free’ water sample was investigated which was a water sample that was filtered through a 55 µm plankton net.

2.9 Sampling and preparation of *Elodea nuttallii* from Lake Baldeney

Additionally the neophyte *Elodea nuttallii* which showed massive growth at Lake Baldeney in 2009 was sampled. Therefore floating plants at the surface of the lake were collected and transported in sterile flasks (according to DIN EN ISO 19458, 2006). For preparation the water plant was washed one time in filter-sterilized lake water and afterwards suspended in filter-sterilized lake water and stirred on a magnetic stirrer for 5 min. The determination of the dry residue and water content was done according to DIN EN 12880 (2000) and the loss of ignition of the dry mass was determined according to DIN EN 12879 (2000).

2.10 Microbiological methods

2.10.1 Determination of total cell count

4 mL of diluted or undiluted sample were mixed with 1 mL DAPI solution (25 µg/mL) in 2 % (v/v) formaldehyde and incubated at room temperature for 20 min in the dark. Afterwards the solution was filtered through a black polycarbonate membrane filter (0.2 µm pore size, Milipore) using a six-fold vacuum filtration apparatus. The filter was stored at 4 °C in the dark until enumeration (Hobbie et al., 1977).

The cells counts were determined using an epifluorescence microscope at 1000-fold magnification with immersion oil. 20 randomly selected fields of view were examined for each filter with the help of a counting grid (100 µm x 100 µm). Results are given as cells/mL.

2.10.2 Determination of heterotrophic plate count (HPC)

Decimal dilutions of water samples and plankton suspensions were prepared in sterile particle-free deionized water and plated in triplicate on R2A agar to determine the HPC (Reasoner and Geldreich, 1985). Colonies were enumerated. After 7 d of incubation at 20 °C colonies were enumerated. Plates with colony numbers between 30 and 300 were considered for enumeration, Results are given as colony-forming units (cfu)/mL or g wet weight.

2.10.3 Determination of culturable *Pseudomonas aeruginosa*

P. aeruginosa was quantified according to the standard DIN EN ISO 16266 (2008) by filtering 10 ml and 1 mL of water samples or plankton suspensions through 47 mm mixed cellulose ester membrane filters with a pore size of 0.45 µm (Pall). Filters were transferred onto CN agar and the plates were incubated at 36 °C for 48 hours. Plates with colony numbers between 20 and 200 were considered for enumeration. Results are expressed as cfu/mL or g wet weight.

Additionally detection by liquid enrichment in malachite green broth in MPN scale was done according to DIN 38411 part 8 (1982). Single and double concentrated broth was used in 3 different volumina in fivefold approach. The analyzed volume amounted 249.75 mL in total.

Table 2.7 Approaches of Malachite green broth MPN method

Approach	Sample volume (mL)	Malachite green broth volume (mL)	Malachite green broth concentration
1	45	45	double
2	4.5	4.5	double
3	0.45	10	single

The broth was incubated at 36°C for 48 hours. All yellow and turbid approaches were subcultured on Cetrimide Agar. Afterwards typical colonies were streaked onto Pseudomonas-Agar P (PAP) and Pseudomonas Agar F (PAF) incubated at 36°C for 48 hours. On PAF and PAP agar plates the colonies were observed for fluorescence under UV light and blue-green (pyocyanine) or red-brownish (pyorubine) pigmentation.

Additionally typical colonies were inoculated in acetamide broth incubated at 36°C for 22 hours. For confirmation a drop of Nessler´s reagent was added to the acetamide broth after incubation, a positive result was proven if a yellow or brick-red precipitate occurred.

By means of positive confirmed results the concentration of *P. aeruginosa* in 250 mL was determined with the help of a MPN table (Klug, M., 2004; Diploma thesis).

2.10.4 Determination of culturable coliforms and *Escherichia coli*

Quantification of total coliforms and *E. coli* was performed using the Colilert-18 Quanti-Tray®/2000 system (IDEXX). One vial of the Colilert-18 reagent was dissolved in 100 mL of the diluted or undiluted water and plankton samples and subsequently transferred into a Quanti-Tray®. The Tray was sealed and incubated at 36 °C for 19 ± 1 h. Positive (yellow colored (coliforms) or fluorescent (*E. coli*) wells were enumerated and the number was converted to MPN/100 mL or per g wet weight according to the manufacturer’s instruction.

2.10.5 Determination of culturable *Enterococcus* spp.

Quantification of *Enterococccus* spp. was carried out according to the standard DIN EN ISO 7899-2 (2000). Sample volumes of 100 mL and 10 mL water samples or plankton suspensions were filtered through 47 mm mixed cellulose ester membrane filters with a pore size of 0.45 µm (Pall).Filters were transferred onto Slanetz & Bartley agar and the plates were incubated at 36 °C for 44 hours. All red or red-brownish colonies were counted and the filters were transferred onto pre-warmed bile-aesculine-azide agar plates and incubated at 44°C for 2 hours. Results were confirmed if typical colonies developed a visible black corona in the agar medium. Plates with colony numbers between 20 and 200 were considered for enumeration. Results are expressed as cfu/mL or g wet weight.

2.10.6 Determination of culturable *Legionella* spp.

Legionella spp. was quantified according to the standard ISO 11731 (1998).

2 ml of the water samples and plankton suspensions were centrifuged for 10 min at 6000 x g and 4 °C. Half volume of the supernatant was discarded and replaced by an equal volume of acid buffer (pH 2.2).

The pellet was resuspended and the suspension was incubated at room temperature for 5 min. After incubation, the suspension was spread-plated in tripiclate and incubated at 36 °C for up to 10 days. Plates with colony numbers between 30 and 300 were considered for enumeration. Results are expressed as cfu/mL or g wet weight.

Additionally 100, 10 and 1 mL of the samples were filtered through black 50 mm mixed cellulose ester membrane filters with a pore size of 0.45 µm (Whatman) and treated with 10 mL of acid buffer (pH 2.2) for 5 min. Afterwards the filters were rinsed with 10 mL of sterile deionised water and placed onto GVPC agar. Plates were incubated for up to 10 days at 36 °C. Plates with colony numbers between 20 and 200 were considered for enumeration. Results are expressed as cfu/mL or g wet weight.

2.10.7 Determination of culturable *Campylobacter* spp.

For qualitative determination of *Campylobacter* spp. (according to ISO 17995 (2005)) volumes of 100 ml and 200 ml water samples and plankton suspensions were filtered through 47 mm mixed cellulose ester membrane filters with a pore size of 0.45 µm (Pall) and transferred into bottles with 100 ml Preston broth. Incubation occurred in anaerobic jars under microaerophilic conditions (Anaerocult C, Merck) at 36°C for 48 hours. Afterwards 10 µL of the Bouillon were streaked onto mCCDA-Agar and incubated under microaerophilic conditions at 42°C for 48 hours.

Grown colonies were streaked in duplicate onto nutrient agar plates and incubated at 42°C for 24 hours, one plate under aerobic and the other under microaerophilic conditions. A positive result is given, when the incubated colonies do not grow under aerobic conditions.

2.10.8 Determination of culturable *Aeromonas* spp.

Quantification of *Aeromonas* spp. was performed according to Havelaar et al. (1987). The water samples and plankton suspensions were spread-plated (volumes 500 µL and 100 µL) on ampicillin dextrin agar and incubated at 30°C for 24 hours. Additionally volumes of 10 mL and 1 mL were filtered through 47 mm mixed cellulose ester membrane filters with a pore size of 0.45 µm (Pall).Filters were transferred onto ampicillin dextrin agar and the plates were incubated at 30 °C for 24 hours. Plates with colony numbers between 30 and 300 (spread-plate method) or between 20 and 200 (membrane filtration) were considered for enumeration. Results are expressed as cfu/mL.

2.10.9 Determination of culturable *Clostridium perfringens* and their spores

C. perfringens was quantified by membrane filtration according to the German Drinking Water Ordinance (TrinkwV, 2001). Water samples and plankton suspension volumes of 100 mL and 10 mL were filtered through 47 mm mixed cellulose ester membrane filters with a pore size of 0.45 µm (Pall). Filters were transferred onto m-CP agar (Armon & Payment, 1988) and incubated in anaerobic jars under anaerobic conditions (Anaerocult A, Merck) at 44°C for 21 hours. All opaque-yellow colonies were counted and afterwards the plates were exposed to ammonium hydroxide steam for 30 seconds. All opaque-yellow colonies which turned to pink were considered for enumeration between 20 and 200. Results are given in cfu/mL or g wet weight.

Additionally the amount of *C. perfringens* spores was determined by pasteurisation of 100 mL of each sample at 80°C for 10 min. Afterwards cultivation occurred similar to those for vegetative cells.

2.11 Characterization of bacterial isolates

2.11.1 Biochemical characterization

P. aeruginosa, *A. hydrophila* and *Campylobacter* spp. were identified using the API® 20 NE system and the API® Campy system (bioMérieux), respectively. Intestinal enterococci and coliforms were identified using API®rapid ID 32 strep and API® 20 E respectively. Test strips were inoculated according to the manufacturer's instruction and incubated at 30 °C for 24 to 48 h (API® 20 NE) or at 36 °C for 24 (API® 20 E) up to 48 h, microaerophilic (API® Campy), or 36°C for 4 h (API®rapid ID 32).

Identification was performed using the software APILAB Plus V 3.3.3.

2.11.2 16S rDNA sequence analysis

2.11.2.1 Isolation of DNA from pure cultures

A concentration of approximately 1.2 x 10^9 cells/mL (Mc Farland standard 4) was prepared in 0.9 % NaCl solution with cell material from 24 h old pre-cultures. By centrifugation (10 min, 5000 x g) the cell material was harvested. The QIAGEN DNeasy® Blood & Tissue Kit was used following the protocol for DNA isolation from Gram-negative bacteria. For the final elution of the DNA from the spin column 2 x 100 µL elution buffer were used. The DNA solution was stored at -20°C.

2.11.2.2 Amplification of 16S rDNA fragments

Polymerase chain reaction (PCR) was performed for the amplification of bacterial 16S rDNA gene fragments by use of the primer fd1 and rp2 (Weisburg et al., 1991). For each reaction the following components were pipetted into a 0.2 mL PCR tube:

Table 2.8: Components and concentrations of the PCR reaction used for bacterial 16S rDNA amplification

Constituent	**Final concentration**	**Volume in 50 µL**
Primer fd1	50 pM	0.5 µL
Primer rp2	50 pM	0.5 µL
MasterMix 2.5x		20 µL
Taq DNA Polymerase	1.25 U	
KCl	50 mM	
Tris-HCl pH 8.3	30 mM	
$Mg(OAc)_2$	1.5 mM (Mg2+)	
Igepal®-CA630	0.1%	
dNTP (each)	200 µM	
Stabilizers		
DNA solution (3.3.2.1.)		1 µL
H2O (molecular biology grade)		28 µL
Total Volume		**50 µL**

A negative control without DNA template was included in all PCR reactions.

Table 2.9 The PCR program parameters

Initial Denaturation	94 °C	120 s
30 Cycles:		
Denaturation	94 °C	60 s
Annealing	59 °C	60 s
Elongation	72 °C	90 s
Final Elongation	72 °C	5 min
Cooling	4 °C	

2.11.2.3 DNA sequencing and comparative sequence analysis

The cleaned PCR products were sent to Sequence Laboratories Göttingen GmbH (Göttingen, Germany). For sequencing 10 µL of DNA with a concentration of 80 ng/µl and 10 µL of the forward primer fd1 with a concentration of 10 pM/µL were sent.

By use of the software Chromas Version 2.01 (Technelysium Pty Ltd, Australia) sequence data were edited and processed. Identification of microbial species was performed using the nucleotide-nucleotide Blast service available at the website of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/).

The nucleotide sequence was compared to sequence databases and the most significant statistical match was considered (Altschul et al., 1997). A sequence match of more than 97% implies relation on species level, a minimum of 95% sequence homology was set to signify relation on genus level (Angenent et al. 2005).

2.12 Molecularbiological methods

2.12.1 Buffers and solutions for polymerase chain reaction (PCR)

dNTP Mix

Commercial ready-to-use reagent mix (5 Prime, 2201200).

10 mM of each dNTP.

MasterMix 2.5x

Commercial ready-to-use reagent mix (5 Prime, 2200100).

Composition: 62.5 U/µL Taq DNA Polymerase, 125 mM KCl, 0.5 % ®-CA630, 75 mM Tris-HCl (pH 8.3), 3.75 mM Mg^{2+} (Mg(OAc)2), , 500 µM dNTP (each), 0.25% Igepal, stabilizers.

Table 2.10 Primers

Primer	Sequence	Reference
fd1	5'-AGA GTT TGA TCC TGG CTC AG-3'	Weisburg et al., 1991
rp2	5'-ACG GCT ACC TTG TTA CGA CTT-3'	Weisburg et al., 1991

Primers were obtained as lyophilisates from Eurofins MWG Operon (Ebersbach, Germany).

Taq buffer advanced

Commercial ready-to-use reagent mix (5 Prime, 2201240).

10x concentrated with 15 mM Magnesium.

Taq DNA Polymerase (5 U/µL; 5 Prime, 2200000).

Taq Master PCR Enhancer

Commercial ready-to-use reagent mix (5 Prime, 2201250).

5x concentrated.

2.12.2 Fluorescence in-situ hybridization (FISH)

2.12.3 Solutions and buffers for fluorescence in-situ hybridization (FISH)

Phosphate-buffered saline (PBS)

Composition in g/L: sodium chloride 8.00, potassium chloride 0.20, sodium hydrogen phosphate dihydrate 1.81, potassium hydrogen phosphate 0.24.

The components were dissolved in Rotipuran water (Roth T143.1). The pH was 7.2 ± 0.2. The solution was autoclaved for 20 min at 121 °C.

Paraformaldehyde solution (4 %)

Composition in g/l: 4 g paraformaldehyde (Merck) were dissolved in 100 ml PBS (pH 7.2) and stirred with a magnetic stirrer for 1 h at 50 °C. The solution was filter sterilized (pore size 0.2 µm).

DAPI solution (1 µg/mL) in PBS

40 µL of the DAPI stock solution (25 µg/mL) were mixed with 960 µL PBS.

0.25 M EDTA

104.50 g Na_4EDTA (Sigma) were dissolved in 1L of Rotipuran water. The solution was autoclaved for 20 min at 121 °C

Hybridisation buffers

Table 2.11 Hybridisation buffers for oligonucleotide probes targeting bacterial 16S rRNA

	EUB338 / NONEUB338	**Psae 16S-182**	**LEG705**	**LEGPNE1**	**AERBOMO**	**Efs 130**
5 M NaCl	0.9 M	0.9 M	0.9 M	0.9 M	0.9 M	0.9 M
1 M Tris pH 7.2	-	-	-	-	20 mM	-
1 M Tris pH 7.6	20 mM	-	20 mM	20 mM	-	-
1 M Tris pH 8.0	-	20 mM	-	-	-	20 mM
10% SDS	0.01 %	0.01 %	0.01 %	0.01 %	0.01 %	0.01 %
Formamide	20%	40 %	20 %	25 %	35 %	35 %

For the preparation of hybridisation buffers, Rotipuran water was used.

5 M NaCl solution

292.2 g sodium chloride were dissolved in 1 L of Rotipuran water. The solution was autoclaved for 20 min at 121 °C.

Table 2.12 Oligonucleotide probes

Probe	Sequence	Specific for	Reference
EUB338	5'-GCT GCC TCC CGT AGG AGT-3'	Eubacteria	Amann et al., 1990
NONEUB338	5'-CGA CGG AGG GCA TCC TCA-3'	negative control	Wallner et al., 1993
Psae16S-182	5'-CCA CTT TCT CCC TCA GGA CG- 3'	*P. aeruginosa*	Wellinghausen et al., 2005
LEGPNE1	5'-ATC TGA CCG TCC CAG GTT-3'	*L. pneumophila*	Grimm et al., 1998
LEG705	5'-CTG GTG TTC CTT CCG ATC-3'	*Legionella* spp.	Manz et al., 1995
AERBOMO	5'-CTA CTT TCC CGC TGC CGC C-3'	*A. hydrophila*	Bomo et al. 2004 Kämpfer et al., 1996
Efs 130	5'-CCCTCTGATGGGTAGGTT-3'	*E. faecalis*	Meier et al. 1997

All oligonucleotide probes were Cy3-labelled at the five prime end and HPLC cleaned. Probes were obtained as lyophilisates from Eurofins MWG Operon (Ebersbach, Germany).

Oligonucleotide probe solutions

Lyophilisates were dissolved in water for molecular biology (Roth) to a final concentration of 1 µg/µL. Then stock solutions of the specific probes were prepared by diluting the dissolved lyophilisates with water for molecular biology to a final concentration of 50 µg/µL.

For preparation of the working solutions stock solutions were diluted 1:10 in the appropriate hybridisation buffer.

10 % (w/v) SDS

10 g SDS (sodium dodecyl sulfate; Riedel de Haen) were dissolved in 100 mL Rotipuran water and the solution was filter sterilized (pore size 0.2 µm).

1 M Tris-buffer pH 7.2/pH 7.6/pH 8.0

121.14 g Tris(hydroxymethyl)-aminomethane (Roth) were dissolved in 1 L of Rotipuran water and the pH was adjusted to pH 7.6 or pH 8.0 ± 0.2 with 7 M hydrochloric acid. The solution was autoclaved for 20 min at 121 °C.

Table 2.13 Washing buffers for oligonucleotide probes targeting bacterial 16S rRNA

	EUB338 / NONEUB338	Psae 16S-182	LEG705	LEGPNE1	AERBOMO	Efs 130
1 M Tris pH 7.2	-	-	-	-	20 mM	-
1 M Tris pH 7.6	20 mM	-	20 mM	20 mM	-	-
1 M Tris pH 8.0	-	20 mM	-	-	-	-
5 M NaCl	225 mM	56 mM	225 mM	160 mM	40 mM	80 mM
0.25 M EDTA	-	5 mM	-	5 mM	5 mM	5 mM
10% SDS	0.01 %	0.01 %	0.01%	0.01%	0.01%	0.01%

For the preparation of washing buffers, Rotipuran water was used.

FISH was performed using the probe EUB338 for the detection of Eubacteria (Amann et al., 1990), Psae16S-182 for *P. aeruginosa*. Leg 705 for *Legionella* spp. and LEGPNE1 for the detection of *L. pneumophila* (Grimm et al., 1998), AERBOMO for *A. hydrophila* (Bomo et al. 2004) and Efs 130 for E. faecalis (Meier et al., 1997).
As a negative control a nonsense probe complementary to the sequence of probe EUB338, NONEUB338 (Wallner et al., 1993) was used.

Fixation. By centrifugation (10 min, 6000 x g, 4 °C) cells were harvested and the supernatant was discarded. The pellet was resuspended in half the volume of 4 % paraformaldehyde in phosphate-buffered saline (PBS), pH 7.2 and incubated for fixation at 4°C for 1 h. Afterwards the suspension was centrifuged (5 min, 6000 x g, 4 °C), the supernatant was discarded and the pellet was washed once in PBS.
After centrifugation (5 min, 6000 x g, 4 °C) the supernatant was discarded and the pellet was resuspended in a mixture of PBS and ethanol absolute (1:1) and stored at -20°C.

FISH procedure. The fixed samples (10 µL) of bacterial suspensions were pipetted onto epoxy-coated 8-well diagnostic slides (Thermo Scientific) and air-dried. By dipping the slides into 50 %, 80 % and 96 % ethanol (3 min for each step) the samples were dehydrated. After air-drying hybridization was conducted by adding 10 µL of hybridisation buffer containing 5 ng/µL of the respective oligonucleotide probe to the samples fixed slides. Hybridization took place in a humid reaction chamber (Vermicon) at 46 °C for 90 min. Afterwards the 8-well diagnostic slides were transferred into reaction chambers containing 25 mL to 46°C pre-heated (48 °C for

Efs 130) washing buffer and incubated at 46 °C for 15 min (5 min. for Efs 130). Then the slides were washed in deionised water, air-dried and bacterial cells were counterstained by adding 10 µL of DAPI (1 µg/mL) to each well and incubating for 20 min at room temperature in the dark. The diagnostic slides were washed in deionised water and stored at 4 °C in the dark until enumeration of cells. For enumeration an epifluorescence microscope at 1000-fold magnification was used and 20 randomly selected fields of view or at least 200 cells were enumerated for each filter with the help of a counting grid (100 µm x 100 µm). The percentage of FISH-positive bacteria was calculated in relation to the number of DAPI stained cells.

2.12.4 Quantitative PCR

2.12.5 Solutions and buffers for qPCR

Reagents

Table 2.14 Ready-made qPCR reagents

qPCR reagent	Manufacturer
Maxima™ SYBR Green/Fluorescein qPCR Master Mix (2X)	Fermentas, St. Leon-Rot, Germany
qPCR probe dilution buffer (10 mM TRIS-Cl, 1 mM EDTA, pH 8)	Eurofins MWG Operon, Ebersberg, Germany
TaqMan Exogeneous Internal Positive Control Reagents (10X Exo IPC Mix, 10X Exo IPC Block, 50X Exo IPC DNA)	Applied Biosystems, Darmstadt, Germany
VeriQuest™ Probe qPCR Master Mix (2X)	USB/Affymetrix, High Wycombe, UK

Oligonucleotides

The following oligonucleotides were used in this study (kit components are not listed):

- Forward primer Pa23FP (Eurofins MWG Operon, Ebersberg, Germany)
- Reverse primer Pa23RPb (Eurofins MWG Operon, Ebersberg, Germany)
- TaqMan probe Pa23FAM (Eurofins MWG Operon, Ebersberg, Germany)

Stock solutions (100 pmol/µL) of the primers Pa23FP and Pa23RPb, and the TaqMan probe Pa23FAM were stored at -20 °C in the dark. The sequences of the oligonucleotides used in this work are listed in Table 2.15.

Table 2.15 Sequences of oligonucleotides

Oligoname	Sequence	Reference
Pa23FP[a]	5'-TCCAAGTTTAAGGTGGTAGGCTG-3'	Schwartz et al., 2006; Volkmann et al., 2007
Pa23RPb[a]	5'-ACCACTTCGTCATCTAAAAGACGAC-3'	Volkmann et al., 2007
Pa23FAM[b]	FAM-5'-AGGTAAATCCGGGGTTTCAAGGCC-3'-TAMRA	Schwartz et al., 2006; Volkmann et al., 2007

[a] Primer.

[b] TaqMan probe.

Enzymes and proteins

Table 2.16 Enzymes and other proteins (enzymes contained in ready-made PCR reagents are not listed)

Enzyme	Concentration	Manufacturer
Bovine serum albumin (BSA)	10 mg ml^{-1}	New England Biolabs, Frankfurt am Main, Germany

KU: Kunitz unit(s).

Isolation and purification of DNA with Aquadien™ DNA extraction Kit (Bio Rad)

According to the protocol for dirty water samples provided by the manufacturer, 50 ml of each sample were concentrated by filtration using white 0.40-µm-pore-diameter polycarbonate membrane filters. Filters were placed in a tube containing lysis buffer. Lysis of cells was performed in the presence of thermal shock by incubating for 15 min at 95 °C. The filter was removed and the lysate was stored for 24 h at 2 °C to 8 °C to allow the resin of the lysis buffer to settle. Using 1 ml of supernatant, DNA was purified by ultrafiltration and adsorption on a silica column, and eluted into a final volume of 100 µl using elution buffer. Centrifugation time was extended to ensure that all supernatant was filtered through the column. Centrifugation speed remained constant for all samples. For samples containing high amounts of plankton material (heavily-loaded samples), 1 ml supernatant was split and processed in two separate columns (500 µl each). After pooling both eluates, a final volume of 200 µl was obtained. These DNA extracts were stored at -20 °C until analysis.
Cells from pure cultures of all bacterial strains each were suspended in deionised water to obtain a bacteria concentration of 10^6 cells litre^{-1} and 5×10^5 cells litre^{-1} for *Legionella* spp. and *Pseudomonas* aeruginosa, respectively. DNA extraction was performed according to the Aquadien™ Kit protocol for drinking water samples.

Z-values

The Z value is a theoretical factor that compensates for DNA loss during DNA extraction and represents the fraction of the initial sample analysed in each PCR assay. It was calculated for every method and was 32 for the Aquadien™ Kit (64 for heavily-loaded samples).

Determination of DNA quantitiy

DNA yield was quantified using the PicoGreen assay Quant-iT™ PicoGreen® dsDNA Kit according to the manufacturer's instructions. DNA extracts were limited in volume, they had to be diluted in TE (1X) buffer 100-fold or 1,000-fold, respectively. Fluorescence was detected using a fluorescence spectrophotometer (SFM 25 A fluorescence spectrophotometer, Kontron Instruments, Mila, Italy).

Quantitative real-time PCR

Quantitative real-time PCR (qPCR) was performed using an iCycler combined with an iQ^{TM}5 Multicolor Real-Time PCR Detection System (Bio Rad). Samples were introduced into the cycler in 96-well plates that were sealed with transparent, self-adhesive film, and analysed in duplicates.

Legionella spp. and *L. pneumophila*

Legionella qPCR was carried out using commercially available iQ-Check^{TM} kits (Bio Rad) for the detection of *Legionella* spp. and *L. pneumophila*, respectively. Each kit provided a target DNA-specific FAM-labelled (FAM-490) molecular beacon probe, and a Texas-Red-labelled (TexasRed-575) probe as the internal control DNA. By mixing the amplification mix and the probe solution in a ratio (8:1) defined by the manufacturer, a PCR reaction mix was created. For each sample, 45 µl of PCR reaction mix were pipetted in a well, and 5 µl sample of extracted DNA were added (according to manufacturers´ instructions). In the event of PCR inhibition detected by validation of the Texas Red signal, samples were diluted in molecular-grade water. Each qPCR run accommodated a no-template control (5 µl molecular-grade water), a positive, and a negative control (both supplied in the kit; 5 µl of each). For quantification, a set of four *L. pneumophila* DNA standards (QS1 – QS4 PCR standards, supplied in the kits; 5 µl of each), corresponding to a DNA concentration range of 15 genome units (GU) to 3×10^4 GU per assay, was applied. A 3-step PCR was run for 50 cycles following initial UDG treatment as well as a denaturing step at 95 °C for 15 min (Table 2.17). Fluorescence emission by the FAM-labelled probe at a wavelength of 530 nm resulting from excitation at 490 nm was measured after each annealing step. The Texas Red signal was monitored simultaneously.

Table 2.17 Temperature protocol used in *Legionella* qPCR.

Cycle	Step	Temperature [°C]	Time [min]
1[a]		50	02:00
2[b]		95	15:00
3 - 52	1	95	00:15
	2[c]	55	00:30
	3	72	00:30

[a] Uracil-DNA Glycosylase inactivation.

[b] Hot-start polymerase activation.

[c] Collect data.

P. aeruginosa

Pseudomonas aeruginosa qPCR was carried out using two different probes: A FAM-labelled TaqMan hydrolysis probe and SYBR Green as a general DNA-binding agent.

TaqMan probe assay. For each qPCR run, a PCR reaction mix was created by mixing primers, probe solution, BSA, VeriQuest™ Probe qPCR Master Mix, and molecular-grade water. Optionally, a TaqMan Exogenous Internal Positive Control (IPC) comprising a VIC-labelled probe and synthetic DNA was administered, adjusting the volume of water used (Table 2.18). In each well, 45 µl of PCR reaction mix were provided. Samples were analysed by adding 5 µl of the respective DNA extract. Template DNA was replaced with *P. aeruginosa* ATCC 10145 pure culture DNA extract as a positive control, or with molecular-grade water as a no-template control, respectively. When using an IPC, additionally a no-amplification control (NAC) had to be applied, controlling the IPC itself. For this, 5 µl of 10X Exo IPC Block solution were added instead of DNA template. Generally, samples were analysed in duplicates. However, when using the IPC, six replicates of NTC respectively NAC had to be run to ensure a 99.7 % confidence level.

Quantification standards were set up using DNA extracted from pure cultures of *P. aeruginosa* ATCC 10145. The DNA content in the extract was measured via PicoGreen assay. The DNA extract was then ten-fold diluted serially to create a set of five standards with a *P. aeruginosa* DNA content ranging from 8 pg ml^{-1} to 80 ng ml^{-1}. In order to obtain results comparable to *Legionella* qPCR, the units of the standards had to be converted to GU. Since no information regarding the genome weight of *P. aeruginosa* had been published so far, the author derived a simplified genome weight by correlating a genome length of approximately 6.3 Mbp and a GC content of 66.6 % (Stover et al., 2000) with a bp weight for GC and AT of 618.14 Da and 617.43 Da (Howe et al., 2009), respectively. A theoretical genome weight of 6.43 fg was obtained. Based on this value, the concentrations of the standards corresponded to a range from 6.22 GU to 6.22×10^4 GU per assay.

A 2-step PCR with a combined annealing and elongation step was run for 40 cycles (Table 2.19), again following an initial UDG treatment and a denaturation step. Fluorescence emission by the FAM-labelled probe at a wavelength of 530 nm resulting from prior excitation at 490 nm was detected after each annealing step. Since restricted calibration of the qPCR instrument did not allow for detecting the VIC signal, the IPC was monitored by using the hexachloro-6-carboxyfluorescein (HEX)

channel instead, which exhibits excitation and emission maxima similar to VIC (VIC: $excitation_{max}$ = 538 nm, $emission_{max}$ = 554 nm; HEX: $excitation_{max}$ = 535 nm, $emission_{max}$ = 556 nm) (Bustin & Nolan, 2004).

Table 2.18 PCR reaction mix for each sample in *Pseudomonas aeruginosa* qPCR using the TaqMan probe.

Component	Final concentration	Volume [µl]
PCR reaction mix	-	45
VeriQuest™ Probe qPCR Master Mix (2X)	1X	25
Forward primer Pa23FP	300 nM	1.5
Reverse primer Pa23RPb	300 nM	1.5
TaqMan probe Pa23FAM	200 nM	1
BSA	200 µg ml^{-1}	1
IPC[a]	-	6
10X Exo IPC Mix[a]	1X	5
50X Exo IPC DNA[a]	1X	1
Water, molecular-grade	-	ad 45
Template DNA / Water / Standard / 10X Exo IPC Block	-	5
Total volume		**50**

[a] Optional.

Table 2.19 Temperature protocol used in *Pseudomonas aeruginosa* qPCR.

Cycle	Step	Temperature [°C]	Time [min]
1[a]		50	02:00
2[b]		95	10:00
3 - 42	1	95	00:15
	2[c]	60	01:00

[a] UDG inactivation.

[b] Hot-start polymerase activation.

[c] Collect data.

SYBR Green assay. *Pseudomonas* qPCR using SYBR Green as a detection system was carried out under the same conditions as with using the TaqMan probe but with an adjusted PCR reaction mix (Table 2.20). Melt curve analysis was performed after each run. Temperature was raised by 0.5 °C every 15 s, starting from 55 °C to 95 °C. Fluorescence signal decrease was measured on-line as a function of temperature. The software automatically constructed a melt curve peak chart by plotting the first negative derivative of the fluorescence against the temperature.

Table 2.20 PCR reaction mix per sample in *Pseudomonas aeruginosa* qPCR using SYBR Green.

Component	Final concentration	Volume [µl]
PCR reaction mix	-	45
Maxima™ SYBR Green/Fluorescein qPCR Master Mix (2X)	1X	25
Forward primer Pa23FP	300 nM	1.5
Reverse primer Pa23RPb	300 nM	1.5
BSA	200 µg ml^{-1}	1
Water, molecular-grade	-	ad 45
Template DNA / Water / Standard	-	5
Total volume		**50**

Data analysis and evaluation

For quantification, the iQ™5 software automatically generates a standard curve based on the C_t values of the standards. PCR benchmarks are expressed in the correlation coefficient R^2 and amplification efficiency (Eq. 1). Results are given as starting quantity (SQ) per well, corresponding to the amount of target GU per assay (5 µl of DNA extract). The baseline threshold value for all wells was manually set to the first cycle in which any amplification could be observed. Also, the fluorescence threshold was adjusted to be just above the baseline noise. These measures were done to improve both the correlation coefficient R^2 and the PCR efficiency, with optimal values being ≥ 0.99 and 75 % to 125%, respectively.

SQ values were translated to GU per litre (Eq. 2) using Microsoft Excel. The unit GU l^{-1} is established in literature for qPCR-based quantifications of bacteria in water. For theoretical considerations, quantitative results obtained for plankton samples (in GU l^{-1}) were further referenced to the wet weight of the plankton biomass analysed. A theoretical water content of the plankton of approximately 100 % was estimated in order to permit a weight-to-volume-based conversion of the results. Plankton sample results were thus expressed as genome units per kilogram plankton (GU kg^{-1}). This

conversion was mandatory in order to permit comparability amongst different plankton samples, and to be able to relate concentrations in the plankton samples to concentrations in the water samples.

$E = e^{ln10/-s} - 1$, (Kuiper et al., 2006) (Equation 1)

E: PCR efficiency.
s: Slope of the regression line.

Detection limits and quantification limits were derived to check whether results were in the range of the standards. The detection limit (Ld) of a qPCR method for the detection of *Legionella* is defined as the lowest number of GU generating a positive result at a 90 % confidence limit (AFNOR XP T90-471, 2006). It corresponds to 5 GU per 5 µl DNA extract (5 GU/assay). Calculation is shown in Equation 5. Although being intended for detection of *Legionella*, it was also used for *P. aeruginosa* qPCR. The lower quantification limit (LQL) corresponds to the lowest number of copies allowing a reliable and accurate quantification as described in the AFNOR XP T90-471 standard (2006). It was calculated for each method (also *P. aeruginosa* qPCR) according to Equation 6. The upper quantification limit on the other hand is defined as the value given by the highest quantification standard (Eq. 5).

$$X = \frac{SQ \times Z \times D}{V} \quad \text{(Equation 2)}$$

$$Ld = \frac{5 \times Z \times D}{V \times 2} \quad \text{(Equation 3)}$$

$$LQL = \frac{Qs1 \times Z \times D}{V} \quad \text{(Equation 4)}$$

$$UQL = \frac{Qs^h \times Z \times D}{V} \quad \text{(Equation 5)}$$

D: Dilution of the DNA in the assay.
Ld: Detection limit [GU l^{-1}].
LQL: Lower quantification limit [GU l^{-1}].
Qs1: Concentration of the lowest standard [GU in 5µl].
Qs^h: Concentration of the highest standard [GU in 5µl].
SQ: Starting quantity in a 5 µl assay [GU].
UQL: Upper quantification limit [GU l^{-1}].
V: Sample volume filtered prior DNA extraction [l].
X: Target genome units per litre of sample [GU l^{-1}].
Z: Factor to compensate for DNA loss during DNA extraction

PCR inhibition was detected using the internal controls (Texas Red for *Legionella* spp. and *L. pneumophila*, VIC for *P. aeruginosa*). In the former case, C_t values of Texas Red revealed partial or complete inhibition, when they were either higher than the C_t of the highest standard or 'N/A' (not applicable - indicated by the qPCR software for the C_t of a sample when fluorescence did not rise above threshold). For *P. aeruginosa* qPCR, IPC validation was done using the qPCR instrument's end-point detection function. NAC wells were labelled as negative controls, whereas NTC wells were labelled as positive controls. Judging from the end-point fluorescence of the controls, samples were allocated to be either positive or negative (inhibited).

2.13 Cultivation of plankton organisms

2.13.1 *Daphnia magna*

Stock cultures of *Daphnia magna* (approximately 20 of them per glass) were raised in glass beakers filled with 1 L of artificial Daphnia medium (ADaM) with saturating concentrations of *Scenedesmus obliquus*. Cultures were maintained static, at room temperature under 12 h light : 12 h dark photoperiod. Every 2 or 3 days the organisms were transferred into fresh ADaM. If the Daphnia clutched, the offspring were separated from the mothers and 20 each of the offspring were transferred into a beaker with freshly prepared ADaM.

2.13.2 *Scenedesmus obliquus*

Cultivation of the unicellular green algae *Scenedesmus obliquus* was performed in WC_g Medium (see 2.3.6) with an 8500 K neon tube (UV spectrum between 450 and 750 nm) and submerged air supply at room temperature. After 7 days the algae were harvested by centrifugation (17700 g, 5 min., 20 °C) and resuspended in ADaM and stored at 4°C until they were used for feeding the daphnids (maximum 1 week).

2.14 Determination of toxicity of pathogens against *Daphnia magna*

In order to use *D. magna* to measure bacterial pathogenitiy, the crustacean was exposed to the bacterial pathogens *P. aeruginosa*, *E. faecalis* and *A. hydrophila* (Le Codiac et al., 2012).

Therefore bacteria were grown on nutrient agar at 36°C for *P. aeruginosa* and *E. faecalis* and at 30°C for *A. hydrophila* for 24 h. The overnight cultures were used to prepare bacterial suspensions in ADaM. According to Le Codiac et al. (2012) daphnids were exposed to bacterial concentrations of $OD_{(600\ nm)}$= 0.4, 0.8, 1.5 and 3.0 for up to 28 h. An OD_{600}= 3.0 was for example determined with approximately 3.96 x 10^9 cells/mL and OD_{600}= 1.5 relates to 1.86 x 10^9 cells/mL respectively. *D. magna* used for these experiments were between seven and ten days old. The ability of the bacteria to have a toxic effect on *D. magna* was assessed by incubating three daphnids in 1 mL of each concentration in a sterile Eppendorf tube under static conditions. Each tube was prepared in triplicate, daphnia in sterile ADaM were used as a control. Each hour live daphnids which were swimming around were discriminated from dead immobile ones that were located at the bottom of the tubes.

2.15 Co-cultivation of *Daphnia magna* with selected bacterial strains

2.15.1 Co-cultivation with *Pseudomonas aeruginosa*

Pre-cultures of the organism *P. aeruginosa* PAO1 were grown on nutrient agar at 36 °C for 24 h. 2 or 3 colonies were inoculated in LB-broth and incubated in a water bath with agitation at 36 °C for 18 h. Cells were harvested by centrifugation and the pellet was washed once in 10 mL sterile filtered ADaM. After centrifugation (1400 g, 10 min., 4 °C) the pellet was resuspended in 10 mL sterile ADaM. The cell concentration was determined by using a Thoma counting chamber.

D. magna used for these experiments were between seven and ten days old. A bacterial concentration of approximately 10^7 cells/mL was used for the co-cultivation with *Daphnia*. In each well of a 6-well plate 10 mL of the PAO1 suspension were transferred and 5 *D. magna* were added to each well (Figure 2.3).

Additionally, wells containing 10 mL sterile ADaM and 5 *Daphnia* were cultivated. As a control bacterial suspension without *Daphnia* and 10 mL sterile ADaM were used.

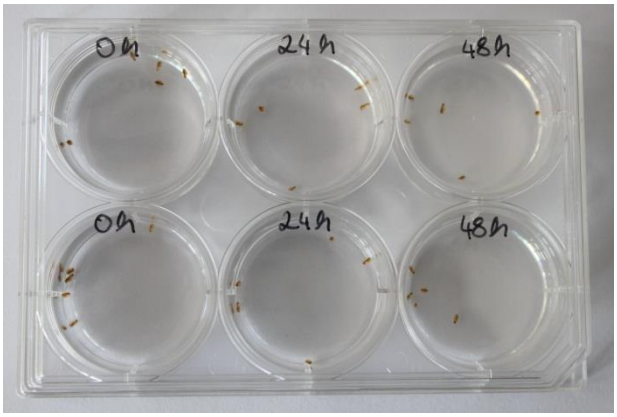


Figure 2.3: Co-cultivation of pathogens with *D. magna*:
6-well plates with 10 mL of a bacterial suspension (10^8 cells/mL) and 5 daphnids in each well. As controls, 5 daphnids in sterile ADaM without bacteria, and bacterial suspension without daphnids. Incubation occurred at roomtemperature (22-24°C) over a period of 48 h.

Sampling of medium and daphnids occurred at time intervals of 0, 24 and 48 h.

At time intervals the 5 Daphnia were collected from the wells, gently washed once in sterile ADaM for 5 minutes to remove nonadherent bacteria. Afterwards the gut of the daphnids was separated from the carapace by the use of two sterile pairs of tweezers (Figure 2.4). The carapace and leftover entrails were minced by use of a mortar, both samples were suspended separately in 1 mL sterile ADaM and diluted in sterile deionized water. By use of a swab the surface of the well was sampled, therefore the medium was removed of the well before and the well was rinsed once with 10 mL of sterile ADaM. The swab was resuspended in 2 mL sterile ADaM and afterwards 1 mL

each of the medium and the rinsingwater were diluted in sterile deionized water (Figure 2.5).

Useful dilutions of all samples (bacterial suspension, Daphnia gut, Daphnia carapace, rinsing water, well surface) were plated onto nutrient agar and incubated at 36°C for 24 h. Colonies between 30 and 300 were considered for enumeration.
Additionally total cell counts were determined by use of the DAPI method. Therefore useful dilutions of all samples were incubated with DAPI (3.2.1).

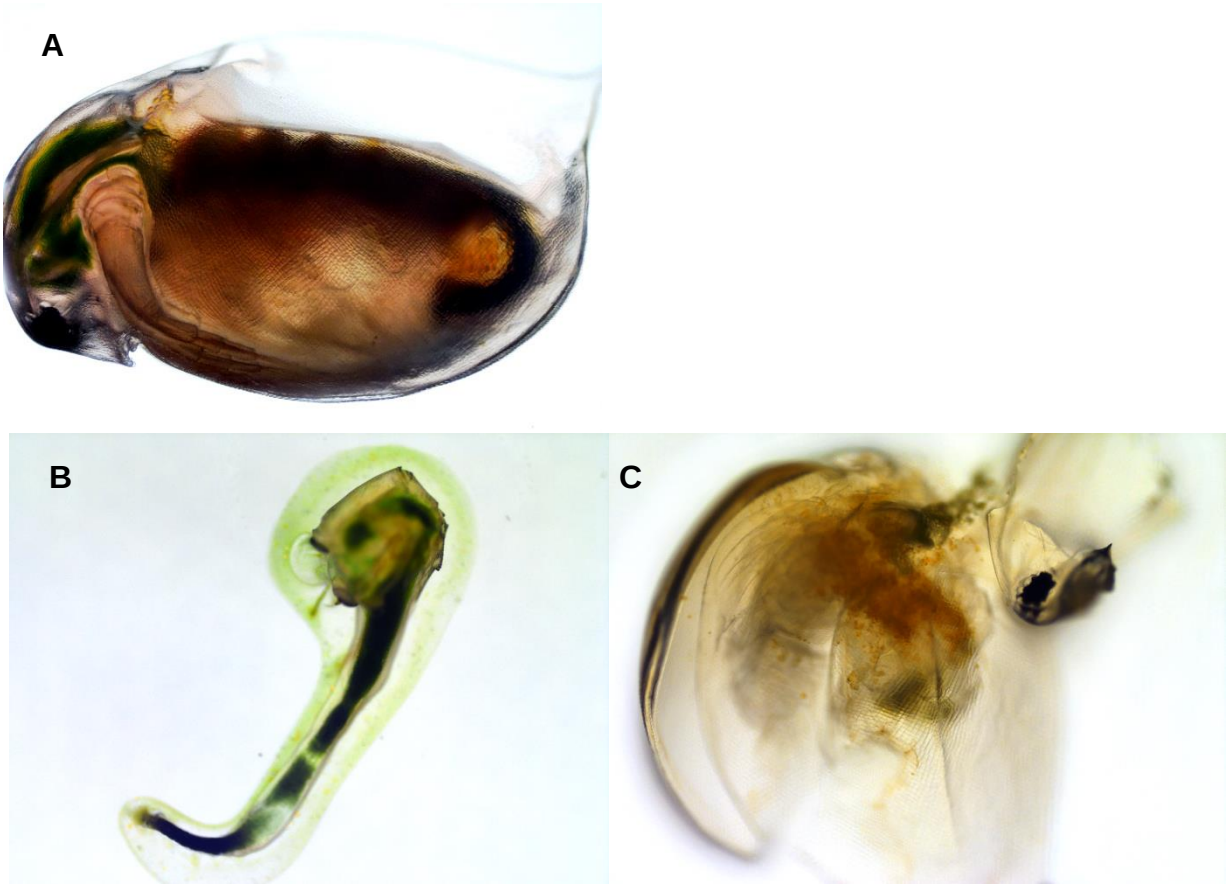


Figure 2.4: Preparation of *D. magna*
Lightmicroscopic pictures (magnification 100x) **A** *D. magna* before preparation; **B** Separated gut of *D. magna*; **C** Carapace of *D. magna* and leftover parts of the entrails. (Source: Miriam Tewes, Biofilm Centre, University of Duisburg-Essen)

2.15.2 Co-cultivation with *Aeromonas hydrophila*

For co-cultivation of *Daphnia magna* with *A. hydrophila* AH-1N pre-cultures were grown on nutrient agar. 2 or 3 colonies were inoculated in LB-broth and incubated in a water bath with agitation at 30 °C for 18 h.

Further procedure see 2.15.1.

Samples were plated onto ampicillin dextrin agar and incubated at 30°C for 24 h.

2.15.3 Co-cultivation with *Enterococcus faecalis*

For co-cultivation of *Daphnia magna* with *E. faecalis* pre-cultures were grown on nutrient agar. 2 or 3 colonies were inoculated in LB-broth and incubated in a water bath with agitation at 30 °C for 18 h.

Further procedure see 2.15.1.

Samples were plated onto Chromocult® Enterococci Agar and incubated at 36°C for 24 h.

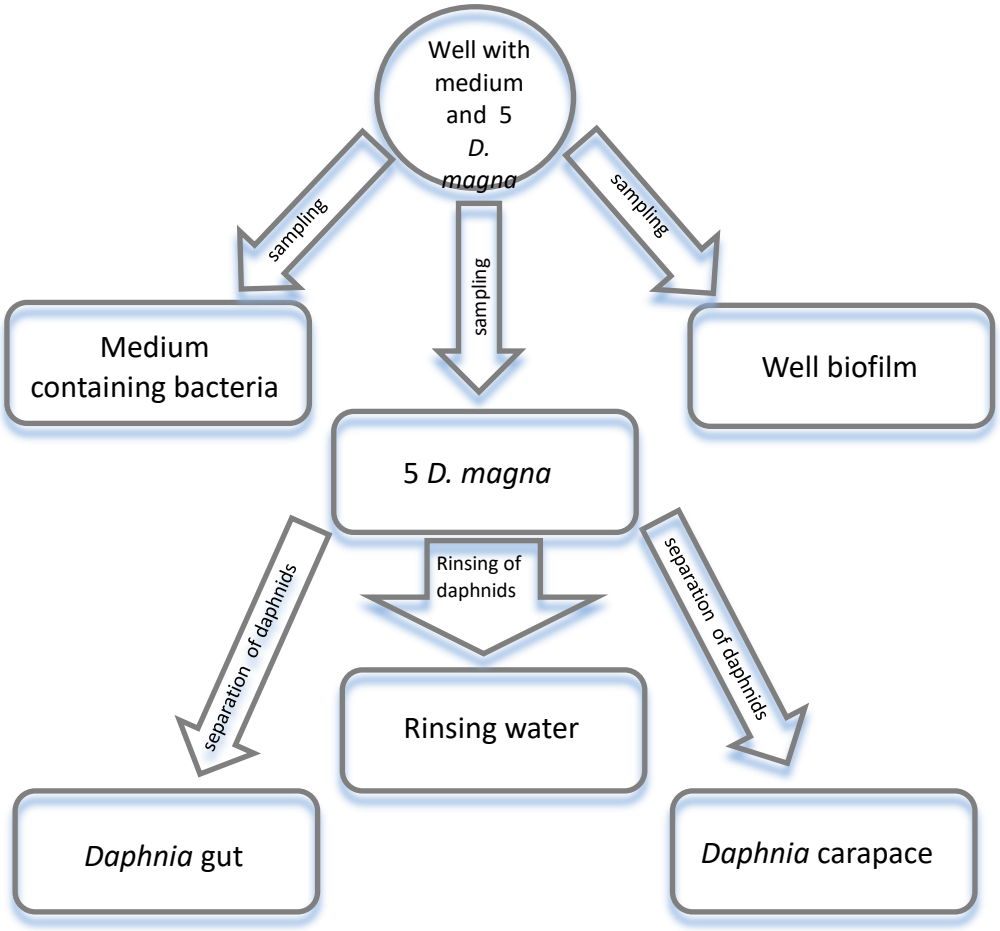


Figure 2.5 Overview oft he sampling procedure of co-cultivation experiments. One well contained 10 mL of medium (ADaM) with bacteri and 5 daphnids.

3 Results

3.1 Association of hygienically relevant bacteria with plankton organisms in Lake Baldeney

In the first part of the present study the association of hygienically relevant organisms with freshwater plankton was investigated in a field study at Lake Baldeney in Essen/Germany. At six sampling dates from April to September in the year 2010 the bacterial abundance, plankton taxa and physico-chemical parameter were investigated monthly. Overall a total of 42 samples (18 each for zoo- and phytoplankton plus 6 water samples) were investigated. Phytoplankton as well as zooplankton samples were taken at three transects across the lake (Figure 3.1).

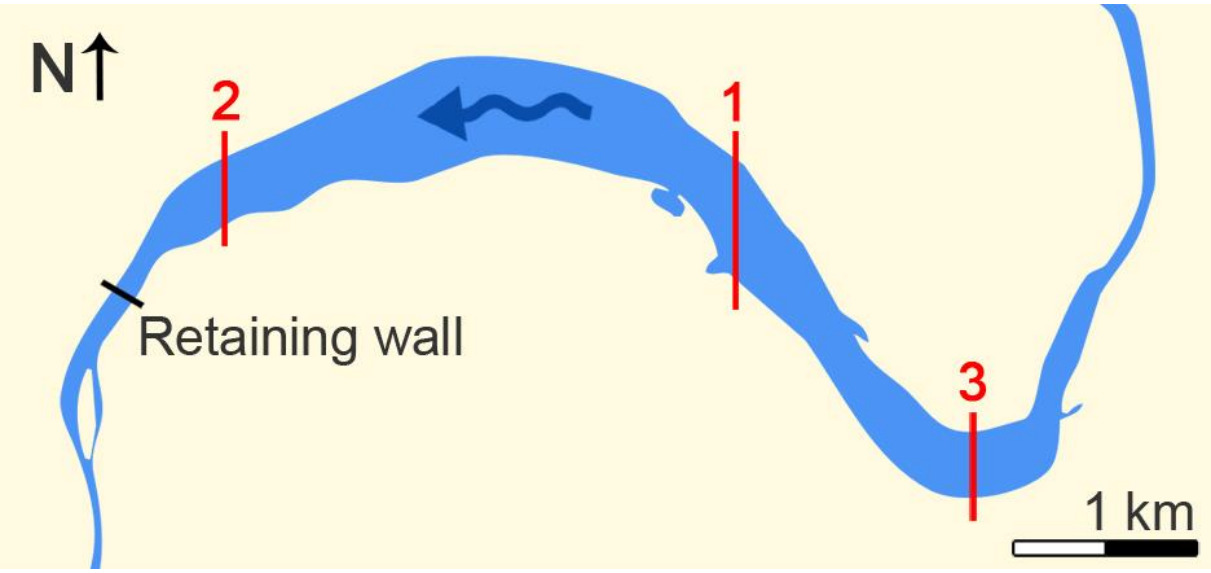


Figure 3.1 Schematic overview of Lake Baldeney. Red lines indicate transects 1, 2, and 3. The retaining wall impounding the River Ruhr is indicated as a black line. Flow direction of the river is from East (right) to West (left).

With respect to the sampling location (T1, T2, T3), plankton samples were referred to as T1Z, T2Z, and T3Z for zooplankton, and T1P, T2P, and T3P for phytoplankton, respectively. A water sample was taken at transect 1 (T1).

Due to the occasion of massive growth of the macrophyte *Elodea nuttallii* in Lake Baldeney in the year 2009, samples of the macrophyte were investigated to discover associations of pathogenic bacteria and the waterplant (n = 3).

3.1.1 Physico-chemical characterization of surface water

Physico-chemical parameters including pH value, water temperature, electric conductivity and oxygen concentration were measured on-site halfway along each transect approximately 30 cm below the water surface.

At the same spots surface water samples were taken and the chemical parameters nitrite, nitrate, ammonia, phosphate, TOC and DOC were determined in the laboratory. Additionally to surface water samples, samples were also taken at the lake bottom. An overview of the physico-chemical parameter of the surface water samples is shown in Table 3.1 and parameter of the water samples taken at the bottom are shown in Table 3.2.

The results indicated that there are no considerable differences between the surface and the ground water samples. Also there is no seasonal dependency, except for the temperature and the oxygen concentration (Table 3.1, yellow framed). The temporal courses of water temperature and oxygen concentration in surface water samples and of the bottom are shown in Figure 3.2. These two parameters are of interest, because they might have an important influence on bacterial abundance.

The water temperature at the surface varied between 10.4°C and 27.1 °C, with its maximum at the sampling date in July. The water temperature at the bottom was between 11.7°C and 20.1 °C with its maximum in May and the lowest temperature in June.

In surface water samples an oxygen concentration with a minimum of 70.2 % and an oversaturation with a maximum of 146.8% in July were determined. In bottom water samples the concentration for oxygen varied between 46.2 % and 95.2%.

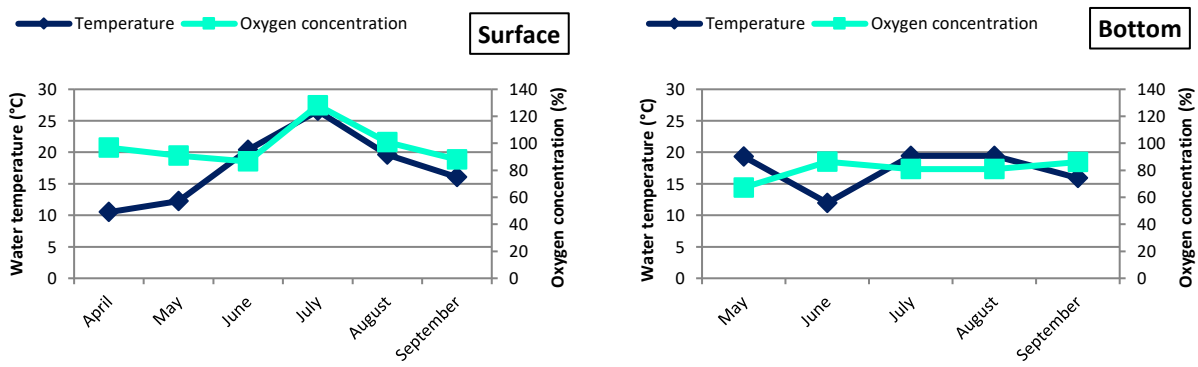


Figure 3.2 Temporal course of mean water temperature and oxygen concentration of surface water samples and water samples taken at the bottom of Lake Baldeney.

Table 3.1 Physico-chemical parameters of surface water samples taken at Lake Baldeney.

Sampling date	Transect	Tempera-ture [°C]	pH	Conduct-ivity [µS/cm at 25°C]	Oxygen concen-tration [mg/L]	Oxygen concen-tration in [%]	Depth of vision [m]	TOC [mg/L]	DOC [mg/L]	Carbonate hardness [mmol/L]	Total hardness [mmol/L]	Chloride [mg/L]	Phosphate [mg/L]	Ammonia [mg/L]	Nitrite [mg/L]	Nitrate [mg/L]
April	**T1**	10.5	7.8	460	10.9	98.0	2.1	2.2	1.9	1.6	1.4	52.0	0.4	0.2	0.1	9.8
	T2	10.4	7.8	455	10.7	95.3	1.2	2.8	2.0	1.8	1.5	50.0	0.3	0.2	0.1	5.5
	T3	10.6	7.9	460	10.7	96.9	4.5	2.0	1.9	1.7	1.2	44.0	0.4	0.2	0.1	12.8
May	**T1**	12.3	6.9	87.5	9.3	88.2	2.1	3.7	3.4	2.0	1.3	42.0	0.5	0.4	0.2	14.2
	T2	12.3	7.7	96.7	10.0	94.6	1.8	4.3	3.5	1.4	1.0	56.0	0.3	0.3	0.1	7.6
	T3	12.1	7.6	89	9.4	89.6	2.5	3.4	3.2	1.4	1.0	56.0	0.4	0.2	0.1	8.7
June	**T1**	20.6	7.6	590	8.9	101.9	1.1	4.2	3.2	2.5	1.5	70.0	0.4	0.2	0.3	13.3
	T2	20.0	8.0	607	7.4	87.4	0.8	4.5	3.2	2.5	1.6	84.0	0.5	0.1	0.0	6.0
	T3	20.6	7.5	586	6.2	70.2	1.2	3.4	3.1	2.5	1.6	82.0	0.5	0.3	0.1	11.8
July	**T1**	26.3	7.7	514	11.7	146.8	0.9	4.2	3.7	2.2	1.5	82.0	0.4	0.2	0.1	10.1
	T2	27.1	7.8	527	9.8	125.3	0.9	4.2	3.7	2.3	1.5	88.0	0.5	0.6	0.1	5.3
	T3	26.3	7.6	509	7.1	112.4	1.1	3.9	3.6	2.1	1.5	82.0	0.4	0.2	0.1	9.8
August	**T1**	19.4	7.4	419	7.1	79.0	1.1	4.2	3.3	1.8	0.9	30.0	0.8	0.1	0.0	12.4
	T2	20.1	7.6	504	7.1	79.0	1.1	3.6	3.1	1.9	0.8	24.0	0.7	0.1	0.0	5.4
	T3	19.4	7.7	431	7.8	85.3	1.0	4.1	3.2	2.0	1.1	30.0	0.7	0.1	0.0	5.3
September	**T1**	16.0	7.2	542	9.5	95.4	1.8	2.5	2.4	1.0	0.7	20.0	0.7	0.1	n.d.	2.1
	T2	16.1	7.7	530	7.6	76.8	2.4	2.4	2.2	2.0	0.7	16.0	0.7	0.2	0.0	3.5
	T3	16.1	7.7	530	9.0	91.9	2.2	2.4	2.3	1.6	0.7	18.0	0.7	0.1	0.0	4.6

n.d., not determined

Table 3.2 Physico-chemical parameters water samples taken at the bottom of Lake Baldeney.

Date	Transect	Depth [m]	Temper-ature [°C]	pH	Conductivity [µS/cm at 25°C]	Oxygen concen-tration [mg/L]	Oxygen concen-tration in [%]	TOC [mg/L]	DOC [mg/L]	Carbonate hardness [mmol/L]	Total hardness [mmol/L]	Chloride [mg/L]	Phosphate [mg/L]	Ammonia [mg/L]	Nitrite [mg/L]	Nitrate [mg/L]
April	**T1**	3.9	n.d.	n.d.	n.d.	n.d.	n.d.	2.5	1.9	1.9	1.4	48.0	0.4	0.2	0.1	9.5
	T2	3.0	n.d.	n.d.	n.d.	n.d.	n.d.	3.0	2.0	2.0	1.5	56.0	0.4	0.2	0.1	17.8
	T3	5.6	n.d.	n.d.	n.d.	n.d.	n.d.	2.0	1.9	2.0	1.4	60.0	0.3	0.1	0.1	5.7
May	**T1**	4.6	17.9	7.3	777	8.10	83.5	7.5	3.5	1.8	1.2	58.0	0.5	0.4	0.2	8.1
	T2	6.0	19.9	7.6	635	6.63	72.0	4.2	3.2	1.5	1.1	58.0	0.4	0.4	0.1	6.9
	T3	5.0	20.1	7.5	611	7.80	46.2	3.4	3.1	1.8	1.3	58.0	0.5	0.3	0.1	11.2
June	**T1**	3.3	12.4	7.3	87,6	8.87	83.5	3.9	3.4	2.4	1.6	78.0	0.4	0.1	0.0	12.6
	T2	2.0	11.8	7.7	96,6	9.37	88.4	4.0	3.5	2.5	1.6	90.0	0.5	0.2	0.1	10.4
	T3	5.0	11.7	7.4	88,2	9.23	87.0	3.4	3.1	2.4	1.7	92.0	0.6	0.3	0.0	12.2
July	**T1**	3.8	19.2	7.5	439	7.48	79.1	4.6	3.3	2.5	1.5	80.0	0.4	0.6	0.1	10.0
	T2	5.8	19.9	7.7	505	7.69	78.8	3.5	3.0	2.2	1.5	80.0	0.5	0.5	0.1	9.8
	T3	6.2	19.2	7.7	433	7.67	85.0	4.0	3.2	2.5	1.5	84.0	0.5	0.6	0.1	11.8
August	**T1**	3.5	19.2	7.5	439	7.48	79.1	4.6	3.3	2.6	1.4	43.0	0.7	0.2	0.0	13.7
	T2	5.9	19.9	7.7	505	7.69	78.8	3.5	3.0	0.8	0.7	30.0	0.6	0.0	0.0	8.2
	T3	4.5	19.2	7.7	433	7.67	85.0	4.0	3.2	1.2	0.9	30.0	0.6	0.1	0.0	6.9
September	**T1**	3.5	15.8	7.1	543	9.38	95.2	2.8	2.2	1.8	0.9	20.0	0.7	0.1	0.0	2.4
	T2	5.0	15.9	7.7	535	7.30	73.4	2.6	2.3	2.0	1.0	30.0	0.7	0.1	0.0	11.5
	T3	6.1	16.0	7.6	531	8.91	89.5	2.4	2.2	1.8	0.6	40.0	0.6	0.1	0.0	4.8

n.d., not determined

3.2 Abundance and identification of plankton organisms

Sampling of plankton was carried out using different plankton nets for phytoplankton (55 µm) and zooplankton (200 µm) along diagonal hauls from the lake bottom up to the surface. The concentrations and the taxa of the phyto- and zooplankton were determined microscopically according to the recommendations of Mischke and Behrendt (2007) and the German Limnological Society (DGL, 2007) for phytoplankton, whereas Kiefer & Fryer (1978) was used for zooplankton.

Table 3.3 shows mean values of individual concentrations determined in phyto- and zooplankton samples during the sampling season. Highest abundance of phytoplankton organisms could be detected in May, June and July with up to 10^{10} individuals/m^3. The temporal course in Figure 3.3 shows an increase of zooplankton according to the decrease of phytoplankton abundnace. Zooplankton was determined with a maximum in July with up to 10^5 individuals/m^3.

This seasonal trend can be explained by zooplankton organisms grazing on phytoplankton unitl the so called clear water state is reached, where phytoplankton is almost eliminated by zooplankton grazing.

Table 3.3 Means of plankton organism abundance in Lake Baldeney during the sampling season (individuals/m^3).

	Phytoplankton	Zooplankton
April	4.4×10^9	9.3×10^1
May	2.0×10^{10}	2.2×10^3
June	1.9×10^{10}	9.6×10^2
July	1.3×10^{10}	3.2×10^5
August	1.4×10^9	3.2×10^4
September	5.2×10^9	8.5×10^1

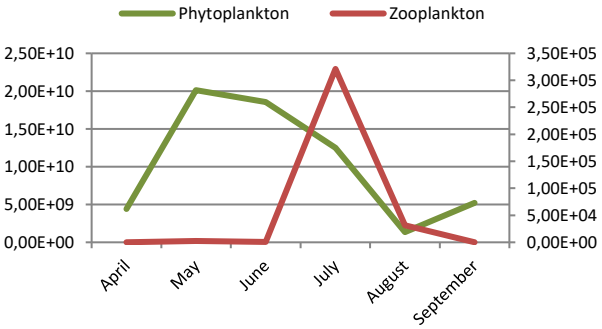


Figure 3.3 Temporal course of phyto- and zooplankton organism abundance in Lake Baldeney (inividuals/m^3).

Identified plankton species are shown in Table 3.4 and 3.5. Among phytoplankton organisms members of the diatoms represented the highest amount. Followed by *Melosira* sp. which was found in all samples. Other diatom species detected in phytoplankton samples were *Fragilaria* sp., *Niztschia* sp., *Asterionella* sp. and *Synedra* sp.

Chlorophyta sp., *Scenedesmus* sp. and *Pediastrum* sp. for example were the most abundant green algae determined in the samples. Cyanobacteria species found in 2010 were *Oscillatoria* sp., *Spirulina* sp. and *Anabena* sp.. Among the class chrysophyceae *Dinobryon* sp. was the only present species of golden algae in phytoplankton samples.

The most abundant species of zooplankton detected in the samples belong to to the group of Rotatoria (Table 3.5). Most of the other detected species were member of the Crustacea, mainly the subclasses Cladocera and Copepoda, e.g. *Daphnia* sp., *Polyphemus* sp., *Cyclopoida* and *Calanoida*.

Table 3.4 Phytoplankton taxa and concentrations determined in phytoplankton samples of Lake Baldeney (individuals/m^3)

Phytoplankton taxa	April			May			June			July			August			September		
	T1	T2	T3	T1	T2	T3	T1	T2	T3	T1	T2	T3	T1	T2	T3	T1	T2	T3
***Oscillatoria* sp.**	4 x 10^8	4x 10^8	1 x 10^9	0	4 x 10^9	3 x 10^9	4 x 10^9	1 x 10^9	9 x 10^8	0	0	0	0	0	1 x 10^8	1 x 10^8	1 x 10^8	0
***Melosira* sp.**	3 x 10^8	2 x 10^8	2 x 10^8	5 x 10^8	1 x 10^9	3 x 10^8	2 x 10^9	4 x 10^8	4 x 10^8	2 x 10^9	4 x 10^8	1 x 10^9	8 x 10^7	3 x 10^8	5 x 10^7	9 x 10^8	6 x 10^8	7 x 10^8
***Fragilaria* sp.**	7 x 10^7	6 x 10^7	2 x 10^8	7 x 10^8	2 x 10^9	9 x 10^8	2 x 10^9	4 x 10^8	7 x 10^8	6 x 10^8	1 x 10^8	3 x 10^8	9 x 10^7	7 x 10^7	2 x 10^7	1 x 10^8	6 x 10^7	2 x 10^8
Chlorophyta non det.	0	5 x 10^7	2 x 10^7	3 x 10^7	2 x 10^8	2 x 10^8	6 x 10^8	4 x 10^7	2 x 10^8	1 x 10^8	3 x 10^9	2 x 10^8	6 x 10^7	6 x 10^7	6 x 10^7	2 x 10^8	2 x 10^8	4 x 10^8
***Asterionella* sp.**	4 x 10^8	6 x 10^8	5 x 10^7	2 x 10^8	1 x 10^9	2 x 10^8	1 x 10^9	4 x 10^8	3 x 10^8	4 x 10^7	0	2 x 10^7	1 x 10^7	3 x 10^7	6 x 10^6	3 x 10^7	1 x 10^8	0
***Scenedesmus* sp.**	0	0	0	5 x 10^7	1 x 10^9	4 x 10^7	1 x 10^9	1 x 10^9	3 x 10^8	0	6 x 10^7	1 x 10^7	0	0	0	0	9 x 10^7	0
***Pediastrum* sp.**	2 x 10^7	0	4 x 10^7	4 x 10^7	1 x 10^8	9 x 10^7	3 x 10^8	8 x 10^7	1 x 10^8	9 x 10^7	1 x 10^9	6 x 10^7	0	5 x 10^7	0	3 x 10^8	1 x 10^8	2 x 10^8
***Micractinium* sp.**	0	0	0	0	0	0	0	0	0	4 x 10^8	2 x 10^9	1 x 10^8	0	0	0	0	0	0
Diatoma elongatum	1 x 10^7	1 x 10^7	3 x 10^7	2 x 10^8	2 x 10^9	9 x 10^7	7 x 10^7	4 x 10^7	3 x 10^7	0	0	0	0	0	0	0	0	0
Pennales non det.	8 x 10^6	4 x 10^6	3 x 10^7	2 x 10^8	5 x 10^8	9 x 10^7	1 x 10^8	7 x 10^7	1 x 10^7	4 x 10^8	4 x 10^7	3 x 10^7	6 x 10^7	8 x 10^7	3 x 10^7	1 x 10^8	8 x 10^7	1 x 10^8
***Synedra* sp.**	2 x 10^6	3 x 10^6	1 x 10^7	9 x 10^7	7 x 10^8	4 x 10^7	5 x 10^8	2 x 10^8	1 x 10^8	6 x 10^7	1 x 10^7	0	0	0	0	5,E+06	0	0
***Spirulina* sp.**	0	2 x 10^8	0	0	0	1 x 10^8	0	0	0	3 x 10^8	0	0	0	3 x 10^7	3 x 10^7	1 x 10^8	0	0
***Dinobryon* sp.**	0	0	0	9 x 10^7	2 x 10^8	5 x 10^7	7 x 10^6	2 x 10^8	0	6 x 10^7	0	0	0	0	2 x 10^7	3 x 10^7	6 x 10^7	0
Nitzschia sigmoidea	3 x 10^6	5 x 10^6	1 x 10^7	2 x 10^7	6 x 10^7	4 x 10^7	1 x 10^7	1 x 10^7	9 x 10^6	4 x 10^6	7 x 10^6	0	2 x 10^7	1 x 10^7	1 x 10^7	4 x 10^7	2 x 10^7	4 x 10^7
***Ceratium* sp.**	0	0	0	0	0	0	4 x 10^6	0	4 x 10^6	4 x 10^6	1 x 10^7	6 x 10^7	2 x 10^6	3 x 10^7	1 x 10^6	5 x 10^6	4 x 10^6	6 x 10^6
***Surirella* sp.**	0	0	1 x 10^6	4 x 10^6	6 x 10^6	6 x 10^6	7 x 10^6	1 x 10^7	0	3 x 10^6	7 x 10^6	0	5 x 10^6	5 x 10^6	3 x 10^6	1 x 10^7	1 x 10^7	2 x 10^7
***Cymbella* sp.**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2 x 10^6	0	0	8 x 10^7
Nitzschia acicularis	0	0	0	5 x 10^6	4 x 10^7	1 x 10^7	7 x 10^6	0	5 x 10^6	3 x 10^6	0	0	0	0	2 x 10^6	0	4 x 10^6	0
***Gyrosigma* sp.**	1 x 10^6	1 x 10^6	2 x 10^6	6 x 10^6	6 x 10^6	7 x 10^6	0	0	0	3 x 10^6	7 x 10^6	0	4 x 10^6	3 x 10^6	3 x 10^6	2 x 10^7	4 x 10^6	9 x 10^6
Meridion circulare	0	0	0	0	0	0	0	0	1 x 10^7	0	0	0	0	0	0	0	0	5 x 10^7
***Anabaena* sp.**	0	5 x 10^7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***Closterium* sp.**	0	0	0	0	0	0	0	0	0	1 x 10^7	0	0	4 x 10^6	1 x 10^6	2 x 10^6	1 x 10^7	7 x 10^6	6 x 10^6
***Cymatopleura* sp.**	0	<1	<1	3 x 10^6	0	3 x 10^6	0	4 x 10^6	3 x 10^6	0	0	0	0	1 x 10^6	3 x 10^6	5 x 10^6	0	0
Centrales non det.	2 x 10^6	3 x 10^6	<1	5 x 10^6	0	6 x 10^6	4 x 10^6	0	3 x 10^6	0	0	0	0	0	0	0	0	0
***Volvox* sp.**	0	0	0	0	0	0	<1	<1	<1	<1	<1	<1	<1	0	0	0	0	0

Table 3.5 Zooplankton taxa and concentrations determined in zooplankton samples of Lake Baldeney (individuals/m³)

Zooplankton taxa	April			May			June			July			August			September		
	T1	T2	T3	T1	T2	T3	T1	T2	T3	T1	T2	T3	T1	T2	T3	T1	T2	T3
Rotatoria non det.	2	1	2	100	200	8	60	300	40	3000	158000	2000	0	0	0	0	0	0
Ciliata non det.	0	0	0	0	0	0	0	0	0	0	140000	0	0	13000	0	0	0	0
***Parapodophyra* sp.**	0	0	0	0	0	0	0	0	0	0	0	0	18000	0	0	0	0	0
Cladocera non det.	7	6	6	200	300	200	40	200	30	3000	12000	400	100	40	300	20	10	10
Cyclopoida non det.	10	10	8	400	0	200	40	100	7	600	2000	100	30	10	50	10	0	20
***Bosmina* sp.**	2	0	0	200	100	50	1	0	0	0	0	0	0	0	0	0	0	0
Nematoda non det.	3	10	8	40	5	50	1	30	20	0	0	0	6	1	8	2	2	2
***Daphnia* sp.**	0	0	0	20	50	10	5	4	3	0	0	0	20	0	40	2	0	2
***Polyphemus* sp.**	0	0	0	0	0	8	0	0	0	0	0	0	8	60	10	0	0	4
Calanoida non det.	2	0	1	0	0	10	5	20	0	0	0	0	0	6	0	0	0	0
Harpacticoida non det.	1	2	1	2	0	20	0	6	1	0	0	0	0	0	0	0	0	0
Acari non det.	0	0	0	2	0	0	5	10	0	0	0	4	0	0	0	1	0	0
Ostracoda non det.	0	0	0	0	0	0	0	6	0	0	0	0	2	1	0	0	0	0

non det. = not determined

3.3 Detection of target organisms with cultural methods in water and plankton samples of Lake Baldeney

In a six month sampling campaign the association of hygienically relevant mircoorganisms with freshwater phyto- and zooplankton was elucidated and compared to organisms in the surrounding water column of Lake Baldeney in Essen/Germany. The investigated organisms with facultative pathogenic properties can be divided in three main groups: faecal indicator bacteria (e.g. *Escherichia coli*, coliforms, intestinal enterococci, *Clostridium perfringens*), obligate pathogens of faecal origin (e.g. *Campylobacter* spp.), and environmental opportunistic bacteria (e.g. some coliforms, *Pseudomonas aeruginosa*, *Aeromonas* spp., *Legionella* spp.). The overall aim was to assess whether the bacteria are more frequently found in a planktonic state in the water phase or in association with plankton.

General bacterial abundance (total cell counts, HPC bacteria) and potentially pathogenic bacteria were detected with cultural methods. Additionally determination of the organisms *P. aeruginosa* and *Legionella* spp were carried out by with molecular methods, such as FISH and qPCR. Furthermore the occurrence and proportions of phyto- and zooplankton organisms were determined.

3.3.1 Distribution and proportion of bacteria in surface water and associated with plankton

The overall bacterial abundance of all surface water and plankton samples was investigated by microscopically determination total cell counts (DAPI method) and culturable HPC bacteria over a period of 6 month (Figure 3.4).

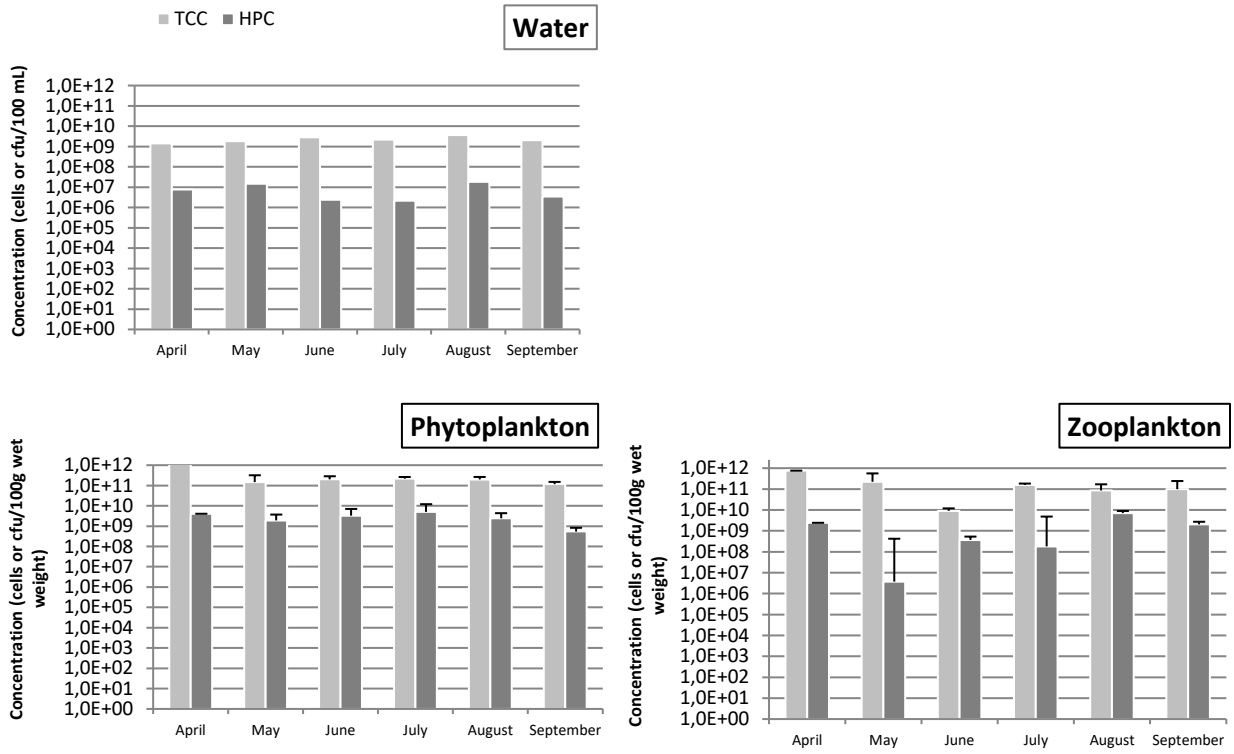


Figure 3.4: Total cell counts (TCC) and colony counts (HPC) of surface water and plankton samples. Investgation of TCC bx microscopically determination using the DAPI method, HPC bacteria were determined by colony counts on R2A medium. Results are given in cells or cfu/100 mL water and cells or cfu/100 g wet weight plankton respectively. Sampling occurred over a period of six month (n = 6), concentrations of the plankton samples are mean values of three transects at each date.

Concentrations of bacteria obviously did not follow a seasonal pattern. Total cell counts as well as colony counts in plankton exceeded the concentrations in water by two to three orders of magnitude when referred to the plankton wet weight.

The percentage of culturable heterotrophic bacteria in water ranged from 0.09 – 0.78 %. The fraction of culturable bacteria on the total counts were significantly higher in the plankton samples compared to the water sample. The culturability varied between 0.29 and 2.30 % in the phytoplankton samples and was between 0 and 8.3 % in zooplankton samples.

The geometric mean values for total cell counts (Table 3.6) were one order of magnitude higher in plankton samples compared to water. Geometric mean values

for heterotrophic plate counts were determined two orders of magnitude higher for plankton than in the water column.

Table 3.6 Geometric mean values for total cell counts (cells/100 mL water or 100 g wet weight plankton) and HPC bacteria (cfu/100 mL water or 100 g wet weight plankton) for water and plankton

	Sample	Geometric mean values
Total cell counts (cells/100 mL or 100 g)	**Water**	2.2×10^9
	Phytoplankton	9.7×10^{10}
	Zooplankton	3.3×10^{10}
Heterotrophic plate counts (cfu /100 mL or 100 g)	**Water**	5.7×10^6
	Phytoplankton	7.8×10^8
	Zooplankton	1.6×10^8

If concentrations of general bacterial abundance were referred to the volume of water which was filtered through the plankton net during sampling (approximately 1250 L phytoplankton, 9000 L zooplankton) (Figure 3.5), the distribution is different compared to results shown in Figure 3.4. Bacteria present in water were enhanced, notably from April till July, exceeding those in plankton up to two log units. In the months July and August, total cell counts in water differed in one order of magnitude compared to those of plankton. However, quantities of culturable bacteria were identical. Concentrations on plankton followed a seasonal variation.

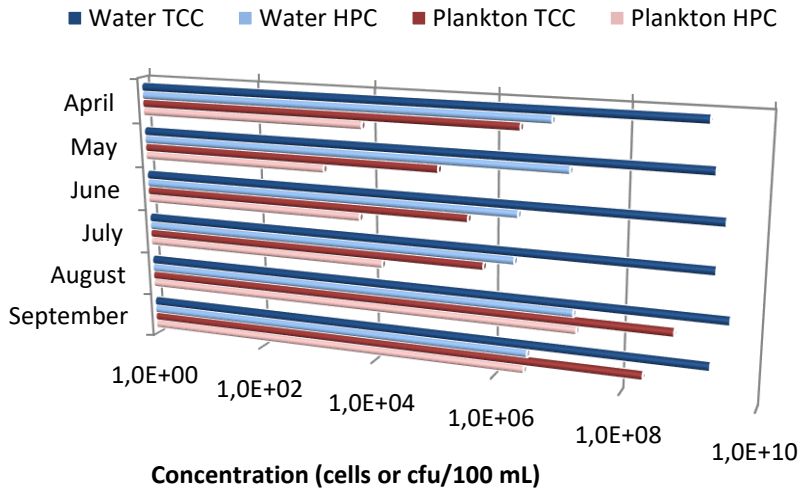


Figure 3.5 Concentrations of total cell counts (TCC) and HPC bacteria in water and plankton samples per 100 mL. Results for plankton were referred to the sampled volume of water which was filtered through the plankton net while collecting plankton (1250 L for phytoplankton and 9000 L for zooplankton)

The results indicated that there is an accumulation of bacteria on plankton, referred to the plankton mass. Also they showed an elevated percentage of culturability compared to those bacteria in water. The culturablilty seems to be enhanced due to favourable nutrient conditions for bacteria living in communitiy with plankton organisms.

If plankton samples are referred to the sampled water volume they reflect only a minor fraction of the general bacterial concentrations when they were compared to the whole water environment of Lake Baldeney. As an example the distribution in percent of total cells between water, phyto and zooplankton in the month August is illustrated in Figure 3.6.

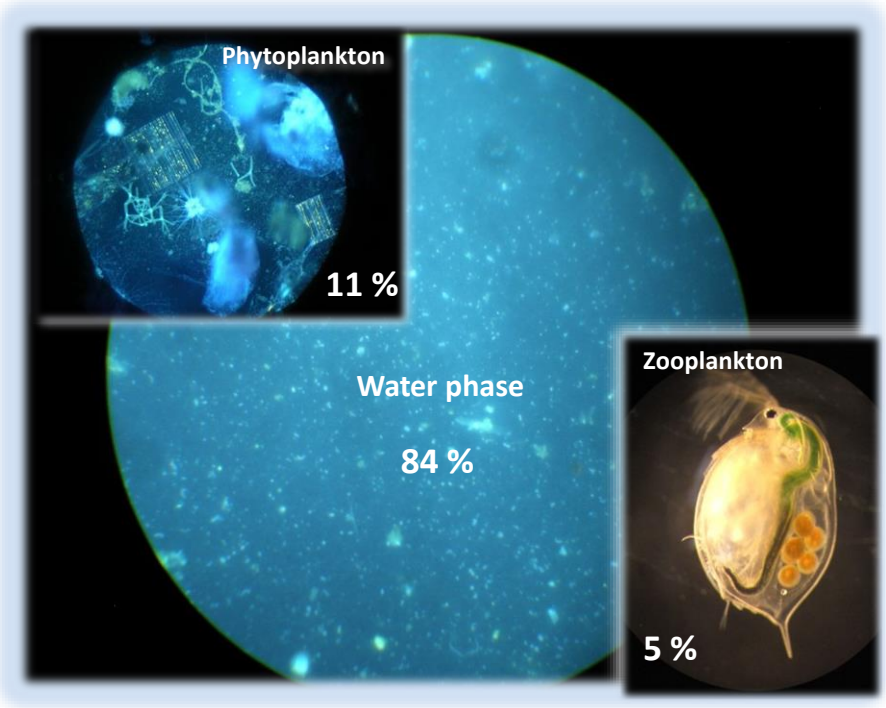


Figure 3.6 Distribution of total cell counts (in %) in the free water, phytoplankton and zooplankton in Lake Baldeney in the month August. (Microscopic pictures by Miriam Tewes)

3.3.2 Detection of organisms of faecal origin in surface water and plankton

Organisms of faecal origin including total coliforms, *E. coli*, intestinal enterococci and *C. perfringens* were determined in surface water and plankton samples. Determination occurred with cultural methods, concentrations are illustrated in Figure 3.7.

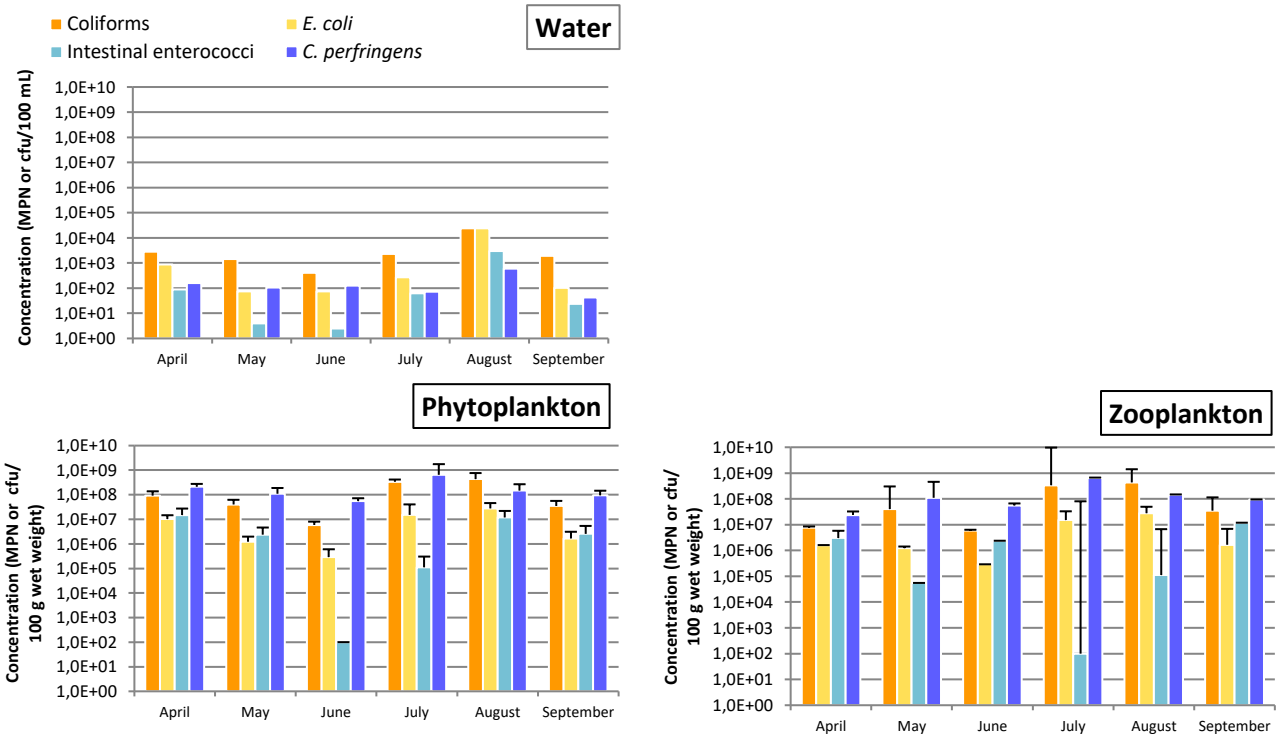


Figure 3.7: Concentrations of organisms with faecal origin.
Concentrations of total coliforms, *E. coli*, intestinal enterococci and *C. perfringens* determined detected by cultural methods in water and plankton samples over a period of six month (n = 6). Bars of plankton samples show mean values from three transects.

The results indicated that plankton displayed higher cell densities of organism with faecal origin compared to the overlying water. In the seasonal course of the six sampling dates, the warmer month July and August showed slightly elevated concentrations.

Geometric mean values calculated for phyto- and zooplankton (Figure 3.8) indicate no differences in bacterial quantities compared to each other. In comparison to the water column the concentrations of plankton samples were enhanced up to six orders of magnitude.

All organisms of faecal origin could be detected in all water as well as plankton samples. Between phyto- and zooplankton samples there are no major differences in the determined concentrations. On theoretical basis the concentrations of the faecal indicator organisms with plankton are higher in comparison to water, which is obvious in the comparison of the geometric mean values in Figure 3.8.

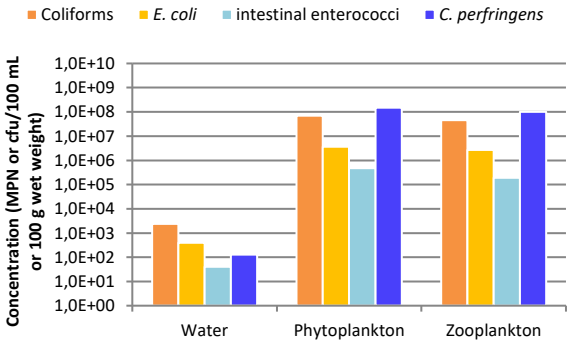


Figure 3.8 Geometric mean values of concentrations of organisms of faecal origin (coliforms, *E. coli*, intestinal enterococci, *C. perfringens*) in water and plankton samples (n = 6)

If plankton samples were referred to the sampled water volume they reflect only a minor fraction of the coliforms, *E. coli* an intestinal enterococci when they were compared to the free water phase of Lake Baldeney except for August and September (Figure 3.9). *C. perfringens* is found in similar concentrations in May and June. However, the values exceeded the amount compared to other sampling months.

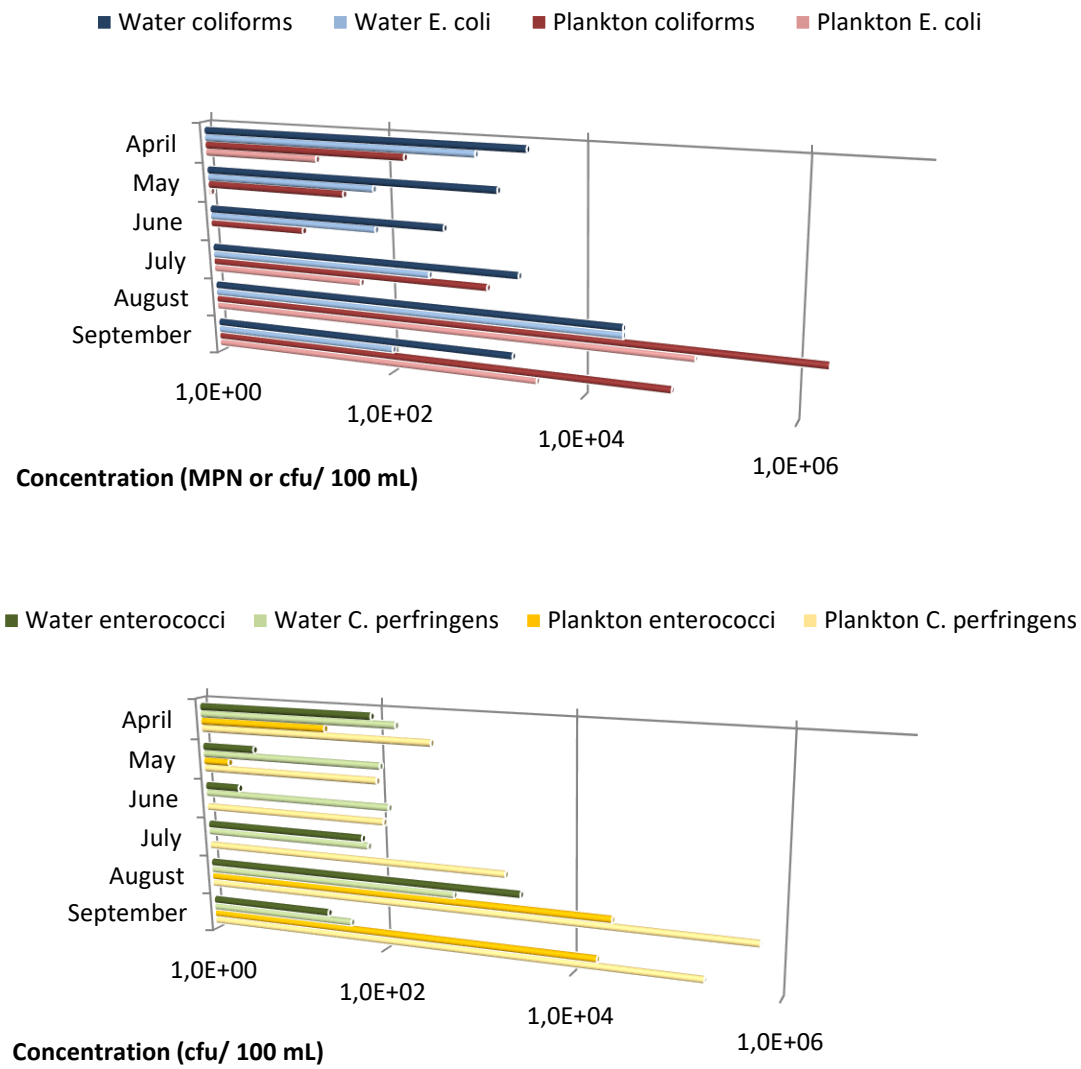


Figure 3.9 Concentrations of organisms with faecal origin in water and plankton samples per 100 mL. Results for plankton were referred to the sampled volume of water which was filtered through the plankton net while collecting plankton (1250 L for phytoplankton and 9000 L for zooplankton)

The results indicated that plankton displayed higher cell densities referred to the wet weight of plankton which represents the high local population of faecal indicator on the plankton organisms. The bacterial concentrations on plankton compared to the situation in the entire surface water, indicate that they reflect only a minor fraction. However, the concentrations of *C. perfringens* were similar or exceeded those in in water during the sampling period. Concentrations of total coliforms, *E. coli* and intestinal enterococci were higher than those in the free water in the month of August and September.

In addition to the quantification of coliforms and intestinal enterococci, the systems API® 20 E and API® rapid ID 32 were used respectively to evaluate the presence of clinically important species. Therefore selected isolates of coliforms and intestinal enterococci from four sampling dates (June, July, August and September 2010) were tested. Identification of coliforms and intestinal enterococci reflected a variety of species (Table 3.7).

Table 3.7 Identified species of coliforms and intestinal enterococci in water and plankton samples of four exemplary sampling dates (June- September 2010). In brackets number of samples which were positive for the species identified. (n.d., not determined)

	Coliforms	**Intestinal enterococci**
June		
Water	*Citrobacter coseri/farmeri* (1) *Enterobacter cloacae* (2) *Klebsiella pneumoniae* (1)	*Enterococcus casseliflavus* (4)
Phytoplankton	*Escherichia coli* (5)	n.d.
Zooplankton	n.d.	n.d.
July		
Water	*Escherichia coli* (3) *Klebsiella pneumoniae* (1)	*Enterococcus casseliflavus* (1) *Enterococcus faecium* (3) *Enterococcus hirae* (4)
Phytoplankton	*Enterobacter cloacae* (6) *Escherichia coli* (6) *Klebsiella oxytoca* (1)	n.d.
Zooplankton	*Escherichia coli* (5)	n.d.
August		
Water	*Enterobacter sakazakii* (1) *Escherichia coli* (5) *Klebsiella pneumoniae* (5)	*Enterococcus casseliflavus (*2) *Enterococcus durans* (1) *Enterococcus faecalis* (5) *Enterococcus gallinarium (*2) *Enterococcus hirae* (4)
Phytoplankton	*Enterobacter cloacae* (4) *Escherichia coli* (2) *Klebsiella pneumoniae* (5)	n.d.
Zooplankton	*Citrobacter freundii (*1) *Enterobacter cloacae* (1) *Enterobacter sakazakii* (1) *Escherichia coli* (2) *Klebsiella oxytoca* (1) *Klebsiella pneumoniae* (3)	n.d.
September		
Water	*Escherichia coli* (7) *Klebsiella oxytoca* (2) *Klebsiella pneumoniae* (4) *Kluyvera spp*. (2)	*Enterococcus casseliflavus* (3) *Enterococcus durans* (1) *Enterococcus faecalis* (1) *Enterococcus faecium* (8) *Enterococcus gallinarium* (1) *Enterococcus hirae* (1)
Phytoplankton	*Enterobacter cloacae* (4) *Escherichia coli* (5)	n.d.
Zooplankton	*Citrobacter braakii* (1) *Enterobacter cloacae* (2)	n.d.

3.3.2.1 Determination of *C. perfringens* spores

In order to examine the presence of *C. perfringens* endospores the samples of water and plankton were pasteurized (80°C, 10 min.). The amount of spores of four sampling dates (June, July, August and September 2010) was determined and results are illustrated in Figure 3.10.

The amount of 100 % of spores corresponds to colony counts on selective agar which include the abundance of vegetative cells and spores.

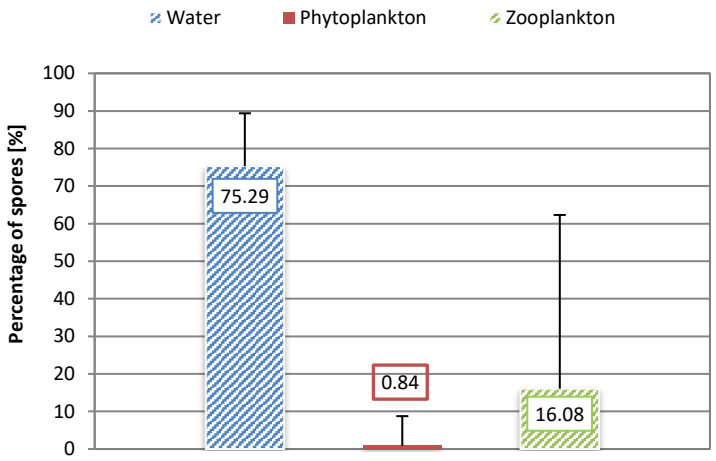


Figure 3.10 Proportion of *C. perfringens* spores.
Amount of *C. perfringens* spores determined by pasteurization (80°C, 10 min.) of water, and plankton of exemplary samples (n =4).

The highest amounts of spores were found in the water samples, whereas the results of plankton were lower with a percentage of 0.84 % for phytoplankton and 16.08 % spores in zooplankton samples.

Table 3.8 shows absolute values of vegetative *C. perfringens* cells and their spores in comparison to endospores alone which were determined by colony counts on m-CP agar after pasteurization. Although quantities of absolute *C. perfringens* concentrations in plankton samples exceeded those of water by two to four log units. Furthermore the percentage of spores was the highest in water.

Table 3.8 Arithmetical mean values of colony counts of vegetative *C. perfringens* cells and endsospores in water and plankton samples

C. perfringens	Water (cfu/100 mL)	Phytoplankton (cfu/100 g)	Zooplankton (cfu/100 g)
Vegetative cells and endospores	2.1×10^2	5.5×10^6	1.0×10^5
Endospores	1.6×10^2	4.6×10^4	1.6×10^4

Presumably *C. perfringens* undergo better nutrient conditions when existing in association to plankton and therefore develop endospores when living in the water column due to environmental stress such as starvation.

3.3.3 Detection of the human pathogen *Campylobacter* spp.

As a member of obligate human pathogens the presence of the organism *Campylobacter* spp. was investigated in water and plankton. Detection occurred qualitatively and isolates of positive samples were analyzed with the API® Campy identification system. Species that were identified (Table 3.9) included *Campylobacter coli* which was found in three out of six water samples and the species *Campylobacter jejuni* which was obtained in one out of six water samples. In plankton samples the presence of *Campylobacter* spp. could not be confirmed.

Table 3.9 *Campylobacter* species identified from six water, phyto- and zooplankton samples. In brackets, number of samples which were positive for the species identified.

Sample	**Identified *Campylobacter* species**
Water	*Campylobacter coli* (3) *Campylobacter jejuni* (1)
Phytoplankton	-
Zooplankton	-

These findings indicate that *Campylobacter* spp. seem to prefer the free water phase as environment, or that they might exist in the VBNC state when they are associated with plankton organisms.

3.3.4 Detection of environmental pathogens in surface water and in association with plankton

The concentrations of the opportunistic pathogens *Aeromonas* spp, *P. aeruginosa* and *Legionella* spp. were determined with cultural methods. The concentrations of the pathogens in surface water and plankton are illustrated in Figure 3.11.

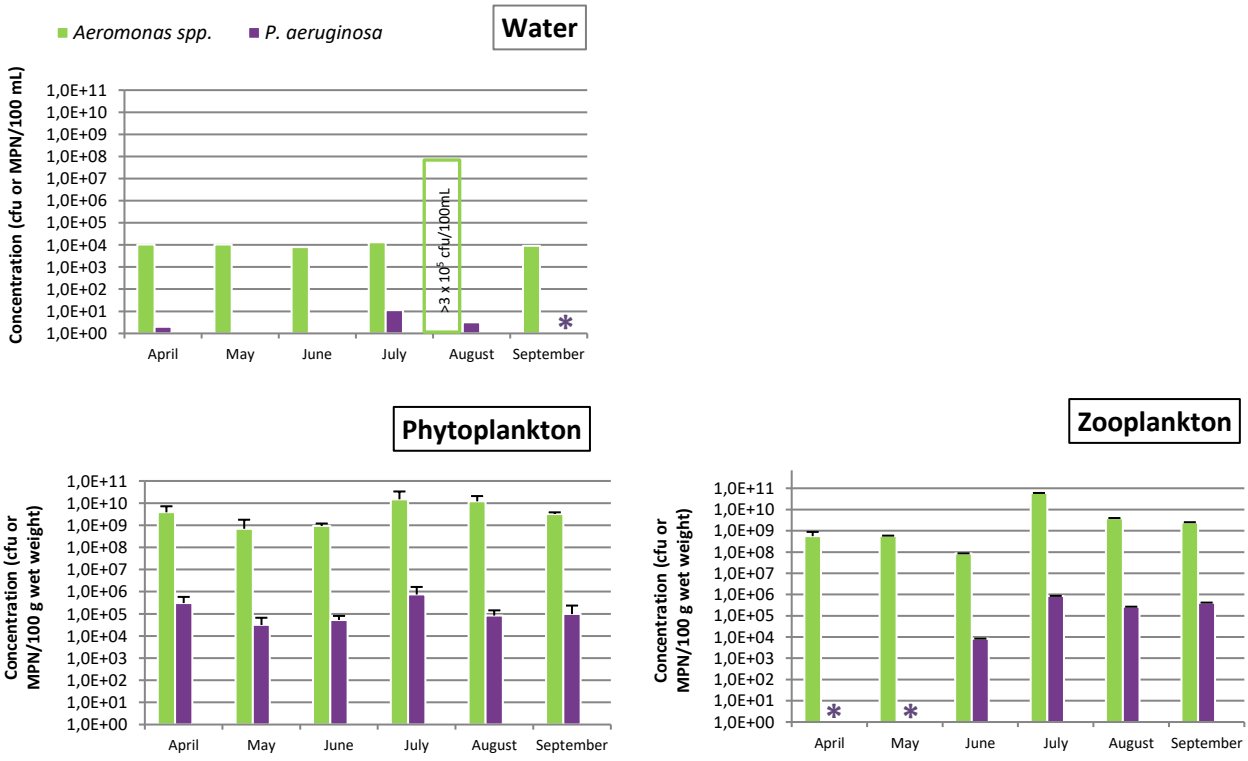


Figure 3.11 Concentrations of *Aeromonas* spp, and *P. aeruginosa* in surface water and plankton samples.
Determination with cultural methods on selective agar over a period of 6 month. Bars of plankton samples show mean values from three transects. (* below detection limit).

The presence of *Aeromonas* spp. in water samples was up to five orders of magnitude lower concentrations compared to the overlying water column and remained constant during the sampling season. In plankton samples an increase of concentration could be observed in July, especially for zooplankton, also in August and September. The determination of *P. aeruginosa* revealed low concentrations in water samples and was below the detection limit in September. In plankton samples the concentration of *P. aeruginosa* was theoretically higher compared to water. Except for July where the determined concentration reached a maximum in all samples, there are no major changes over the sampling period.

In zooplankton samples *P. aeruginosa* was observed also two times below the detection limit which was calculated in relation to the determined plankton mass with 6.5 x 10^4 MPN/100 g wet weight and 1.7 x 10^5 MPN/100 g wet weight.

The geometric mean values (Figure 3.12) show that the local density of the two investigated opportunistic pathogens on plankton, particularly on phytoplankton, was higher compared to the water column.

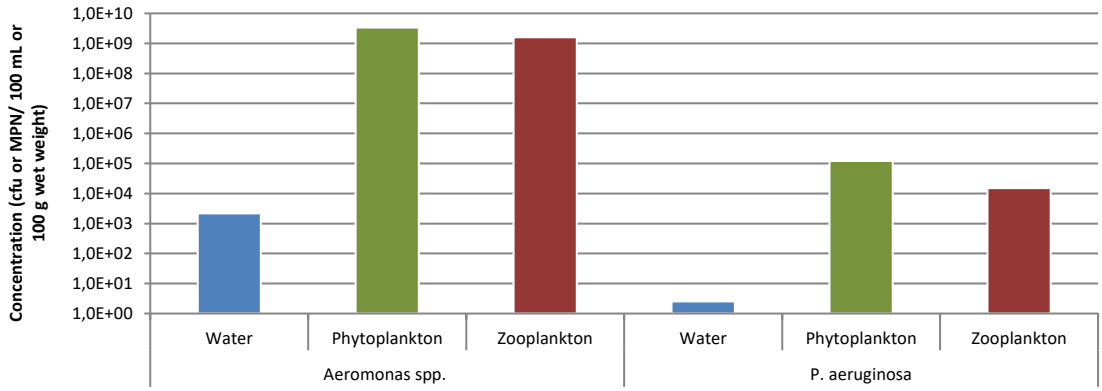


Figure 3.12 Geometric mean values for *Aeromonas* spp. and *P. aeruginosa* in water and plankton samples (n = 6)

If plankton samples were referred to the sampled water volume the concentrations of *Aeromonas* spp. and *P. aeruginosa* showed compared to the free water less differences in the months April till June (Figure 3.13). *Aeromonas* spp. quantities in plankton samples exceeded those in water in July, August and September. The same could be detected for *P. aeruginosa* in August and September.

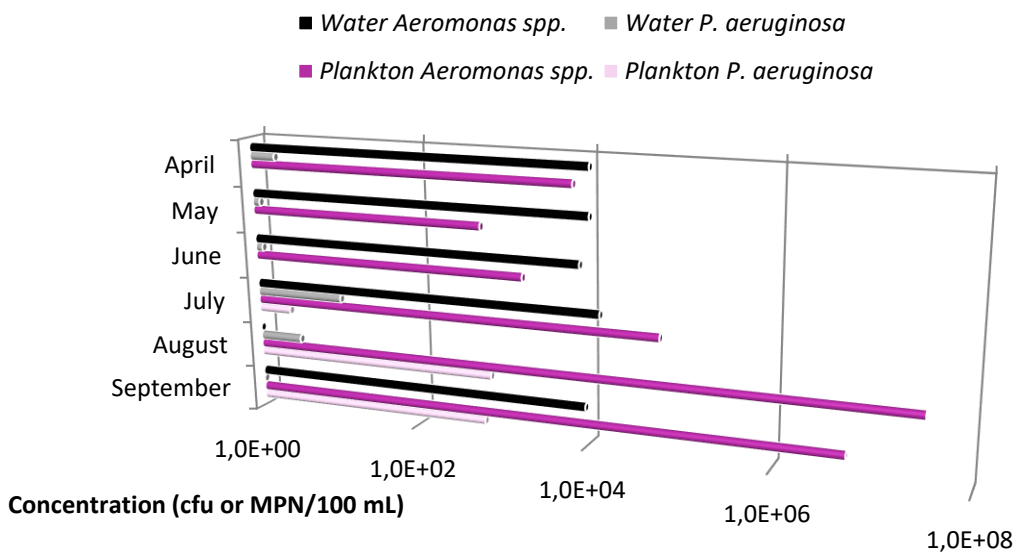


Figure 3.13 Concentrations of *Aeromonas* spp. and *P. aeruginosa* in water and plankton samples per 100 mL. Results for plankton were referred to the sampled volume of water which was filtered through the plankton net while collecting plankton (1250 L for phytoplankton and 9000 L for zooplankton)

When bacterial concentrations of all samples on plankton were referred to the total abundance in the aquatic environment the findings were different compared to those referred to the plankton wet weight. The plankton microhabitats represent enhanced concentrations of pathogens, although they account only for a minor fraction in the entire surface water. Remarkable are the elevated concentrations in the samples of August and September where the quantities exceeded those in water for instance *Aeromonas* spp., coliforms, *E. coli*, enterococci and *C. perfringens.*

Moreover 13 isolates of *Aeromonas* spp. from water and plankton samples were analyzed by 16S rDNA sequencing (Table 3.10). The analysis identified 11 isolates as *A. hydrophila* with a probability of 94 – 99%. This species showed homology to ATCC type strain 7966. Furthermore, one species isolated from phytoplankton was identified as *A. salmonicida* (probability 96%) and one isolate could not be identified and is nominated as 'none'.

Table 3.10 Aeromonas species identified by 16S rDNA sequencing analysis of 13 isolates from surface water and plankton. In brackets, number of samples which were positive for the species identified

Sample	**Identified *Aeromonas* species**
Water	*Aeromonas hydrophila* (5)
Phytoplankton	*Aeromonas hydrophila* (2) *Aeromonas salmonicida* (1) 'None' (1)
Zooplankton	*Aeromonas hydrophila* (4)

Legionella spp. was never detected neither in water nor in plankton over the whole sampling period of six months with cultural methods. Detection limits for *Legionella* spp. were calculated on the basis of the assumption that at least 1 cfu/100 mL or 100 g plankton might have been detected. Hence, the detection limit in water was 1.0 cfu/100 mL. Detection limits on plankton were calculated in relation to the sampled plankton mass and were 3.7 x 10^4 cfu/100 g wet weight for phytoplankton and 2.2 x 10^5 cfu/100 g wet weight of zooplankton.

3.3.5 Distribution of pathogens in a filtered water sample

In order to determine if there is a significant difference of bacterial abundance in a water sample compared to a plankton-free water sample, at three sampling dates water was filtered through a plankton net (mesh size 55 µm) and therefore considered as plankton-free.

The exemplary investigation of total cell counts, heterotrophic plate counts, coliforms and *E. coli* in plankton-free water showed no differences compared to the water samples (data not shown) and was therefore neglected in further sampling.

3.3.6 Detection of target organisms with culture independent methods

Since many opportunistic pathogens, such as *P. aeruginosa* and *Legionella* spp., are known to enter the viable but nonculturable state, it is of interest to determine their number with molecular methods, like FISH and quantitative PCR, additionally to cultural methods.

Figure 3.14 shows results for *P. aeruginosa, Legionella* spp. and *L. pneumophila* quantified with qPCR in water and plankton in comparison to total cell counts and culturable HPC bacteria.

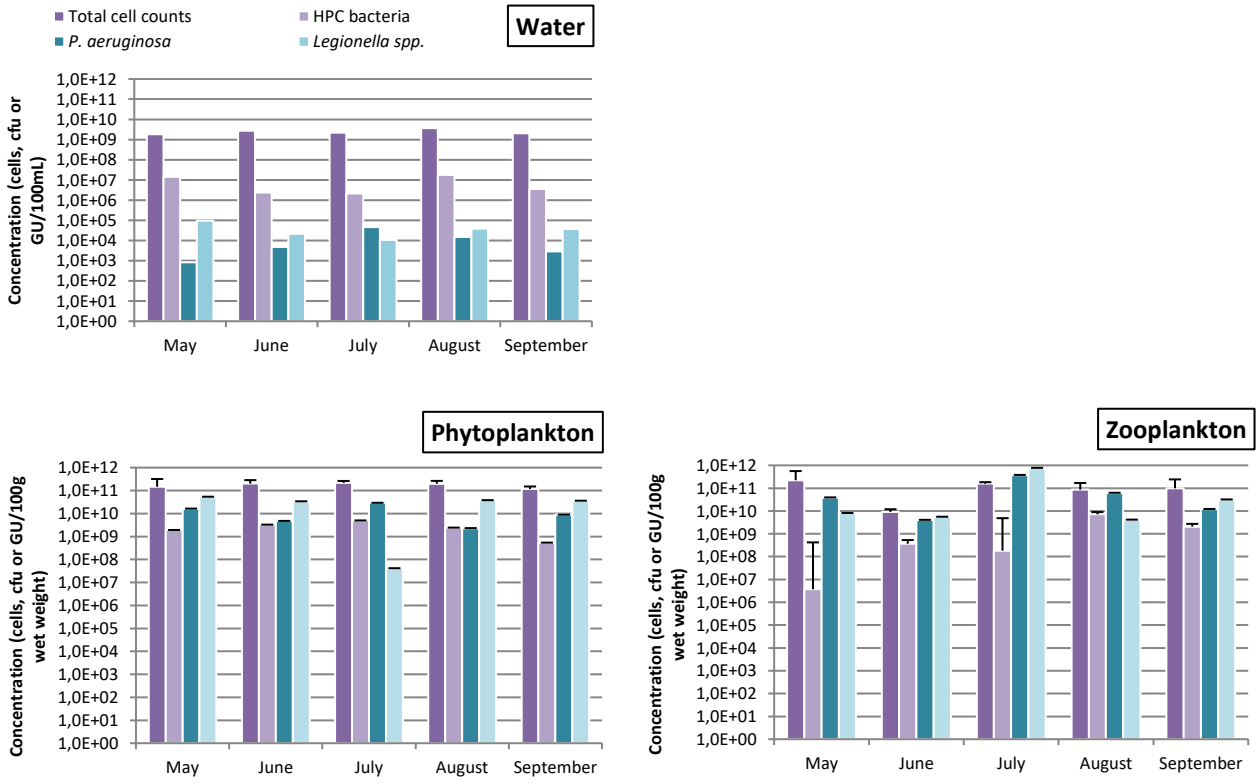


Figure 3.14 Quantification of *P. aeruginosa* and *Legionella* spp. in water and plankton samples with q PCRin comparison to total cell counts (TCC) and colony counts (HPC bacteria).
Bars of plankton samples show mean values of three transects (n = 5) (GU = genomic units).

The results show that by the use of culture independent methods the determined bacterial concentrations of *P. aeruginosa* and *Legionella* spp. were higher in both water and plankton samples compared to the quantities of cultural detection. *Legionella* spp. was never detected with cultural methods, but in high densities with qPCR. If the concentrations of the pathogens in water and those associated with plankton are assumed equal for hypothetical comparison, the local densites of the bacteria asoociated with plankton were magnitudes higher than those of bacteria

living in the water column. A slightly peak was also notable in zooplankton in comparison to phytoplankton samples, especially in the month July.
If qPCR results are compared to total cell counts, the difference displays about five orders of magnitude in water and about 2 orders of magnitude in plankton samples.

FISH-results of the opportunistic pathogens *P. aeruginosa*, *Legionella* spp. and *L. pneumophila* could be detected and quantified in all water samples during the six month of sampling. Concentrations of the three organisms were around 10^6 cells/100 mL of water (Figure 3.15).

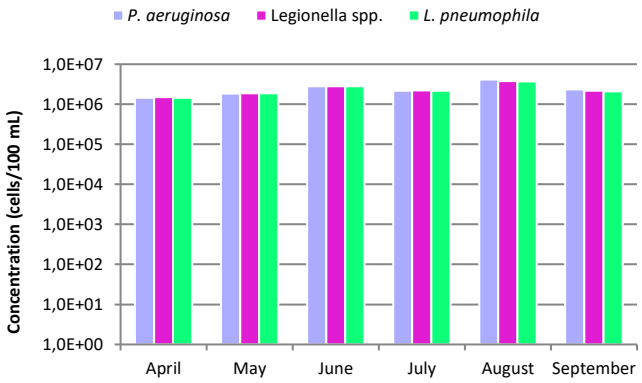


Figure 3.15 Concentrations of FISH positive cells of the opportunistic pathogens *P. aeruginosa* (probe Psae 16S-182), *Legionella* spp. (probe LEG705) and *L. pneumophila* (LEGPNE1) in water samples (n = 6)

In Figure 3.16 the comparison of cultural and molecular detection of *P. aeruginosa*, *Legionella* spp. and *L. pneumophila* in water samples is shown.

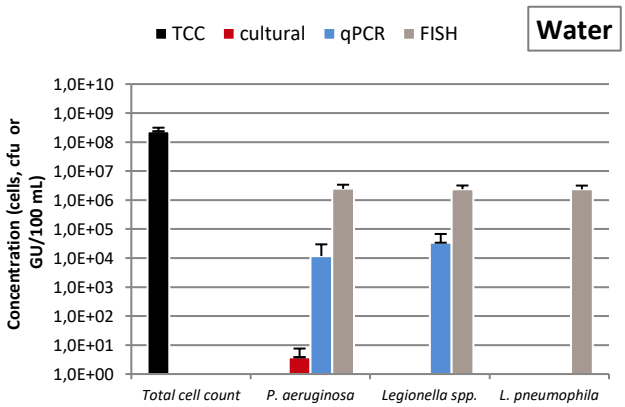


Figure 3.16 Comparison between cultural and molecular methods of the water samples.
Total cell count of the water samples and concentration of *P. aeruginosa* using cultivation methods (enrichment in malachite green broth), FISH (probe Psae-16S – 182) and qPCR (Taqman probe), *Legionella* spp. using cultivation (GVPC-Agar) and FISH (probe LEG 705) and qPCR (FAM-490) and *L. pneumophila* using cultivation (GVPC-Agar), FISH (probe LEGPNE 1) and qPCR (FAM-490). (n = 6 for total cell counts, cultural detection and FISH; n = 5 for qPCR).

The results show that the molecular methods revealed higher concentrations with up to 2 log units with qPCR and up to 5 log units with FISH compared to cultural detection for *P. aeruginosa*. *Legionella* spp. and *L. pneumophila* which were not detected with cultural methods, were found in high concentrations by use of culture independent methods.

3.3.7 Microorganisms associated with the macrophyte *Elodea nuttallii*

In aquatic environments there are different surfaces available for bacteria to attach and for biofilm formation. Due to the massive growth of the macrophyte *Elodea nuttallii* in the year 2009 it was obvious to investigate whether there is an accumulation of bacteria on the surface of the waterplant. Figure 3.17 shows the overall bacterial abundance with total cell counts and heterotrophic plate counts associated with the macrophyte compared to planktonic bacteria in the water phase.

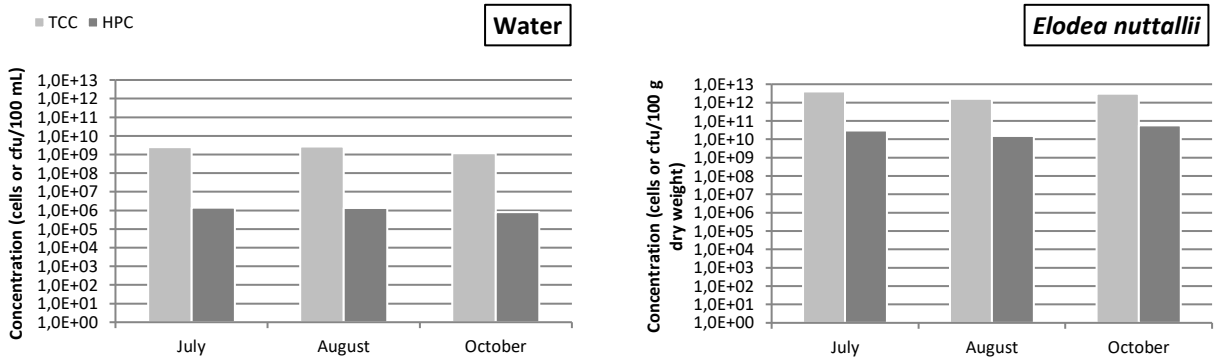


Figure 3.17 General bacterial abundance in surface water and on *Elodea nuttallii*.
Total cell counts (TCC) and colony counts (HPC) in water and on the macrophyte *Elodea nuttallii* (n = 3).

Water temperature during *Elodea* sampling ranged between 21.3 °C in July, 22.5 °C in August and 11.3 °C in October.

The bacterial abundance, determined by total cell counts and heterotrophic plate counts, was enhanced with one to three orders of magnitude by presence of *E. nuttallii* compared to the results of the overlying water. This indicates that the nutrient supply by the macrophyte is favourable for bacterial attachment.

In Figure 3.18 the concentrations of the organisms with faecal origin, thus total coliforms, *E. coli*, intestinal enterococci, *C. perfringens* and the amount of the opportunistic pathogen *Aeromonas* spp. are shown in water samples and samples with *Elodea nuttallii*.

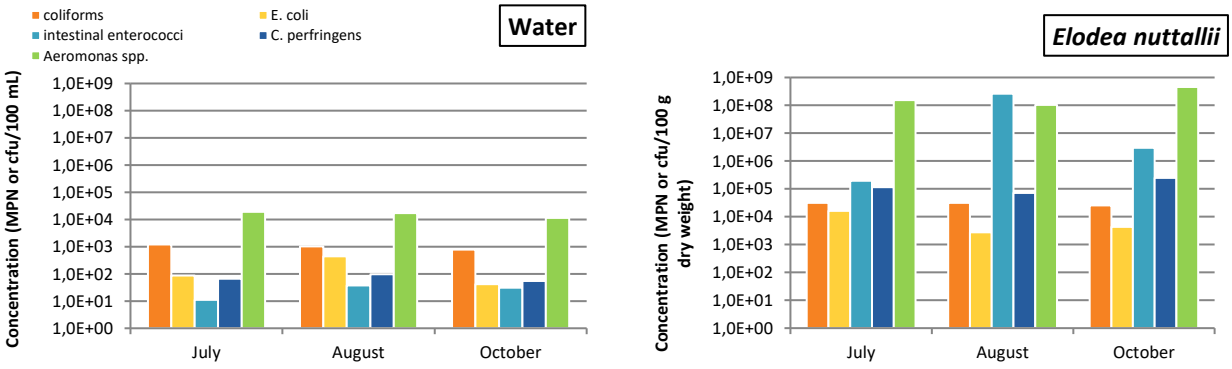


Figure 3.18 Concentrations of organisms with faecal origin (coliforms, *E. coli*, intestinal enterococci, *C. perfringens)* and the pathogen (*Aeromonas* spp.) in surface water and on *Elodea nuttallii*. Determination of the pathogens occurred with cultural methods, samples were taken in July, August and October (n = 3). Results are given per 100 mL of water and per 100 g of dry weight for *Elodea nuttallii*.

Densities of the investigated organism were found elevated in association with *Elodea nuttallii* compared to the water. The group of intestinal enterococci seemed to prefer the interaction with the macrophyte, notably in August where the concentration was about seven orders of magnitude higher than in water sample. The organisms *Aeromonas* spp. and *C. perfringens* showed also increased concentrations on the macrophyte compared to water with a difference of three to four log units.

As an important member of obligate human pathogens, *Campylobacter* spp. was investigated in water samples and in association with *Elodea nuttallii*. Identification of positive samples with the API® Campy identification system confirmed the presence of *Campylobacter coli* in one of the three macrophyte samples, but never in water samples.

Hygienically relevant organisms were present in the water column as well as in samples associated with the macrophyte *Elodea nuttallii*. When milliliters of water and grams of dry weight of the waterplant are assumed equal for hypothetical comparison, there is a notable accumulation of bacteria on *Elodea nuttallii*.

The comparison of bacterial abundencies determined on *Elodea nuttallii* are compared to those in plankton samples, indicated an enhancement in total cell counts as well as HPC bacteria around one order of magnitude (Figure 3.19).
Organisms with faecal origin as well as *Aeromonas* spp. were also found in higher concentrations in plankton samples, except for intestinal enterococci which where determined with higher quantity on *Elodea*.

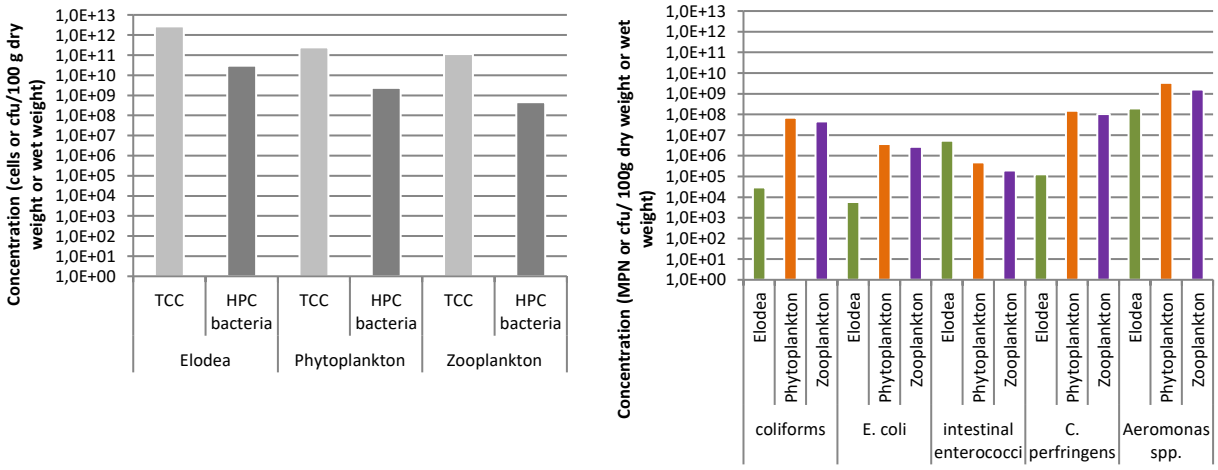


Figure 3.19 Geometric mean values of bacterial abundencies (total cell counts and HPC bacteria, left) and organisms with faecal origin (coliforms, *E. coli*, intestinal enterococci, *C. perfringens*, right) as well as the opportunistic pathogen *Aeromonas* spp. determined in *Elodea nuttallii* samples (n = 3, given per 100 g dry weight) compared to concentrations on plankton (n = 6, given per 100 g wet weight).

The macrophyte *Elodea nuttallii* seems to represent another surface for hygienically relevant microorganisms for attachment and biofilm formation or symbiosis. Where the general abundance of bacteria was found higher on the macrophyte, the organisms of faecal origin and the opportunistic pathogen *Aeromonas* spp. seem to prefer the interaction with plankton. Except for the group of intestinal enterococci, they were found in elevated concentraions on *Elodea nuttallii*.

3.4 Interaction between *Daphnia magna* and pathogens in laboratory microcosms

As an example for associations of hygienically relevant organisms with zooplankton organisms, the interaction of the opportunistic pathogens *Pseudomonas aeruginosa*, *Aeromonas hydrophila* and *Enterococcus faecalis* with the zooplankton model organism *Daphnia magna* was investigated in laboratory microcosms.

3.4.1 Determination of toxicity of *Pseudomonas aeruginosa*, *Aeromonas hydrophila* and *Enterococcus faecalis* to *Daphnia magna*

To determine bacterial toxicity on *D. magna* according to Le Codiac et al. (2012) the zooplankton organism was exposed to 4 different concentrations each of the organsims *P. aeruginosa* PAO1, *A. hydrophila* AH-1N and *E. faecalis* DSMZ 20478 over a period of up to 28 h.

This was conducted to determine a bacterial concentration that does not lead to death of daphnids which was important for the following co-cultivation experiments.

In each batch culture three daphnids were exposed to bacterial densities with an OD (600 nm) of 0.4, 0.8, 1.5, and 3. An OD_{600} of 3 corresponded to a cell density of approximately 10^9 cells/mL for all of the pathogens. Bacterial suspensions were prepared in sterile *Daphnia* medium (ADaM) and incubation occurred at room temperature.

D. magna was assumed to be dead when they were immobile, thus sinking to the bottom of the Eppendorf tubes and not swimming anymore after turning the tube upside down (Le Codiac et al., 2012).

As a control three *D. magna* individuals were cultivated per Eppendorf tube containing sterile Daphnia medium (ADaM).

Results of toxicity are illustrated in Figure 3.20. With increasing bacterial concentration a faster death of daphnids occurred.

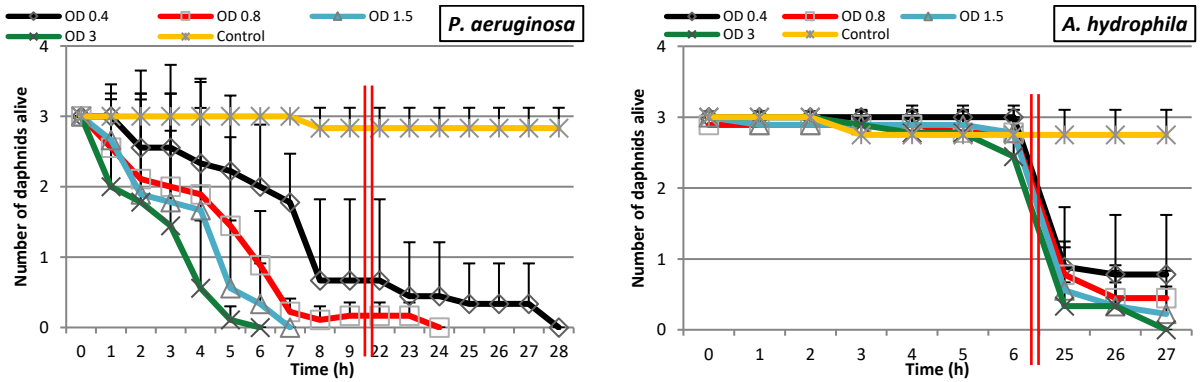


Figure 3.20 Toxicity of *P. aeruginosa* and *A. hydrophila* on *D. magna.*
In each Eppendorf tube three *D. magna* were exposed to 1mL bacterial suspension of the pathogens *P. aeruginosa* PAO1 and *A. hydrophila* AH-1N with bacterial densities of OD_{600}= 0.4, 0.8, 1.5, and 3 for up to 28 h (n = 3). Incubation occurred at room temperature (22-24°C). As a control, three *D. magna* were incubated in sterile ADaM.

In the Eppendorf tube with an OD_{600} of 3 the three daphnids died within 6 hours, after 7 hours none of three *D. magna* were alive in the batchculture with a bacterial density of OD_{600} = 1.5. After 24 hours the tube with a bacterial concentration of OD_{600}= 0.8 yielded no *Daphnia* alive and an OD_{600} of 0.4 led to death of all three daphnids after 28 hours.

In the microcosms with *A. hydrophila* the first daphnids were found to be dead after 27 hours within the tubes with a bacterial density OD_{600} of 3. In all other tubes a few *D. magna* were still alive.

For *E. faecalis* the daphnids survived completely and no death was observed within 28 h (data not shown).

Acute toxicity on *D. magna* dependent on bacterial concentration could only be observed for *P. aeruginosa*.

3.4.2 Co-cultivation of *Daphnia magna* with *Pseudomonas aeruginosa*

Since associations between hygienically relevant microorganisms and plankton are described in literature and were approved with investigations of Lake Baldeney, this phenomenon was examined in laboratory experiments. The aim was to elucidate wheter the bacteria prefer to live free in the water phase or associated to the zooplankton organisms. In the latter case a differentiation between the proportion of organisms found on the integument or in the gut of the daphnids was carried out. This separation was conducted to determine the ratio of bacteria that were attached to the daphnids´ surface and the part which was ingested by the plankton organism. Furthermore the quantities of pathogens existing in a nonculturable state where determined with FISH.

In co-cultivation experiments *D. magna* was exposed to defined concentrations (~ 10^7 cells/mL) of bacterial suspensions. The association was determined by use of total cell counts, colony counts on selective media and with FISH at the times of 0, 24 and 48 h. Co-cultivation was performed in 6-well culture plates composed of polystyrene. Bacterial suspensions were prepared in sterile Daphnia medium (ADaM). As a control, the bacterial suspension was incubated without daphnids. Incubation occurred at room temperature between 22° and 24°C.

To observe the distribution of the organisms in batch cultures, samples of different compartments were obtained.

Compartments are:

- the ‘well biofilm’, bacterial biofilms adhering to the surface of the cultivation well
- the ‘planktonic phase’, consisting of the culture medium and loosly associated bacteria, which could be washed off (rinsing water) of the daphnids surface
- ‘Daphnia-associated’, therefore the daphnids were separated under a microscope by the use of tweezers into the gut and the carapace, including leftover entrails and the rest of the organisms´ body (Figure 3.21).

As a control, *D. magna* was incubated without bacteria and same samples were processed (data not shown). The investigated opportunistic pathogens were never detected and therefore concluded that they were not member in the natural bacterial flora of the daphnids tested in this study.

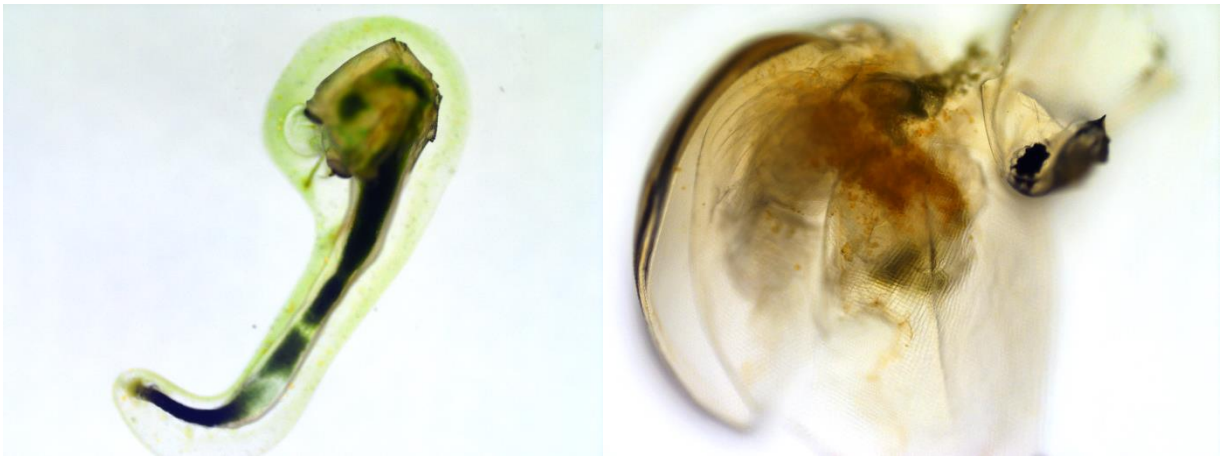


Figure 3.21 *D. magna* separated into gut (left) and the carapace with leftover entrails. (Lightmicroscopic pictures, magnification 100x, source: Miriam Tewes, Biofilm Centre, University of Duisburg-Essen)

Results of the planktonic phase are given in mL, results of *Daphnia* are given per one *D. magna* organism and results of the biofilm are calculated for the surface of one well in the 6-well plates (which was determined with 13.47 cm^2) and are given per cm^2.

The association of *D. magna* with the pathogen *P. aeruginosa* was investigated in a co-culture system. The distribution of the bacterium onto the different mentioned compartments was investigated with total cell counts (DAPI-method), colony counts on CN agar and FISH with the gene probe Psae-16S-182 (Figure 3.22). The control, thus the inoculation medium, was determined with total cell counts around 3.0 x 10^7 cells/mL and colony counts of 8.8 x 10^6 cfu/mL.

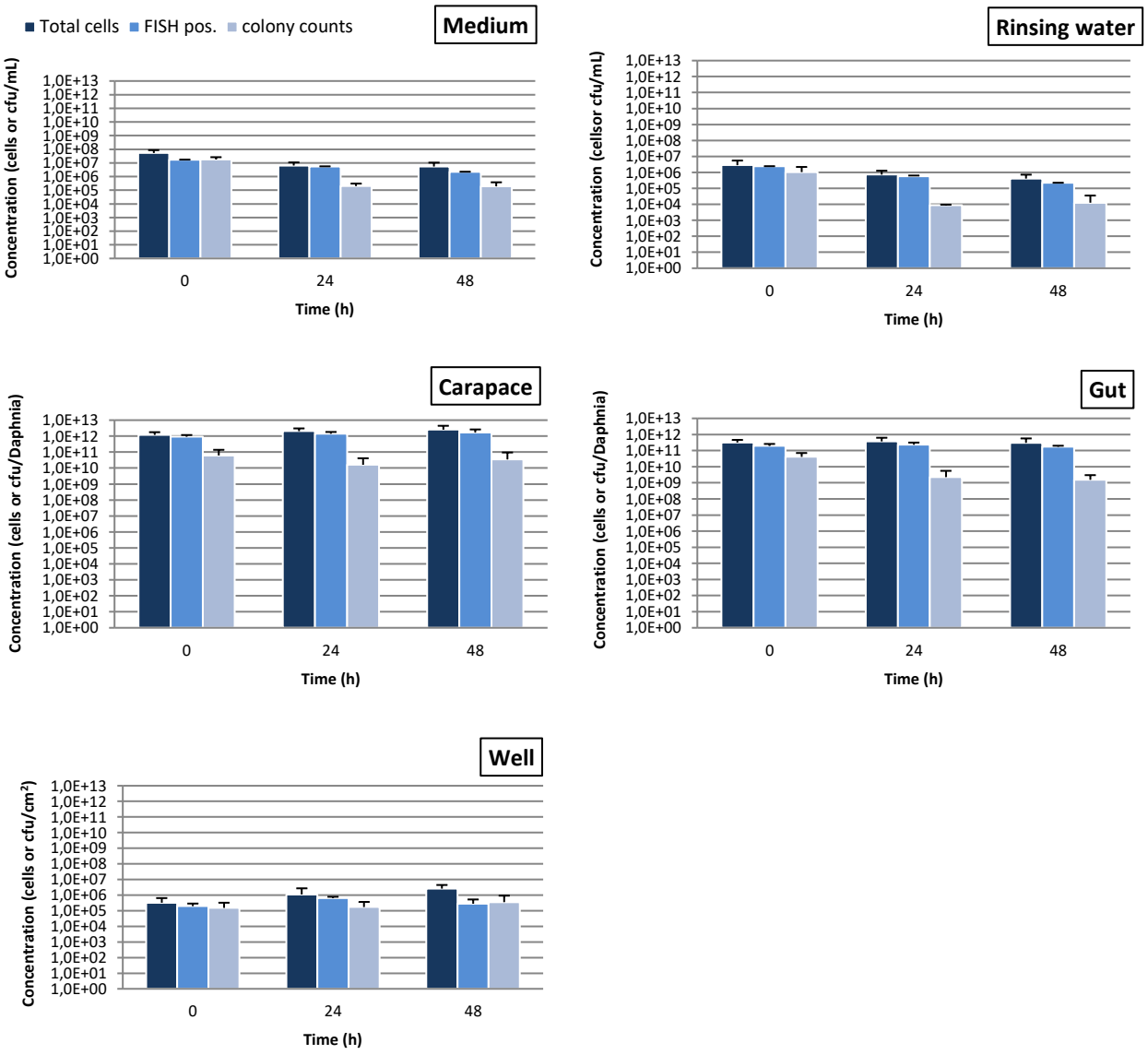


Figure 3.22 Total cell counts, determined with the DAPI method, colony counts on CN selective agar and FISH positive cells (probe PSAE-16S-182) of *P. aeruginosa* in association with *D. magna* (n =2).
In a co-culture with 5 *D. magna* in each well the distribution of the pathogen *P. aeruginosa* was observed over a time period of 48 h. Incubation occurred in ADaM medium with a bacterial concentration of 10^8 cells/mL at room temperature (22-24°C). As a control, bacterial suspension without *Daphnia*.

Total cell counts in all compartments were found to be fairly constant over the experimental period, with slight decreases in the medium and slight increases in the well biofilm. It has to be considered that total cell counts comprise the natural bacterial flora of the daphnids and the investigated organism. Culturable bacteria were detected decreasing in all compartments, except in the well biofilm where the concentrations remained constant. FISH concentrations in all compartments were comparable to total cell count quantities and exceeded the cultural cells with up to two orders of magnitude. *P. aeruginosa* was detectable in association with the daphnids as well as on the well surface within short time.

The results indicated that *P. aeruginosa* was found in all compartments during the co-cultivation experiment, whereas a fraction was not detectable with cultural methods. Attachment to the surface of the well and the *Daphnia* occurred within short time and *P. aeruginosa* was filtered by the *Daphnia* since it was found inside the gut.

The culturability decreased in all compartments over time (Table 3.11), except in the well biofilm which correlates to the increasing concentrations of colony counts.

Table 3.11 Culturability (in %) of *P. aeruginosa* in the different compartments in a co-culture with *D. magna* over a time period of 48 h (n=2)

Time	Medium	Rinsing water	Carapace	Gut	Well
0	100.0	43.2	6.7	21.0	77.5
24	3.8	1.5	1.1	1.0	26.3
48	8.6	5.7	2.1	0.9	100.0

To observe the distribution in between the different compartments in the co-cultivation system the percentages of culturable *P. aeruginosa* are shown in Figure 3.23.

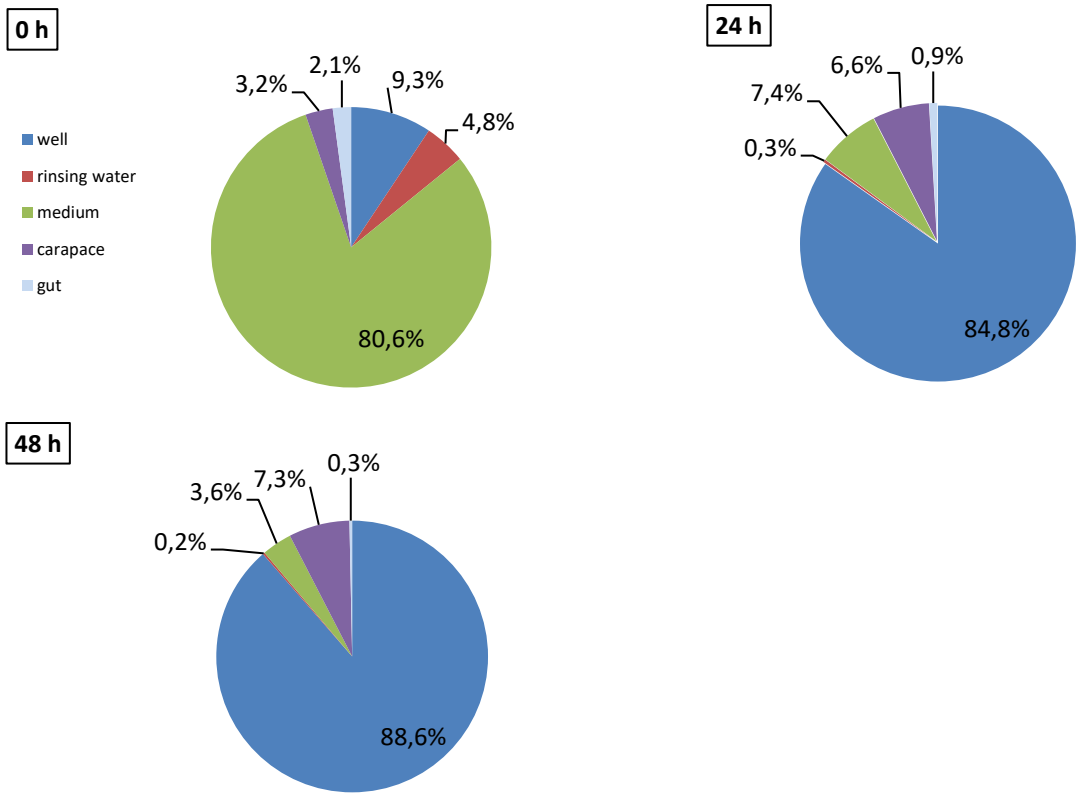


Figure 3.23 Balance of colony counts of *P. aeruginosa* in association with *D. magna.*
Distribution (in %) of *P. aeruginosa* in the different compartments of the co-cultivation system; the well, rinsing water, medium, carapace and gut. (n=2).

The major shift in the distribution of culturable *P. aeruginosa* within the co-cultivation compartments was observed within the inoculation medium, where it decreased from 80.6 % to 3.6 % onto the surface of the well, where culturable cells increased from 9.3 % to 88.6 %. *Daphnia*-associated *P. aeruginosa* showed a very slight increase in colony counts.

The distribution of FISH positive cells within the five compartments, showed that the proportion of *P. aeruginosa* is the highest in the association with the daphnids, in particular with the carapce. The concentration was continously decreasing in the planktonic compartments, the medium and the rinsing water, and increasing in association with *Daphnia* within 48 h (Figure 3.24).

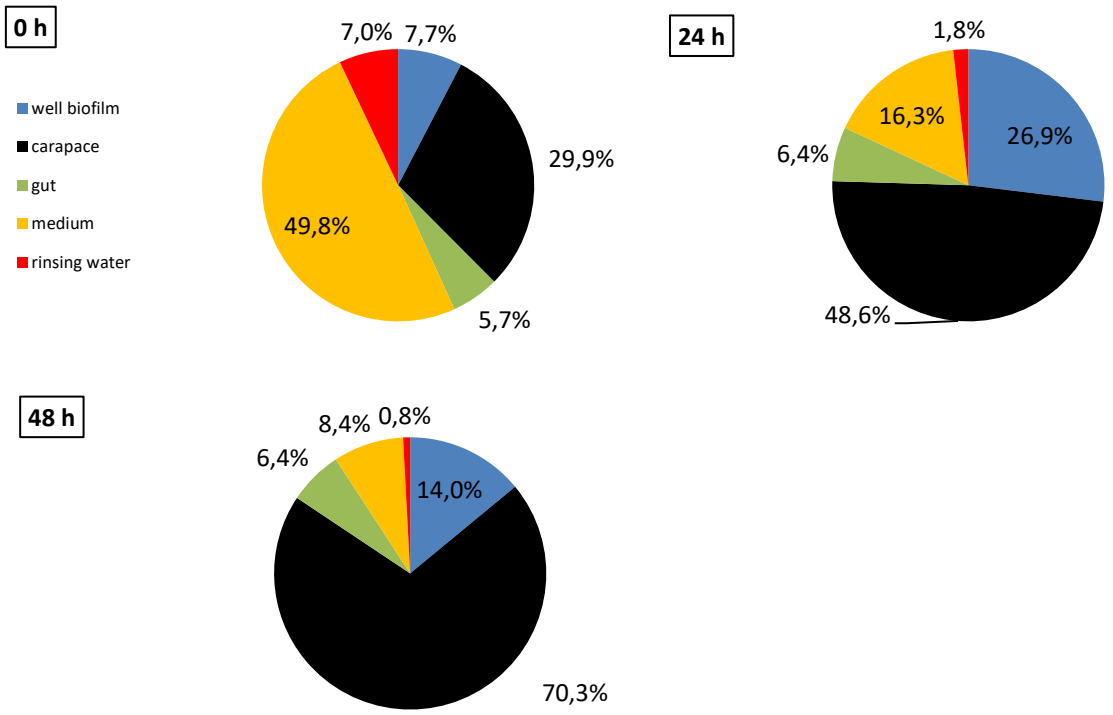


Figure 3.24 Balance: Distribution of FISH positive cells of *P. aeruginosa* in a co-cultivation experiment *with D. magna* (n = 2)

P. aeruginosa was detected in all compartments during the co-cultivation experiment, whereas a fraction was not detectable with cultural methods. Attachment to the surface of the well and the *Daphnia* occurred within short time since the organism was found inside the gut at time point 0 it has to be assumed that the organism was filtered by the *Daphnia* immediately. Co-cultivation of *P. aeruginosa* with *D. magna* indicated that within 48 h the opportunistic pathogen attached with preference to the daphnids surface and to the well of the culture plate. The culturability of *P. aeruginosa* decreased in association with the *Daphnia* and increased in the well biofilm. This might indicate that *P. aeruginosa* passed into the VBNC state in interaction with *D. magna.*

3.4.3 Co-cultivation of *Daphnia magna* with *Aeromonas hydrophila*

In a co-cultivation experiment the association of *A. hydrophila* with the zooplankton organsim *D. magna* was investigated using total cell counts, colony counts on ampicillin-dextrin agar and the FISH method (probe AERBOMO) (Figure 3.25).

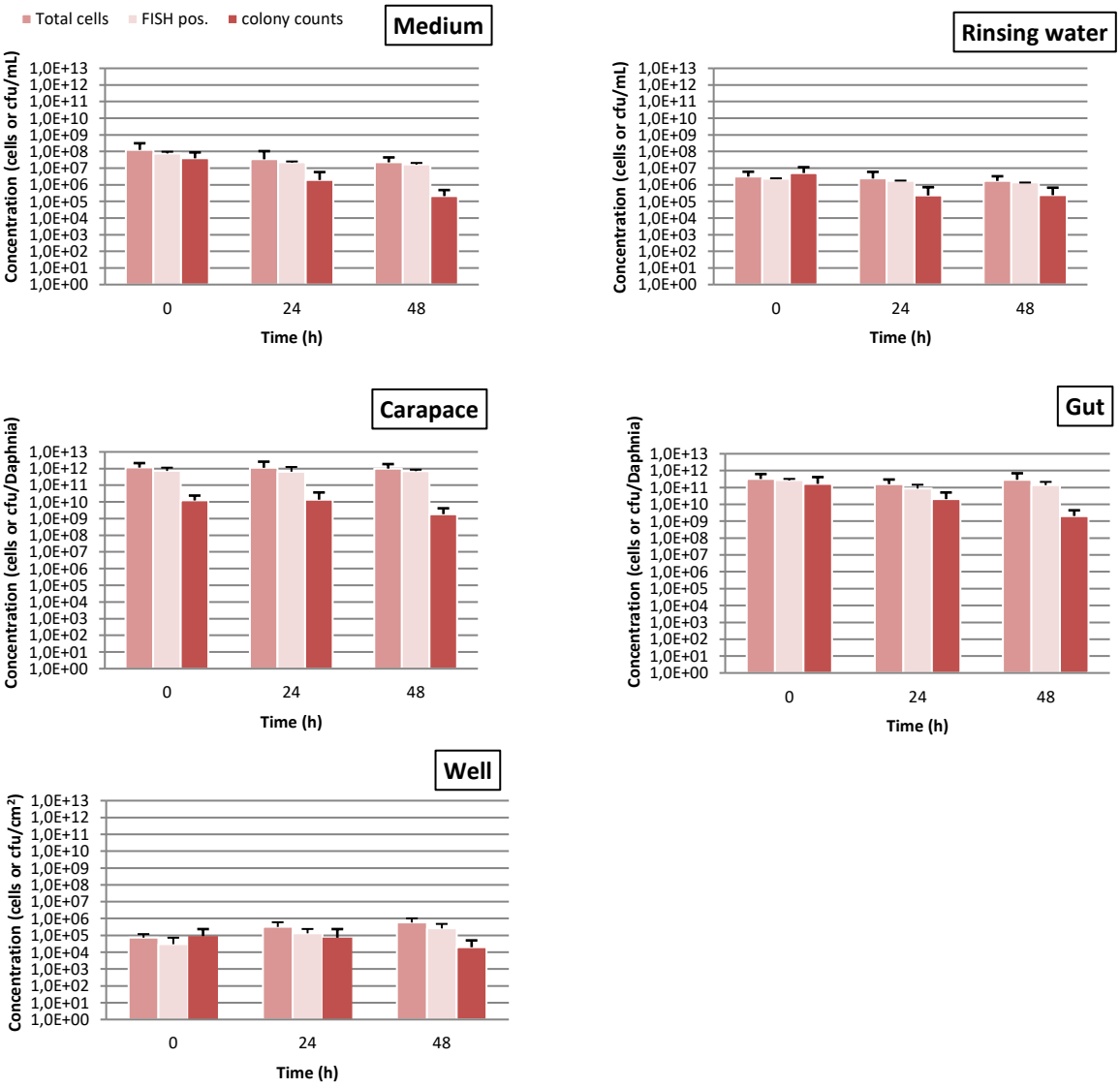


Figure 3.25 Total cell counts (DAPI method), colony counts (ampicillin-dextrin agar) (n=6) and FISH positive cells (probe AERBOMO) (n=2) of A. hydrophila in association with D. magna.
In a co-culture with 5 *D. magna* in each well the distribution of the pathogen *A. hydrophila* was observed over a time period of 48 h. Incubation occurred in ADaM medium with a bacterial concentration of 10^8 cells/mL at room temperature (22-24°C). As a control, bacterial suspension without *Daphnia*.

The control, thus the inoculation medium of *A. hydrophila* without the influence of *Daphn*ia was remaining constant over time with total cell counts of 8.4 x 10^7 cells/mL and colony counts of 8.9 x 10^7 cfu/mL.

Total cell counts as well as culturable bacteria showed decreasing tendencies in the medium, wheras the concentrations were slightly increasing in the well biofim and remained fairly constant in the rinsing water, the carapace and the gut. Concentrations of *A. hydrophila* determined with the FISH method were constant over time and about one to two orders of magnitude higher than with cultural methods. In the well biofilm, FISH positive cells were detected increasing with one log unit within 48 h.

The culturability of *A. hydrophila* in the association with *D. magna* was decreasing in all compartments over time (Table 3.12).

Table 3.12 Culturability (in %) of *A. hydrophila* in the different compartments in a co-culture with *D.*

Time	Medium	Rinsing water	Carapace	Gut	Well
0	51.2	100.0	7.7	61.0	100.0
24	8.4	13.4	2.1	23.6	54.8
48	1.3	17.4	0.3	1.5	7.7

magna over a time period of 48 h (n=6)

Percental distribution of *A. hydrophila* over the different compartments available in the co-cultivation system observed over a time period of 48 h are shown in Figure 3.26.

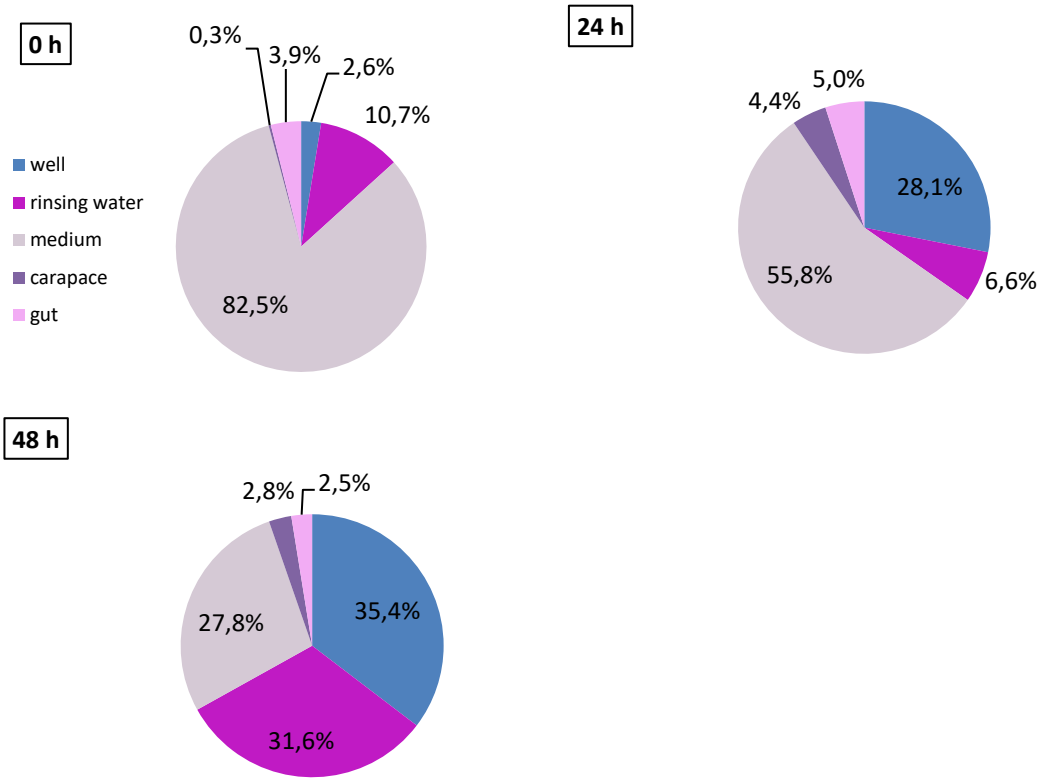


Figure 3.26 Balance of colony counts of *A. hydrophila* in association with *D. magna*. Distribution (in %) of *P. aeruginosa* in the different compartments of the co-cultivation system; the well, rinsing water, medium, carapace and gut (n=6)

Changes of culturable *A. hydrophila* in the inoculation medium of the experiment with 82. 5 % in the beginning to the compartments rinsing water (31.6 %) and the well biofilm (35.4 %) in the end of the co-cultivation. The percentage of culturable *A. hydrophila* associated with the daphnids (carapace and gut) was decreasing over time.

Aeromonas hydrophila was detected in all compartments during the co-cultivation experiment, whereas a fraction was not detectable with cultural methods. Attachment to the surface of the well and the *Daphnia* occurred within short time since *A. hydrophila* was found inside the gut at time point 0 it has to be assumed that the organism was filtered by the *Daphnia* immediately.

Similar results could be detected in *P. aeruginosa* co-cultivation experiments, with the exception ofculturability of *A. hydrophila* which was decreasing in all compartments within 48 h.

The distribution of FISH positive *A. hydrophila* in co-cultivation compartments, showed the highest in the *Daphnia* compartment (Figure 3.27). With decreasing percentages of FISH positive cells in the planktonic phase, an increase in the well biofilm and with preference in association with the carapace of *D. magna* could be detected.

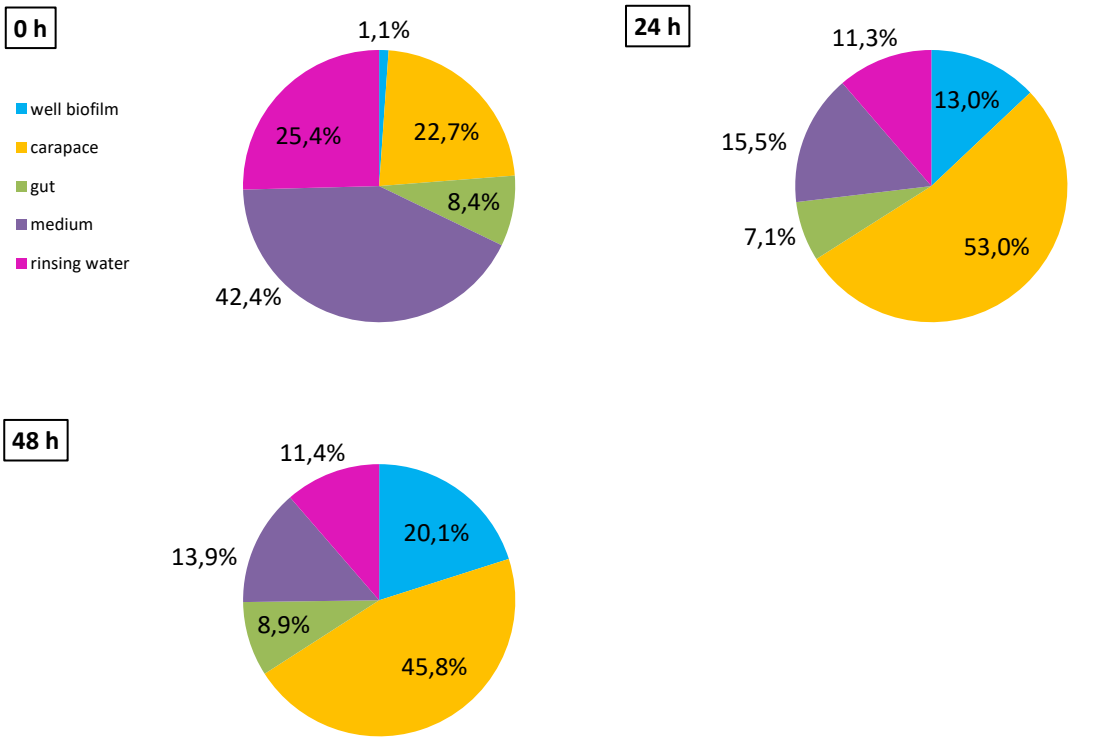


Figure 3.27 Balance: Distribution of FISH positive cells of *A. hydrophila* in a co-cultivation experiment *with D. magna* (n = 2)

Co-cultivation of *A. hydrophila* with *D. magna* indicated that within 48 h an increased attachment of the opportunistic pathogen to the daphnids surface and to the well of the culture plate were favoured. Whereas the culturability decreased in association with the *Daphnia*, it increased in the well biofilm. This might indicate that *A. hydrophila* passed into the VBNC state in association with *D. magna.* These observations were analogical to findings in *P. aeruginosa* co-cultivation.

3.4.4 Co-cultivation of *Daphnia magna* with *Enterococcus faecalis*

The comparison of total cells, colony counts and FISH results of *E. faecalis* is shown in Figure 3.28.

The control, thus the bacterial suspension without Daphnia, remained constant over 48 h with concentrations of total cells with 4.0 x 10^8 cells/mL and colony counts of 3.8 x 10^7 cfu/mL.

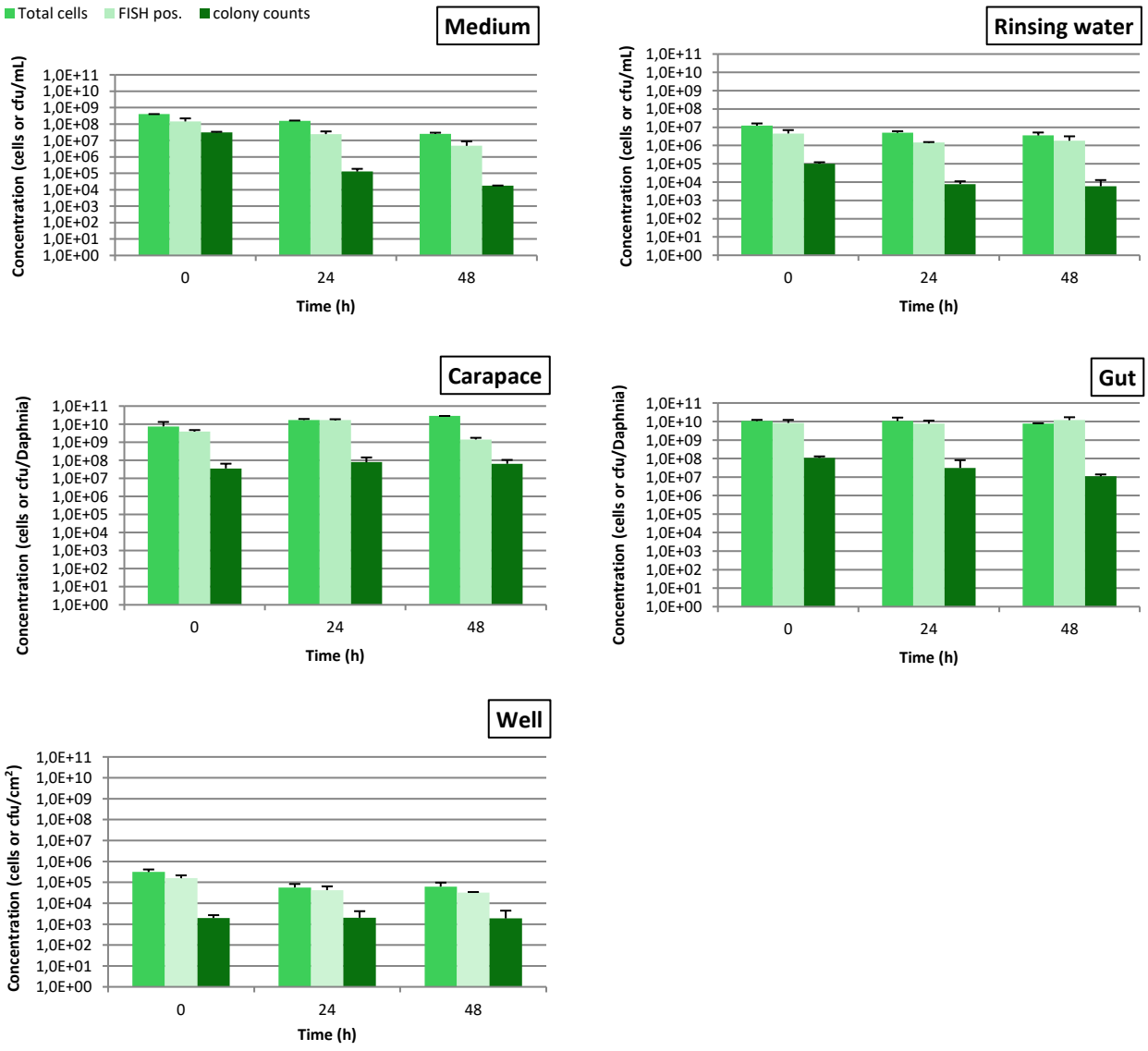


Figure 3.28 Overview of total cell counts, FISH positive cells and colony counts of *E. faecalis* in association with *D. magna*.
In a co-culture *D. magna* was observed in association with *E. faecalis* over a time period of 48 h. Determination of total cells occurred with the DAPI-method (n = 6), FISH with the gene probe Efs 130 (n = 2) and colony counts were obtained on Chromocult Enterococci Agar (n = 6).

Determination of *E. faecalis* cells in the planktonic phase which was consisting of the two compartments, medium and rinsing water, showed both a decrease in concentrations.

The two compartments consisting of *E. faecalis* associated with *D. magna* (the carapace and the gut) showed opposed variations in the bacterial concentrations. Whereas the concentration on the carapace increased, the abundance of *E. faecalis* in the gut decreased slightly.

The results indicated that the concentrations of *E. faecalis* detected by the cultural method were up to 3 log units lower than those determined by FISH. Obvious is that the concentrations in the planktonic phases are decreasing over time and slightly increase in the *Daphnia*-associated compartments, the carapace and the gut.

The culturability of *E. faecalis* in association with *D. magna* decreased in all compartments, except of the well, where the percentage of culturable cells increased slightly from 1.2 % to 5.8 % within 48 h (Table 3.13).

Table 3.13 Culturability (in %) of *E. faecalis* in the different compartments in a co-culture with *D. magna* over a time period of 48 h (n=6)

Time	Medium	Rinsing water	Carapace	Gut	Well
0	29.9	3.4	1.1	0.3	1.2
24	0.5	0.2	0.3	0.5	4.8
48	0.1	0.3	2.4	0.0	5.8

In the co-cultivation experiments of *D. magna* and *E. faecalis* the distribution of the microorganism between the different compartments in the batch cultures was compared to each other. In Figure 3.29 on the left side the percentages of total cell counts of *E. faecalis* in the medium, the washwater, the carapace, the gut and the well are shown. The circular charts on the right side indicate the distribution of the bacterium between free-living in the planktonic phase, in the biofilm attached to the wall of the well and associated with the zooplankton organism.

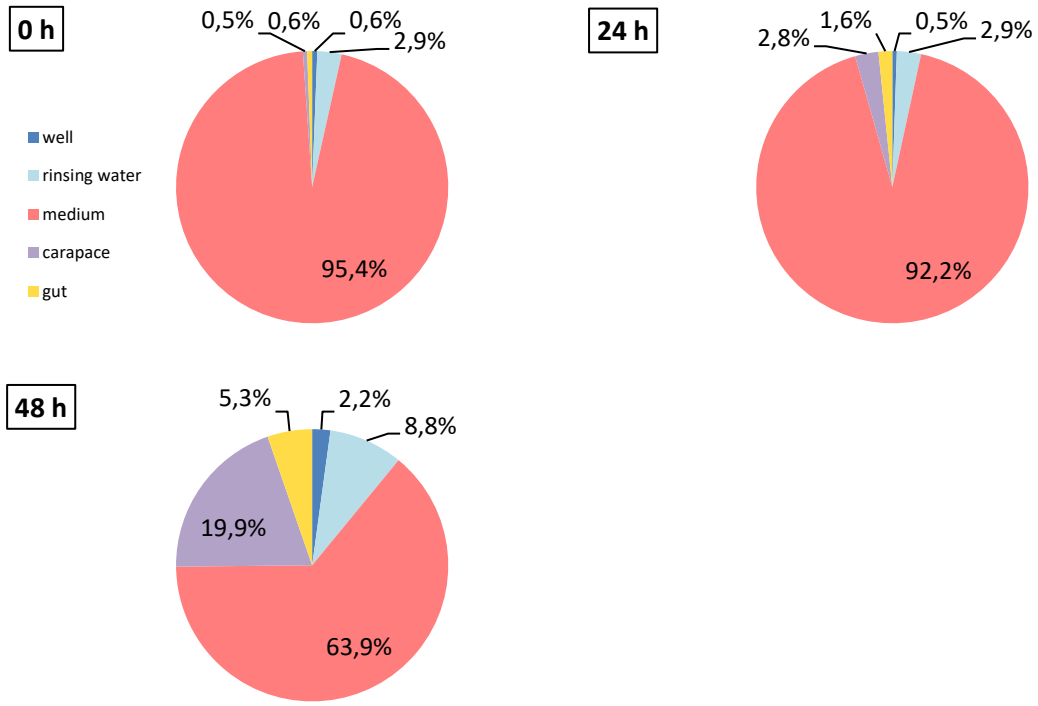


Figure 3.29 Balance of colony counts of *E. faecalis* in association with *D. magna*.
Distribution (in %) of *E. faecalis* in the different compartments of the co-cultivation system; the well, rinsing water, medium, carapace and gut (n=6)

Co-cultivation experiments resulted in a shift in the dispersal of *E. faecalis* from the inoculation medium onto the other compartments. The circular charts show that the percentage of colony counts in the medium decreased over time. The abundance of culturable *E. faecalis* on the carapace of *D. magna* increased, whereas all other compartments increased.

The distribution of FISH positive *E. faecalis* in co-cultivation compartments, showed that the proportion was the highest in the *Daphnia* compartment (Figure 3.30). When the percentage of FISH positive cells decreased in the planktonic phase, an increase in the well biofilm and in association with the gut of *D. magna* were determined.

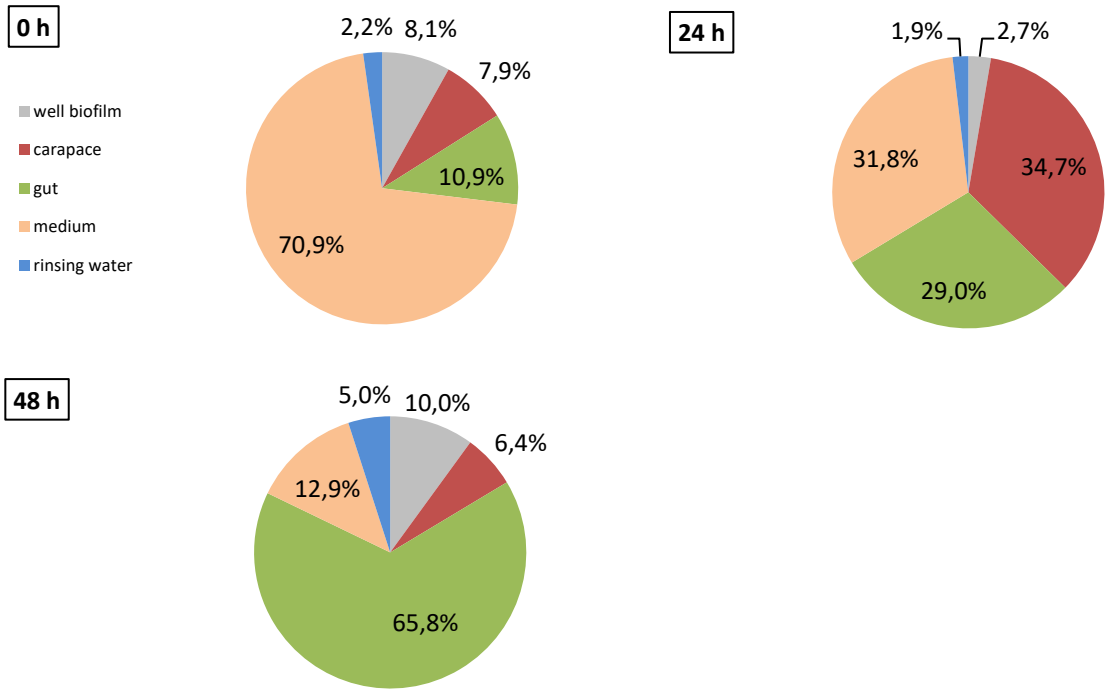


Figure 3.30 Balance: Distribution of FISH positive cells of *A. hydrophila* in a co-cultivation experiment *with D. magna* (n = 2)

E. faecalis was detected in all compartments during the co-cultivation experiment, whereas a fraction was not detectable with cultural methods. Attachment to the surface of the *Daphnia* occurred within 24 h and decreased afterwards. Accumulation in the gut was observed after 48 h. The culturability decreased in all compartments, hence *E. faecalis* seemed to undergo the transition into the VBNC state in association with the zooplankton organism.

Compared to *P. aeruginosa* and *A. hydrophila* the preferred accumulation side is different. They attached with preference to the carapace of *D. magna* or the well surface, whereas *E. faecalis* accumulated in the gut. Decreasing culturability was observed in all co-cultivation experiments which indicates the possibility of a transition into the VBNC state for all of the three organisms.

4 Discussion

4.1 Association of potentially pathogenic bacteria with plankton organisms

In the present study the occurrence of pathogenic bacteria with freshwater phyto- and zooplankton was elucidated and compared to organisms in the surrounding water column in Lake Baldeney. The investigated organisms are ubiqutious bacteria in aquatic environments with facultative pathogenic properties. The faecal indicator bacteria (e.g. *Escherichia coli*, coliforms, intestinal enterococci, *Clostridium perfringens*), obligate pathogens of faecal origin (e.g. *Campylobacter* spp.), and environmental opportunistic bacteria (e.g. some coliforms, *Pseudomonas aeruginosa*, *Aeromonas* spp., *Legionella* spp.). Furthermore microcosm experiments with *Daphnia magna* and selected organisms were applied to study possible associations in detail. The overall aim was to evaluate the role of plankton in a freshwater environment and observe interactions between pathogens and a zooplankton model organism in microcosms, considering the following questions:

(i) is plankton acting as a vector for those pathogens

(ii) is there an evidence of pathogens occuring viable but non-culturable?

4.1.1 Effects observed in Lake Baldeney

In the field study the general bacterial abundance (total cell counts, HPC bacteria) and potentially pathogenic bacteria were detected with cultural methods. Additionally determination with molecular methods, FISH and qPCR for certain selected organisms were determined in water and associated with plankton. Occurrence and proportions of phyto- and zooplankton organisms were determined.

In natural aquatic environments pathogens can enter in a viable but non-culturable (VBNC) state due to environmental stresses. The VBNC state is considered to be a survival strategy and includes the capability to remain viable, conserve their pathogenic characteristics, to increase metabolic activity and regain growth again (Kell et al., 1998; Oliver 2000; Pruzzo et al., 2002; Whiteside and Oliver, 1997). Thus the determination of pathogens with cultural methods alone will underestimate the real load of hygienically relevant bacteria. The VBNC state is described for various pathogens, such as *V. cholera, Aeromonas* spp. or *E. faeaclis,* and some report that the organisms were found attached to plankton in the VBNC state (Binzstein et al.,

2004; Heim et al., 2002; Shukla et al., 1995; Signoretto et al., 2005). Resuscitation is possible when the environmental stress factors are reduced (Kell et al., 1998). The pathogens can regain infectivity after resuscitation what was reported for *P. aeruginosa.* The organism was resuscitated after copper stress by use of a copper chelator, and regained infectivity to human lung cells (Dwidjosiswojo et al., 2011). This is siginificant to human health if the water body is used for recreational purposes or drinking water production.

To start with a quintessence, it can be mentioned that it succeeded to show that freshwater plankton organisms provide microhabitats which support attachment and proliferation of potentially pathogenic bacteria. Furthermore there are signs for bacteria occurring in a VBNC state. These findings will be discussed in detail in the following chapters. Table 4.1 shows geometric mean values of bacterial concentrations in water and plankton samples.

Table 4.1 Geometric mean values for hygienically relevant microorganisms in water and plankton (n.d.; not determined)

Parameter	Detection	Unit	Water	Phytoplankton	Zooplankton
Total cell counts	Microscopically	Cells/100 mL or 100 g	2.3×10^{7}	2.4×10^{11}	1.1×10^{11}
HPC bacteria	cultural	cfu/100 mL or 100 g	8.0×10^{4}	2.4×10^{9}	4.6×10^{8}
Coliforms	cultural	MPN/100 mL or 100 g	2.4×10^{3}	7.0×10^{7}	4.6×10^{7}
E. coli	cultural	MPN/100 mL or 100 g	3.9×10^{2}	3.7×10^{6}	2.7×10^{6}
Enterococci	cultural	cfu/100 mL or 100 g	4.0×10^{1}	7.8×10^{5}	2.0×10^{5}
C. perfringens	cultural	cfu/100 mL or 100 g	1.3×10^{2}	1.5×10^{8}	1.0×10^{8}
***Aeromonas* spp.**	cultural	cfu/100 mL or 100 g	2.2×10^{3}	3.4×10^{9}	1.6×10^{9}
P. aeruginosa	cultural	MPN/100 mL or 100 g	2.5	1.2×10^{5}	1.5×10^{4}
	FISH	Cells/100 mL or 100 g	2.5×10^{6}	n.d.	n.d.
	qPCR	GU/100 mL or 100 g	6.1×10^{3}	8.6×10^{9}	3.4×10^{9}
***Legionella* spp.**	cultural	cfu/100 mL or 100 g	n.d.	n.d.	n.d.
	FISH	Cells/100 mL or 100 g	2.4×10^{6}	n.d.	n.d.
	qPCR	GU/100 mL or 100 g	3.2×10^{4}	1.0×10^{10}	2.2×10^{10}
L. pneumophila	cultural	cfu/100 mL or 100 g	n.d.	n.d.	n.d.
	FISH	Cells/100 mL or 100 g	2.4×10^{6}	n.d.	-n.d.
	qPCR	GU/100 mL or 100 g	n.d.	n.d.	n.d.

4.1.1.1 Abundance of plankton in Lake Baldeney

Determination and quantification of plankton organisms collected during the sampling season in 2010 displayed species according to determinations of the Ruhrverband (2010). Concentrations of phytoplankton were found between 10^9 to 10^{10} individuals/m^3 with a maximum from May to July. The abundance of diatoms was the highest among phytoplankton, followed by green algae. The diatom species *Melosira* sp. was found in most of the phytoplankton samples. Other diatoms identified in phytoplankton samples were *Fragilaria* sp., *Niztschia* sp., *Asterionella* sp. and *Synedra* sp.

Chlorophyta sp., *Scenedesmus* sp. and *Pediastrum* sp. were the most abundant green algae determined in the samples. Cyanobacteria species found in 2010 were *Oscillatoria* sp., *Spirulina* sp. and *Anabaena* sp. In phytoplankton samples one species of the golden algae was determined as *Dinobryon* sp. The Ruhrverband reported phytoplankton concentrations in 2010 varying between 9.0 x 10^9 to 1.9 x 10^{10} cells/m^3 whereas in the 1990´s more than 1.0 x 10^{11} cells/m^3 were found. The spectrum of phytoplankton individuals has changed in the last years. The typical individuals of the green algae were found seldom, but diatoms were increasing and dominated the phytoplankton in the summer of the year 2010 (Ruhrverband, 2010). The Ruhrverband investigated that diatoms represented the highest amount with about 94% of the whole biovolume of algae, the species *Melosira* was dominating. This is confirmed by the results reported in this study. Since a few years it could be observed that the abundance of cyanobacteria and green algae is considerably decreasing, whereas the occurrence of golden algae increased. The improvement of the trophic level of the lower part of the River Ruhr could be the reason fot that (Ruhrverband, 2010). The temporal course and intensity of phytoplankton growth is effected by grazing of zooplankton and other abiotic factors, such as river flow and total irradiation.

Zooplankton organisms were determined with concentrations ranging between 10^1 and 10^5 individuals/m^3 with a peak in July and August. The increase in zooplankton individuals from 9.62 x 10^2 individuals/m^3 in the month June to 3.21 x 10^5 individuals/m^3 in July and 3.17 x 10^4 individuals/m^3 in August might be explained by the clearance rate of algivore zooplankton organisms which graze on phytoplankton.

The most abundant species of zooplankton detected in the samples belonged to the group of Rotatoria, e.g. the species *Brachionus*. Other dominant species identified for zooplankton are in the group of Crustaceans, mainly the subclasses Cladocera and Copepoda, e.g. *Daphnia* sp., *Polyphemus* sp., *Cyclopoida* and *Calanoida*. Copepods accounted for 86.8% among all zooplankton organisms in a marine coastal zone by Maugeri et al., (2004).

It was observed that the plankton abundencies follow a seasonal trend. With declining phytoplankton concentrations the abundance of zooplankton was rising.

This seasonal trend can be explained by zooplankton organisms grazing on phytoplankton until the so called clear water state is reached, where phytoplankton is almost eliminated by zooplankton grazing.

For evaluation of surface areas provided by plankton organisms in the samples in this study, calculation was done as follows.

Assumption:

The phytoplankton samples consist of the diatom species *Fragilaria capucina* and zooplankton is composed of *Daphnia magna.*

The specifications for these organisms (length, width and height) are given in Hoehn et al. (1998). For the calculation of the surface areas it was assumed that *Fragilaria capucina* can be seen as a cuboid form and *Daphnia magna* as an elliptical cylinder (Table 4.2).

Table 4.2 Surface areas calculated for the plankton organisms *Fragilaria capucina* and *Daphnia magna* (μm^2)

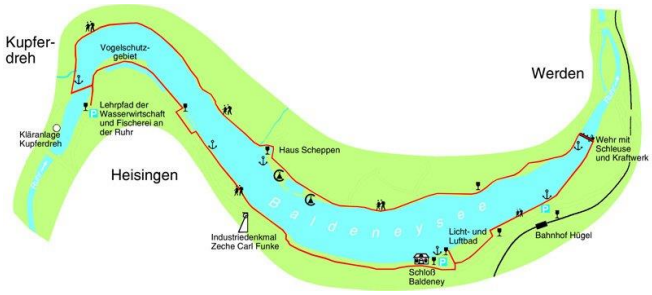
Individual	Formula	Surface area (μm^2)
Fragilaria capucina	Cuboid: 2 x length x width	161
Daphnia magna	Elliptical cylinder: 2 x π x length x width + 2 x π x length x width x height	2×10^9

As an example, the surface areas for phyto- and zooplankton samples of the month April and July (Table 4.3) were calculated due to the amount of individuals per m^3.

Table 4.3 Surface areas of phyto- and zooplankton in the samples of April and July (m^2/ m^3)

Surface area of plankton in the samples	Phytoplankton (m^2/m^3)	Zooplankton (m^2/m^3)
April	0.2	0.2
July	0.7	447

The surface of zooplankton in the month July is by far huge compared to the month April and in comparison to phytoplankton.



Volume	7.6 x 10^6 m^3
Surface area	2.64 km^2
Mean width	355 m
Mean length	7.8 km
Mean depth	3.14 m

Figure 4.1 Lake Baldeney data (source: Ruhrverband, 2010)

When the surface areas of plankton are related to the volume of Lake Baldeney (7.6 x 10^6 m^3; Ruhrverband, 2010) it becomes apparent, that the surface area of zooplankton is enormous with 3400 km^2 (Table 4.4). In comparison, Lake Baldeney has a surface area of 2.64 km^2 (Ruhrverband, 2010), this is less than 1 % of the zooplankton surface in August.

Table 4.4 Surface area of plankton (km^2) in relation to the volume of Lake Baldeney

Surface area related to Lake Baldeney (volume 7.6 x 10^6 m^3)	**Phytoplankton (km^2)**	**Zooplankton (km^2)**
April	2	1
July	5	3400

In one Liter of water the plankton surface areas are below 1 cm^2. In August the surface of zooplankton in one Liter of water is 45 cm^2 (Table 4.5).

Table 4.5 Surface area of plankton in one Liter sample (cm^2/L)

Surface area of plankton in one Liter of the samples	**Phytoplankton (cm^2/L)**	**Zooplankton (cm^2/L)**
April	0.02	0.02
July	0.07	45

All these calculations are based on the assumption that both samples consist of one species with a mean size. The results should be handled with care, since they are more or less estimations with a fault of approximately +/- a half order of magnitude. However, it is possible to get a valuation of plankton surface sizes.

The results indicate that plankton, especially zooplankton in the month July, represents a large surface which can be colonized by bacteria.
With respect to the surfaces per Liter (Table 4.5) the concentrations of bacterial abundance were calculated per square metre of the plankton surface (Table 4.6).

According to a schematic detection range for bacterial densities (Flemming et al., 2000) the colonization of plankton with bacterial biofilms can be characterized (Table 4.7). The general bacterial abundance on plankton seems to be a multilayer biofilm, since there are 10^8 or more cells/cm^2. Whereas the distribution of the pathogenic species is either a monolayer biofilm, microcolonies or single cells. Phytoplankton surfaces seem to be more densly colonized by bacteria than zooplankton. This is due to the difference in the calculated surface areas, since phytoplankton surfaces were less, the distribution of bacteria is of higher density.

Table 4.6 Bacterial abundance on 1 m^2 of plankton (cells, cfu or MPN/m^2)

Phyto-plankton	**Total cell counts**	**HPC bacteria**	**Coliforms**	***E. coli***	**Intestinal enterococci**	***C. perfringens***	***Aeromonas* spp.**	***P. aeruginosa***
April	1×10^{14}	6×10^{11}	1×10^{8}	2×10^{7}	2×10^{7}	3×10^{8}	5×10^{9}	4×10^{5}
July	2×10^{13}	6×10^{11}	3×10^{7}	4×10^{5}	4×10^{5}	9×10^{6}	2×10^{10}	2×10^{5}
Zoo-plankton	**Total cell counts**	**HPC bacteria**	**Coliforms**	***E. coli***	**Intestinal enterococci**	***C. perfringens***	***Aeromonas* spp.**	***P. aeruginosa***
April	7×10^{13}	2×10^{11}	7×10^{6}	2×10^{6}	3×10^{6}	2×10^{7}	5×10^{8}	6×10^{4}
July	3×10^{9}	9×10^{7}	4×10^{4}	3×10^{2}	2×10^{4}	3×10^{6}	2×10^{7}	1×10^{2}

Table 4.7 Bacterial densities (bacteria/cm^2) on a surface (Flemming et al., 2000)

Bacteria/cm^2	10^4	10^5	10^6	10^7	10^8
Quantitiy of bacteria, or biofilm	1	10	microcolonies	monolayer biofilm	multilayer biofilm

4.1.1.2 General abundance of bacteria in lake water and in association with plankton

The general microbial populations in water and plankton were quantifed by the determination of total cell counts and the fraction of culturable (HPC) bacteria. The water and plankton samples revealed total cell counts of 10^9 cells/100 mL and up to 10^{12} cells/100 g wet weight and colony counts with up to 10^7 cfu/100 mL and 10^9 cfu/100 g wet weight. Culturability in water was below 1 % and in plankton samples between 0 and 8 %. These concentrations of total cell counts and HPC bacteria were identical to the results of Balzer et al. (2010) who investigated water, epilithic biofilms and sediments in a river (e.g. River Ruhr). Bacteria associated with plankton were found in slightly higher concentrations on both phyto- and zooplankton in comparison to the water column.

The results indicated that plankton displayed high cell densities of the autochthonous bacteria compared to the surrounding water. In the literature, total cell counts and HPC bacteria in surface water and also associated with freshwater plankton have not been considered yet. Only Griebler et al., (2001) determined total cell counts in sediments of different freshwater environments (lakes, rivers) and found high bacterial densities in the sediments compared to the surrounding water.

The culturability in water was low. The elevated culturability in plankton samples can be explained by favourable nutrient conditions for bacteria living associated to plankton organisms. Bacteria have nutritial advantages when living associated to copepods (Heidelberg et al., 2002) or if they can use algal exudates (Byappanahalli et al., 2003). Furthermore the low culturability can be explained by unfavourable culture conditions, or the bacteria occur in a viable but non-culturable state (VBNC). A correlation effect between cell densities and water temperature or oxygen concentration was not observed, this may be due to the limited number of sampling events.

4.1.1.3 Occurence of organisms with faecal origin associated with plankton and in the free water

Surface waters can be contaminated with faecally derived bacteria. This can occur through point sources, such as sewage effluents, or non-point sources, such as agricultural or urban run-off. The faecal indicator organisms investigated in this study were total coliforms, *E. coli*, intestinal enterococci and *C. perfringens.* Coliform bacteria can also be of environmental origin (Leclerc et al. 2001). All these organisms were detected in water as well as plankton samples during sampling from April to September. Slightly elevated concentrations of these organisms in the water column were observed in August. Generally the investigated faecal indicator organisms seem to be more abundant adhering to plankton than in the water column when referred to the plankton mass.

4.1.1.3.1 Abundance of total coliforms and *E. coli* in lake water and associated with plankton

Total coliforms and *E. coli* were all detected in water and plankton samples. Total coliform concentrations were quantified with 4.0×10^2 to 2.4×10^4 cfu/100 mL in lake water and the abundance in plankton samples varied between 10^6 to 10^8 cfu/100 g. The concentrations of *E. coli* ranged from 7.4×10^1 to 2.4×10^4 MPN/100 mL in water and between 10^5 and 10^7 MPN/100 g wet weight for plankton. Balzer et al. (2010) reported similar concentrations for river water and epilithic biofilms. The elevated concentrations of coliforms and *E. coli* in plankton samples compared to those in water indicate that the bacteria persist in these environments and may meet favourable nutrient conditions in the interaction with plankton.

The aim of the Ruhrverband (2010) is to not exceed a target value of coliforms with 1.5×10^4 per 100 mL in mean, which was not fulfilled with the detected values. The River Ruhr is no bathing water but it is intensively used for other recreational purposes. Araujo et al., (1989) reported faecal coliforms with 10^7 to 10^9 cfu/100 mL in freshwater environments (rivers) in the South of Barcelona/Spain, this confirms observations made in association with plankton in Lake Baldeney. *E. coli* concentrations in river water were reported with 4.9×10^1 cfu/100 mL to 2.2×10^4 cfu/100 mL (Rodriguez & Araujo, 2010). Maugeri et al. (2004) found *E. coli* in seawater with 1.0×10^1 to 1.5×10^4 cfu/100 mL. Less is considered about plankton associations of coliforms or *E. coli* in literature yet, only by Signoretto et al. (2004;

2005) *E. coli* has been linked to plankto. However there are reports about sediments. For freshwater and coastal sediments the presence of *E. coli* was reported several times. Elevated concentrations of coliforms and *E. coli* as well as long-term survival and multiplications in sediments was assumed to be likely compared to the overlying water in freswahter streams as well as coastal waters (Byappanahalli et al., 2003; Craig et al. 2002, 2004; Obiri-Danso & Jones, 1999; Valiela et al., 1991). However, the survival of these bacteria seemed to be limited by competition with the natural microflora and predation by protozoa (Marino & Gannon, 1991). A variety of species was found by identification of coliform isolates from water and plankton. They can be divided into three ecological categories according to Leclerc et al., (2001). The identified species *Citrobacter*, *Klebsiella* and *Enterobacter cloa*cae belong to the ubiquitous group of coliforms. They may be of faecal origin, but can also be found in natural environments such as surface waters, soil or vegetation. The organism *E. coli* was isolated from both water and plankton samples and is representing the thermotrophic group of faecal origin. Member of the environmental group that were detected was *Kluyvera* sp.

Some of these species identified are known as oportunistic pathogens, such as *K. pneumonia*, *Enterobacter* spp., *Citrobacter* spp. (Guentzel, 1996; Sanders & Sanders, 1997; Struve & Krogfeldt, 2004). *E. coli* which is usually harmless commensals for humans, also include pathogenic variants such as enterohaemorraghic *E. coli* O157:H7 (Leclerc et al., 2001).

In this study it succeeded to determine total coliforms as well as *E. coli* in water and plankton samples. The organisms showed preferences to be plankton-associated, since they were found with four orders of magnitude higher concentrations in plankton samples than in the free water. In August the abundance of total coliforms was elevated and showed plankton preference even when the concentration was related to the sampling volume.

4.1.1.3.2 Intestinal enterococci in water and plankton samples

In water samples enterococci varied between 2.5 and 3.0 x 10^3 cfu/100 mL. These findings are similar to those and quantities in river water with Enterococci were reported with 2.0 to 8.0 x 10^2 cfu/100 mL (Maugeri et al., 2004) or 6.0 to 2.4 x 10^2 cfu/100 mL (Balzer et al., 2010).

E. faecalis has been linked to plankton, in Lake Garda it was found adhering to plankton and in water. Enterococci were reported more often associated with zooplankton, than *E. coli*, especially in the winter month, where zooplankton may provide an overwintering site for enterococci (Maugeri et al., 2004). Signoretto et al. (2004) found numbers of enterococci in a freshwater lake to decrease in the water phase and concurrently increase in zooplankton during summer. In this study, during the sampling season from April to September, *E. coli* was more abundant in plankton samples as enterococci.

The organisms identified from enterococci isolates of water were *Enterococcus hirae*, *Enterococcus durans*, *Enterococcus faecalis* and *Enterococcus faecium*. These organisms represent species of true faecal origin (Pinto et al., 1999), whereas *E. faecalis* and *E. faecium* are known to cause a variety of infections in humans (Jett et al., 1994). Other species identified were *Enterococcus casseliflavus* and *Enterococcus gallinarum* which are regarded as environmental organisms (Pinto et al., 1999). *E. faecalis* and *E. faecium* were the most frequently isolated species from environmental samples (Pinto et al., 1999), this correlates to the inicidence in Lake Baldeney. Similar to investigations in this study Mote et al. (2012) described up to 95% higher abundencies of culturable enterococci in the plankton-associated state than in the bulk water. Several authors investigated associations of enterococci with specific types of plankton such as copepods and green algae in freshwater and marine environments (Signoretto et al., 2004, 2005, Whitman et al., 2003).

In this study an association of enterococci with plankton was clearly observed. In August and Septmeber the abundance of intestinal enterococci exceeded those in the free water significantly, although plankton represents only a small fraction in the bulk water.

4.1.1.3.3 *C. perfringens* and their endospores, can both be determined in the free water as well as plankton-associated?

The faecal indicator *C. perfringens* was quantified with 10^1 to 10^2 cfu/100 mL in water samples of Lake Baldeney and between 10^7 and 10^8 cfu/100 g in plankton samples.

Endospores of *C. perfringens* were consistently recovered in water samples at about 75.3 % and with less percentages in plankton samples (16.1 % for zooplankton; 0.8 % for phytoplankton). Abundance of *C. perfringens* in freshwater sediments were

reported with quantities of 3.0 x 10^5 cfu/100 g to 1.1 x 10^6 cfu/100 g dry weight, the presence of *C. perfringens* spores was found to be constant at high levels in Lake Michigan (Mueller-Spitz et al., 2010). *C. perfringens* levels above 10 cfu/100 mL can be associated with human health risks when bathing in fresh recreational waters (Wiedenmann et al., 2006). In literature associations of *C. perfringens* and their spores with plankton are not considered yet. Hence, the results of this study can only be reverted to findings in sediments and sewage effluents. Medema et al. (1997) found *C. perfringens* deriving from sewage was persistent in natural waters. The organism survived longer than oocysts and may therefore be proved as an indicator for the presence of *C. parvum*. Recovery of spores in freshwater sediments had been reported by several authors (Edwards et al., 1998; Lisle et al., 2004). Mueller-Spitz et al. (2010) considered freshwater sediments and sewage inputs into freshwater habitats as reservoirs of enterotoxin-carrying *C. perfringens* spores. However, the accumulation of *C. perfringens* to particles or sediments indicates the possibility of creating reservoirs for this potential pathogen organism in aquatic environments (Mueller-Spitz et al., 2010).

New in this study was the determination of *C. perfringens* associated with plankton organisms, as well as the balancing of endopspore distribution between the free water and plankton-associated state. It succeeded to detect both vegetative *C. perfringens* cells and their spores. Whereas the vegetative form seemed to prefer the plankton association, most of the spores were found in the water column. The high concentration of endospores in the free water compared to plankton in this study can be referred to elevated nutrient conditions for *C. perfringens* vegetative cells living attachted to plankton surfaces.

The results of organisms of faecal origin indicate that plankton displayed higher cell densities compared to the surrounding water column. In the warmer months July and August the oxgen concentration was elevated and correlated to higher abundancies of coliforms, *E. coli* and *C. perfringens* in plankton samples. Compared to total cell counts and HPC bacteria the concentrations of the faecal indicator bacteria reflect that they join better nutrient conditions when associated to plankton than living in the free water (Table 4.1). If the results were referred to the sampled volume the abundencies of the organisms on plankton seem to be lower than in the free water

phase. However in the months August and September the organisms were found to be more associated with plankton than in the free water.

4.1.1.3.4 Abundance of the human pathogen *Campylobacter* spp. in the freshwater environment of Lake Baldeney

The obligate human pathogen *Campylobacter* spp. can contaminate surface waters via point sources, like sewage effluents, or non-point sources, like agricultural or urban run-off and wild bird excretion. *Campylobacter* spp. was determined qualitatively in four out of six water samples, but was never present in plankton samples. *Campylobacter* spp. isolates were identified as the species *C. coli* and *C. jejuni.* Both are the most common human enteric pathogens among campylobacters and cause acute bacterial diarrhea (Frost 2001).

In this study the human pathogen of faecal origin was determined sporadically in water, but never in plankton samples. However in previous studies *Campylobacter* spp. were found more frequently associated with zooplankton than with phytoplankton in seawater (Maugeri et al., 2004). In this study several factors may have contributed to the apparently low *Campylobacter* isolation,

(i) a low prevalence of the organism in the sampled water, presumably below the detection limit,
(ii) predation by and competition with other microorganisms which led to reduced survival times for *Campylobacter* spp. and might explain the absence in plankton samples (Korhonen et al., 1991),
(iii) unfavourable culture conditions, or
(iv) *Campylobacter* species may have entered into a VBNC state due to starvation or other environmental stress and therefore fail to grow on culture media.

Hence molecular detection assays may be useful to elucidate potential epidemiological sources and reservoirs (Moore et al., 2001; Rollins & Colwell, 1986). In river waters in Greece *C. jejuni* and *C. coli* were the most abundant species identified (Arvanitidou, et al., 1995). These identified species may originate from sewage effluents, run-off or wild bird populations and have been found in freshwater bathing sites in the UK (Obiri-Danso & Jones, 1999). In river water of the Mediterranean area *Campylobacter* counts were low and determined with 1.3

MPN/100 mL, whereas especially in the summer many samples were negative (Rodriguez & Araujo, 2010). The sampling period in this study occurred from April to September which might explain the low concentrations of the human pathogen due to the elevated temperatures which not favour their survival and growth. *Campylobacter* spp. were found in higher occurrence in the winter month and with lower concentrations or none in the warmer summer months by several authors (Brennhovd et al., 1992; Carter et al., 1987; Jones et al., 1990). Survival seemed to be enhanced in water microcosms at temperatures below 4 °C (Rollins & Colwell, 1986; Terzieva & McFeters., 1991).

Campylobacter spp. was detected in the bulk water, but seldom. An association with plankton was not confirmed, although there is a reference in literature. The identified species in water belong to the most common enteric human pathogens. The reason for the absence of *Campylobacte*r spp. in plankton samples is not clear, and different possibilities to explain the absence were mentioned before. If it is assumed that the human pathogen occurs in a VBNC state when associated to plankton, this can pose a health threat for humans. The VBNC-*Camplyobacter* spp. may regain their pathogenic characteristics after resusucitation due to amelioration of environmental conditions. Therefore it should be considered to use cultivation independent methods to elucidate the true abundance of the human pathogen.

4.1.1.4 Opportunistic pathogens in Lake Baldeney

Determination of opportunistic pathogenic bacteria in association with plankton confirmed that all organisms, except for *Legionella* spp., were found in both phyto- and zooplankton samples by cultural methods. Remarkable are the high concentrations of *Aeromonas* spp. in water as well as plankton and the low culturability of *P. aeruginosa*.

4.1.1.4.1 *Aeromonas* spp. – the most abundant organism determined in Lake Baldeney

Aeromonas spp. were found with 8.0 x 10^3 and 3.0 x 10^5 cfu/100 mL in the water phase of Lake Baldeney, with elevated concentrations in August. In plankton samples abundance of *Aeromonas* spp. varied between 10^8 to 10^{10} cfu/100 g of

phytoplankton and between 10^7 to 10^{10} cfu/100 g in zooplankton samples. In both samples an increase in colony counts in August could be observed due to elevated water temperatures with 26°C. Species identified from isolates of water and plankton included the organisms *A. hydrophila* and *A. salmonicida*. Both has been reported to cause infections in humans (e. g. Galindo et al., 2006; Janda 2001), whereas the latter is also well-known as a fish pathogen (Austin & Austin 1993).

Maugeri et al. (2004) determined *Aeromonas* spp. with 4 x 10^3 cfu/100 mL in seawater. Abundance during the sampling period showed seasonal pattern in the water samples of the marine coastal zone in Italy. The species *A. hydrophila* was quantified with concentrations of 10^4 cfu/100 mL in a River in Poland (Niewolak & Opieka, 2000). Concentrations of *Aeromonas* spp. reported by Araujo et al. (1989) in rivers in Spain were up to 4 log units (10^2 to 10^9 cfu/100 mL) higher than those presented in this study. *Aeromonas* spp. has been linked to plankton colonization (Maugeri et al., 2004) and was recently found in association with the cladoceran *B. coregoni* (Grossart et al., 2009). The concentrations of Aeromonas spp. determined in this study exceed the findings of 6.0 x 10^2 cfu/100 mL in phytoplankton and 1.9 x 10^{10} cfu/100 mL in zooplankton reported by Maugeri et al. (2004). Omar et al. (2002) found *A. hydrophila* in siginificant numbers in surface water as well as in association with copepods in the Straits of Malacca, Malaysia.

Dumontet et al. (2000) reported that the abundance of *Aeromonas* spp. often exceeds those of coliform bacteria, whereas Araujo et al. (1989) described a correlation between *Aeromonas* spp. and faecal coliforms. In Lake Baldeney the concentrations of *Aeromonas* spp. were often higher than those of coliforms in water samples as well as in plankton samples.

In this study *Aeromonas* spp. was determined with with the highest abundance among all investigated organisms, by use of cultural methods. Abundance in the plankton samples was determined with six orders of magnitude higher concentrations than in the free water. The amounts correlated to those of HPC bacteria. For *Aeromonas* spp. the preference seem to be a plankton-associated life state, since they showed elevated culturability in plankton samples compared to water. Plankton organisms seem to be an attractive microhabitat for *Aeromonas* spp. in a freshwater environment. During the sampling period from April to September *Aeromonas* spp. was consistently abundant in all samples and with higher concentrations in plankton samples. In the warmer months July, August and September the concentrations were

slightly elevated when they were referred to the wet weight of plankton. Also when they were referred to the plankton sampling volume, *Aeromonas* spp. associated with plankton exceeded the concentrations in water in July, August and September.

4.1.1.4.2 *P. aeruginosa* was less abundant than *Aeromonas* spp.

Detection of *P. aeruginosa* in water samples of Lake Baldeney revealed 10 MPN/100 mL, whereas in plankton samples the colony counts varied between 10^4 to 10^5 MPN/100 g wet weight, with the highest abundance among all samples, in the month with the warmer water temperature, in July. Bacterial loads, including *P. aeruginosa*, were reported as significantly higher at elevated water temperatures and with the occurrence of algal blooms, e.g. in the Woluwe River in Belgium (Hoadley, 1977; Pirnay et al., 2005). Cultivation-based quantification of *P. aeruginosa* in river water provided results in the range of 10^3 cfu/100 mL to 10^4 cfu/100 mL (Hardalo & Edberg, 1997; Pirnay et al., 2005; Ziegert & Stelzer, 1986). Seyfried & Cook (1984) reported much lower concentrations in lakes in Canada with quantities ranging from 2 to 33 cfu/100 mL, and similar values with 3 to 32 cfu/100 mL were found in a river in Poland (Niewolak & Opieka, 2000). In Tokio Bay *P. aeruginosa* was determined with 7.0×10^1 cells/mL (Kimata et al. 2004). They suggest that *P. aeruginosa* is commonly present in Tokio Bay, but that only a small percentage of those is culturable. The environmental pathogen *Pseudomonas* spp. is one of the most common bacteria in aquatic habitats (Pearce et al., 2005) and was reported in association with marine phytoplankton (Berland et al., 1976), previously *P. aeruginosa* had been found in association with *Daphnia* (Qi et al., 2009).

In this study it succeeded to detect *P. aeruginosa* associated with plankton, until now this phenomenon is not mentioned in literature. *P. aeruginosa* was found to be less culturable than all other investigated organisms. Especially in water the concentrations were low. The concentrations referred to the sampling volume of plankton were found to be elevated in August and September, when compared to the free water. The difference in the concentrations between water and plankton samples was four to five orders of magnitude. This allows the assumption of favourable conditions for *P. aeruginosa* when existing associated with plankton organisms.
This supports the assumption that, apart from sediments, surface-associated biofilms, like on plankton surfaces, appear to represent a reservoir of *P. aeruginosa*

in natural waters (Pirnay et al., 2005). Pellett et al. (1983) found *P. aeruginosa* to be present at highest numbers when associated with submerged surfaces of rocks, macrophytes, and fish, whereas concentrations were lower in the water.

Both organisms with facultative pathogen properties, *Aeromonas* spp. and *P. aeruginosa* showed slightly elevated cell densities in the warmer months July and August, where the oxygen saturation in the lake peaked. The results indicate higher concentrations of the pathogens associated with plankton compared to the surrounding water. The carapace of zooplankton organisms is mostly made up of chitin. Therefore the question remains, whether pathogens can degrade the chtin and use it as carbon, nitrogen and energy source *A. hydrophila,* for instance employs extracellular chtinases and is able to degrade chtin. Although *P. aeruginosa* is also known to produce a chitinase, the organism is reported not to grow with chitin (Jagmann et al., 2010). The hypothesis is that this phenomenon explains the high abundance of *Aeromonas* spp. and the low frequency of *P. aeruginosa* abundance.

4.1.1.4.3 Abundance of *Legionella* spp. and *L. pneumophila*

Legionella spp., or *L. pneumophila,* the medically most important species among legionellae, were never detected in water as well as plankton samples with cultural methods during the sampling period. Culture-based detection frequently underestimates the true number of legionellae (Behets et al., 2007) and was reported repeatedly (e.g. Carvalho et al., 2007). Reasons might be that the culture media were not favourable and the organisms may have entered into the VBNC state due to environmental stress, such as starvation. Presumably legionellae suffered by competition with other microorganisms, that the plates were overgrown by other bacteria (e.g. Ng et al., 1997).

4.1.1.5 Are *P. aeruginosa, Legionella* spp. and *L. pneumophila* occurring in a viable but nonculturable state in Lake Baldeney?

The hypothesis is that a large proportion of the hygienically relevant microorganisms found in both lake water and plankton samples are viable but nonculturable.

This assumption was confirmed using the culture-independent qPCR technique and the FISH method. *P. aeruginosa* was determined in very low concentrations, whereas *Legionella* spp. as well as *L. pneumophila* were never detected with cultural methods. With molecular methods the target organisms were found to be present at much higher quantities, and in virtually every sample, suggesting that both *Legionella* spp. and *P. aeruginosa* were present in significant concentrations throughout the sampling period.

In comparison with FISH and qPCR results, cultivation provided a recovery of less than 0.1 %, detecting concentrations of *P. aeruginosa* with 10^6 cells/100 mL water by FISH and up to 10^4 GU/100 mL water by qPCR. The organism *P. aeruginosa* was determined in concentrations up to three or five log units higher than compared to cultural methods. The number of studies dealing with the quantification of *P. aeruginosa* in environmental aquatic samples using qPCR is low. Various qPCR assays have been developed for clinical (e.g. Qin et al., 2003) or wastewater-related applications (Schwartz et al., 2006; Volkmann et al., 2007), but no attempt has been made to quantify *P. aeruginosa* abundance in natural surface water or on plankton. The same applied for the FISH method. *P. aeruginosa* was found to show signs of metabolic acitivity such as presence of ribosomal RNA which was detected by the FISH method using the gene probe PSAE-16S-182. Since rRNA is known to remain stable for a long time after cell death, it is not an appropriate viability marker. Hence, FISH positive cells should not directly be regarded as VBNC cells (Tolker-Nielsen et al. 1997, Prescott et al.,1999). A FISH positive signal should critically be seen as, at most, a first sign of the possibility of VBNC.

P. aeruginosa concentrations in the water showed significant seasonal differences. The highest concentrations were observed in the hottest months of the sampling period, in July and August. This is confirmed by observations of Pirnay et al. (2005) who found the microbial load in river water, including *P. aeruginosa* abundance, to peak during the warmest period of a year. Reasons for this increase are probably higher water temperatures and algal blooms, which both have been reported to support multiplication of *P. aeruginosa* (Hoadley, 1977).

P. aeruginosa is known to produce a chtinase and a chitin-binding protein, but it was found not biodegrade chitin by Jagmann et al., 2010. Whereas *A. hydrophila* could grow with chitin, *P. aeruginosa* could not. In co-cultures of *P. aeruginosa* and *A. hydrophila* in association with chitin, Jagmann et al. (2010) observed oxidation of chtin by *A. hydrophila* with acetate as end-product. This supported the growth of *P. aeruginosa* which influenced *A. hydrophila* in parasitic way.

Legionella spp. which was not detectable by culture based methods, where found in high amounts in water as well as plankton samples in Lake Baldeney by use of molecular methods. Culture-based detection frequently underestimates the true number of legionellae (Behets et al., 2007). In this case, underestimation might have occurred due to the following reasons: (i) the plates were rapidly overgrown by other microorganisms, a phenomenon observed regularly (e.g. Ng et al., 1997). Evaluation of these plates was impossible. (ii) preparation steps such as acidification of the samples in order to reduce this contamination or sample concentration by filtration probably have led to a decrease of *Legionella* viability of 50 % to 90 % (Boulanger & Edelstein, 1995; Levi et al., 2003). Furthermore, another explanation might be that no viable cells were present in the samples.

Numerous studies concerning the detection of legionellae, have shown that qPCR is more sensitive than conventional cultures (Behets et al., 2007; Bonetta et al., 2010; Fiume et al., 2005; Palmer et al., 1995; Wellinghausen et al., 2001; Yaradou et al., 2007). In the present study, concentrations of *Legionella* spp. in the water samples determined by qPCR averaged 4.0 x 10^4 GU/100 mL, whereas *L. pneumophila* could not be detected. These findings confirm previously reported concentrations of legionellae in surface waters detected by qPCR. Parthuisot et al. (2010) found *Legionella* spp. with 4.7 x 10^4 GU/100 mL for the majority of their samples along the Tech River in France. They were also not able to detect the most important species among *Legionella* spp. concerning human health, *L. pneumophila*. Similar *Legionella* spp. concentrations of 1.0 x 10^5 cells/100 mL were reported for rivers and open storage basins by Wullings & van der Kooij (2006) who used a semi-quantitative PCR method. Declerck et al. (2007) found *Legionella* spp. and *L. pneumophila* both to be present in natural aquatic environments (e.g. lakes, creeks) at concentrations of up to 10^1 GU/100 mL.

Carvalho et al. (2007) found *Legionella* spp. failed to grow on routine culture media, but detected DNA sequences with PCR which were homologous to the 16S ribosomal DNA gene of *Legionella pneumophila* and other *Legionella* species. Recently, qPCR has become a popular technique for the detection of legionellae in aqueous systems (Behets et al., 2007; Declerck, 2010). However, the studies dealing with qPCR have been mostly restricted to man-made systems like drinking water distribution systems or cooling towers (Behets *et al*., 2007; Bonetta *et al*., 2010; Chang *et al*., 2009; Joly et al., 2006; Levi et al., 2003; Wellinghausen et al., 2001; Wéry et al., 2008; Yáñez et al., 2005; Yaradou et al., 2007). Only few attempts have been carried out to use qPCR for the enumeration of legionellae in surface waters (Declerck et al., 2007; Parthuisot et al., 2010; Wullings & van der Kooij, 2006).

The FISH method revealed high concentrations of *Legionella* spp. as well as *L. pneumophila* (~ 10^6 cells/100 mL), which indicates metabolical activity, however literature does not provide comparative studies.

A seasonal pattern in *Legionella* concentrations resulting in a summer and autumn peak was reported elsewhere (Fliermans et al., 1981; Parthuisot et al., 2010; Wéry et al., 2008), but could not be confirmed in this study. In fact, concentrations appeared to decrease with increasing water temperature. This might indicate the presence of legionellae with a relatively low temperature optimum for growth. Concentrations of *Legionella* spp. in the plankton samples remained constant over time. A summer peak was observed in only one sample collected in August and therefore did not appear to be significant.

In this study, the medically most important species among legionellae, *L. pneumophila* could be detected with FISH but never with qPCR. Parthuisot et al. (2010) were also not able to detect *L. pneumophila* with qPCR. The high amount of FISH positive *L. pneumophila* cells indicates that this species accounts for almost all of the FISH positive *Legionella* spp. cells.

In contrast to cultivation, qPCR also detects viable but non-culturable (VBNC) cells, contributing to the observed discrepancy between culture and molecular methods (Signoretto et al., 2004). Entry to VBNC state is triggered by environmental stressors such as starvation or altered temperature and promotes survival of the cell in unfavourable conditions. Both Legionella spp. and *P. aeruginosa* are described to become VBNC (Dwidjosiswojo et al., 2010; Oliver, 2005). VBNC *Legionella* have

been detected in natural waters (Delgado-Viscogliosi et al., 2005), and their presence is considered to greatly affect the magnitude of qPCR results (Bonetta et al., 2010). VBNC *P. aeruginosa* were assumed to be present in marine environments, but little is known about the abundance of VBNC cells in freshwater (Khan et al., 2007). Co-detection of VBNC cells, however, can be important with respect to health risk assessment of contaminated water systems. The detection of these bacteria with PCR (Wellinghausen et al., 2001; Declerck et al., 2009; Felföldi et al., 2009) or FISH (Långmark et al., 2005; Lehtola et al., 2007) has been shown to be more efficient compared to culture-based methods.

It is not known whether all bacteria detected by FISH using oligonucleotide probes which are targeted at intact rRNA are still viable. Ribosomal RNA can still remain stable, although the bacteria are already dead. This would lead to false-positive results.Therefore FISH-positive cells should not be seen as an evidence for VBNC cells, but rather as a hint for VBNC possibility (Tolker-Nielsen et al. 1997, Prescott et al.,1999). The possibility to detect false-negative results can arise from degradation of rRNA due to environmental stress which induces weak or absent fluorescent signals (Bjergbæk & Roslev, 2005; Lehtola et al., 2007). Based on the assumption that detection of rRNA with fluorescent oligonucleotide probes indicated viability, the low ratio of culturable cells to FISH-positive cells suggests that *P. aeruginosa* and *Legionella* spp. may occur in the VBNC state in freshwater environments, in the water column. These organisms are known to enter the VBNC state (Oliver 2010), but the determination of these organisms with the FISH method is not reported for surface waters in literature up to now.

Even though qPCR might overestimate the true number of viable and culturable target organisms present in a sample, VBNC cells still may pose a potential health threat. Health significance of various pathogens in VBNC state has been demonstrated before (McFeters et al., 1986), proving them to regain virulence after resuscitation. The same applies to legionellae. In environmental water systems, they are able to resuscitate from VBNC state within ubiquitous amoebae (Steinert et al., 1997; Oliver, 2005). Therefore, disregarding non-culturable cells in the detection of pathogenic bacteria may lead to an underestimation of health risk and thus to a false evaluation of water safety. Since qPCR also detects free DNA that may originate from dead, lysed cells or is released during horizontal gene transfer. It is possible that

the true number of target genomes is overestimated, (Declerck et al., 2007; Ng et al., 1997; Yanez et al., 2005). Therefore, quantitative results must be considered critically with respect to health risk evaluation (Bonetta et al., 2010). However, it remains unknown whether the proportion of DNA which was present in the samples as extracellular DNA was large enough to significantly bias quantification.

4.1.1.5.1 Is the macrophyte *Elodea nuttallii* an appealing habitat for hygienically relevant bacteria?

Investigations of bacterial abundance in association with the macrophyte *Elodea nuttallii* compared to the water phase occured in July, August and October in the year 2009. Total cell counts were about 10^{12} cells/100 g dry weight and culturable HPC bacteria were detected with 10^{10} cfu/100 g dry weight. The distribution of total cells as well as HPC bacteria on macrophytes has not been reported yet. However Hempel et al. (2008) determined total cell concentrations with 10^9 cells/100 g dry mass on submerged macrophytes in Lake Constance. Compared to other aquatic surfaces, cell densities found on *Elodea nuttallii* are similar to those of sediments in a river (Balzer et al. 2010). Faecal indicator bacteria, total coliforms *E. coli* and enterococci abundencies were determined with 10^3 to 10^4 MPN/100 g dry weight and between 10^6 to 10^8 cfu/100 mL for the latter one. These concentrations are up to two log units higher compared to those found in sediments in the river Ruhr (Balzer et al., 2010). *E. coli* and enterococci were reported to be associated with the macrophytic green alga *Cladophora* which harbored high densities (up to 10^8 cfu/100 g dry weight) in Lake Michigan (Byappanahalli et al., 2007; Olapade et al., 2006; Whitman et al., 2003, 2006). Determination of *Aeromonas* spp. yielded concentrations of 10^8 cfu/100 g dry weight in *Elodea* samples. The human pathogen *Campylobacter* spp. was found associated with the macrophyte *Elodea nuttallii* quantitatively in one of three investigated samples. The species identified was *C. jejuni.* It is known as one of the most common human enteric pathogens among the thermotolerant campylobacters (Frost 2001). No information exists in literature about the association between *Elodea nutallii* with hygienically relevant bacteria. However, there is evidence for pathogens existing associated with macrophytes. Ishii et al. (2006) determined the pathogens *Salmonella*, *Shigella* and *Campylobacter* in samples of *Cladophora* in Lake Michigan. Furthermore associations with bacteria of the

Cytophaga-Flavobacteria-Bacteriodetes group and alpha- and betaproteobacteria in freshwater and marine habitats are often reported (Eiler et al., 2004; Riemann et al., 2000; Sapp et al., 2007). Although submerged macrophytes produces secondary metabolites, such as polyphenols, which may have antimicrobial activity, some bacteria seem not be influenced in their attachment to the plant and their survival when associated with the plant (Hempel et al., 2008; Scalbert, 1991).

New in this study was the investigation of bacteria with hygiencal relevance in association with the macrophyte *Elodea nuttallii*. The local density of general bacterial abundance as well as concentrations of pathogens living attached to *Elodea nuttallii* were four to six orders of magnitude higher than those in the water column. The hypothesis is that these organisms may overcome the polyphenol-based plant defences and are able to profit from the released inorganic and organic nutrients. One question remains, how high is the amount of potentially pathogens occurring in a VBNC state when existing associated with the macrophyte, due to stress by antimicrobial metabolites? To evaluate the true amounts of pathogens associated with *Eldoea nuttallii,* the detection by molecular methods should be considered.

4.1.1.5.2 Estimation and reliability of the results referred to plankton volume and wet weight

Bacterial concentrations determined for plankton samples, which were referred to the wet weight of plankton mass, indicate accumulations of the potentially pathogens in comparison to the surrounding water. If the results of plankton are referred to the sampling volume from which the plankton organisms were collected, then the bacterial abundance on plankton represents only a small proportion in the whole aquatic environment. In August the incidence was different. The absolut concentrations of most of the pathogens were higher in association with plankton than in the free water. This might be in relation with the clear water state, where abundencies of zooplankton exceed those of phytoplankton due to grazing. These findings seem to indicate that there exists a stronger association of hygienically relevant microorganisms with zooplankton than with phytoplankton, due to less amounts of phytoplankton available.

The important fact is that plankton as well as *Elodea* microhabitats often only account for a minor fraction of the total bacterial abundance in the surface water, although

they represent dense local populations of hygienically relevant bacteria. It has to be considered that these accumulations on plankton can spatially increase the bacterial concentrations up to those of infectious doses and therefore pose a health concern for humans (Omar et al., 2002).

However, as a drawback to this finding, it has to be acknowledged that comparisons of concentrations of bacteria free-living in the water column, of plankton-associated bacteria and macrophyte-associated bacteria (expressed per 100 mL of water; 100 g wet weight plankton and 100 g dry weight of *Elodea nuttallii* respectively) are restricted and, at best, estimates (Heidelberg et al., 2002).

(i) this is due to the sampling method. Since the plankton was concentrated during sampling, the true distribution of the plankton within the original water column was lost, whereas sampled water was analysed undiluted. The relatively low weight of the sampled plankton biomass could not be estimated correctly, which is why quantitative results of the plankton samples lost precision during unit conversion from mL to g. However, normalizing bacterial concentrations in plankton to the sampled biomass was essential for theoretical considerations. Since this allowed a comparison of all plankton samples to a comparable reference value.

(ii) the nature of the reference media (water and plankton) differs widely, which complicates weight-to-volume comparisons. The water content of the sampled plankton is unknown and also may differ between phyto- and zooplankton. Plankton must then be considered as a colonisable phase boundary of unknown surface area which includes not only the outer surface, but also the guts of, for instance, crustacean zooplankton (Carli et al., 1993).

4.1.1.5.3 Associations of pathogens with plankton in freshwater environments

In this study it was successful to demonstrate that there are strong associations of hygienically relevant bacteria with phyto- and zooplankton organisms, as well as with the macrophyte *Elodea nuttallii*. These findings were absolutely new for most of the bacterial species.

Microbial diversity associated with plankton is partly species-specific to the zooplankton characteristics. Furthermore dependend on the environment, for instance to the ambient bacterial communities. Associations between bacterial communities and zooplankton, whether if they are permanent or transient can affect ecological and biogeochemical pathways in the water column (Grossart et al 2009). Colonization of plankton by bacteria seems to be a widespread phenomenon. There are different possibilities in the association of bacteria with plankton. The microorganisms can colonize and attach to phytoplankton or zooplankton organisms by direct contact to its surface (Carman and Dobbs, 1997), or enter the gut of a zooplanktor by ingestion. In the case of ingestion, the host can release the organism by defecation of the gut flora into the environment (Tang, 2005). This leads to an active exchange of bacteria between plankton organisms and the surrounding water. The question is if they are released unharmed and active after gut passage or in a VBNC state. There is evidence that some bacteria survive the passage through the gut, whereas others are digested or biodegraded. Copepod-bacteria associations seem to occur regardless of the oligotrophic or eutrophic state of the surface water (Nagasawa, 1988). However in case of eutrophy, the abundance of plankton will dramatically enhance the association with pathogens and therefore the proliferation within the surface water.

4.1.2 *Daphnia magna* as a habitat for hygienically relevant bacteria

D. magna is known to be a well-established model organism and is used in biological research for ecotoxicology, ecology and evolution studies since the 18th century (Ebert, 2008; Lampert, 2011; Routtu et al., 2010; Schaffer 1755). The cladoceran is a key herbivors in many freshwater ecosystems and efficiently consumes heterotrophic bacteria (Brendelberger at al., 1991; De Mott 1986; Gophen & Geller, 1984). *Daphnia* sp. was found to be abundant in Lake Baldeney samples. To investigate the fate of *Daphnia magna* in association with the pathogens *Pseudomonas aeruginosa*, *Aeromonas hydrophila* and *Enterococcus faecalis,* toxicity experiments according to Le Codiac et al. (2012) were performed.

The selected organisms of hygienical relevance were chosen, with the following reasons, since all of the three were found in association with plankton in Lake Baldeney:

- *P. aeruginosa* was less abundant in lake water, but indicated a clear association to plankton. The organism is known to persist and proliferate in biofilms, for instance in drinking water systems. *P. aeruginosa* is assumed to be VBNC in association with plankton, as observed in Lake Baldeney.
- *Aeromonas* spp. was found to be the most abundant organism in Lake Baldeney, with respect to those which were included in this study. Preferencially it was found in association with plankton.
- The organism *E. faecalis* has faecal origin and was found to be associated with plankton and occurring in a VBNC state by Signoretto et al. (2004; 2005).

The experiment according to Le Codiac et al. (2012) was conducted to determine a concentration of the pathogens that seemed to be harmless to the health of *D. magna*. Since the zooplankton organism should survive the co-cultivation experiments with the pathogens over 48 h.

The experiments with *P. aeruginosa* showed that with increasing bacterial concentration more rapid death of daphnids occurred. For *A. hydrophila* different results were obtained and the first zooplankton organisms died after 25 h. This was observed independent of the bacterial densities. With *E. faecalis* no toxic effects on *D. magna* were observed, no death occurred within 28 h.

D. magna can be used to assess acute pathogenity of organisms relevant to human health, such as *P. aeruginosa*. The toxicity to *D. magna* might be caused by secretion of various toxic compounds by *P. aeruginosa* (e.g. rhamnolipids, elastase; Le Codiac et al., 2012). Some strains of *Pseudomonas* spp. are able to produce secondary metabolites that have the characteristic to inhibit or kill invertebrates, including *Daphnia* (Padmanabhan et al., 2005), or to inactivate other pathogens, like *A. hydrophila* (Jagmann et al., 2010). Tan et al. (1999) found *P. aeruginosa* accumulating in the intestine of nematodes and killing their hosts slowly by an infection-like process. *D. magna* incubated with a virulent strain of *P. aeruginosa* (PT 894) died within 6 hours and with the wild-type virulent strain *P. aeruginosa* PAO1 daphnids died over a period of 7 h in experiments of Le Codiac et al. (2012).
Daphnia can be seen as a model organism to analyze associations with several bacterial environmental pathogens in a natural context and also mounting opportunistic infections in humans (Ebert, 2008; Le Codiac et al., 2012).

Co-cultivation experiments were investigated with *D. magna* in association with the pathogens, *P. aeruginosa*, *A. hydrophila* and *E. faecalis* under defined conditions over a period of 48 h. The aim was the examination of the question whether the bacteria prefer the free-living state in the inoculation medium (1), if they are attached to the integument of the cladoceran (2), infiltrated and accumulated in the gut (3) or if they prefer the adhesion to the surface of the polystyrene well (4) (Figure 4.2).

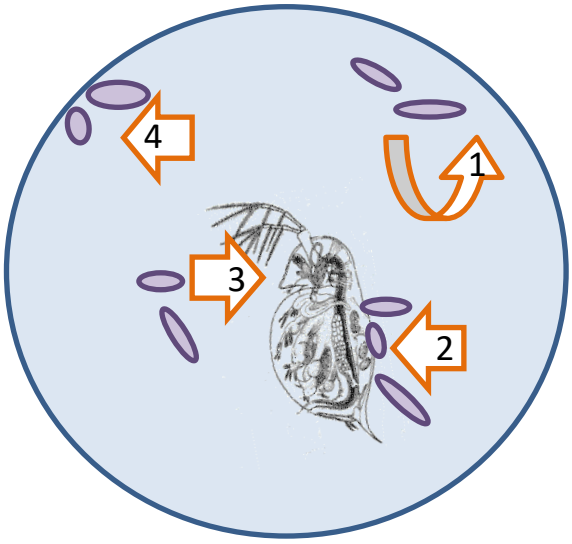


Figure 4.2 Schematic overview of attachment sites for hygienically relevant organisms in a well of the co-cultivation system with *D. magna*. (1) Bacteria free-living in the medium, (2) Attachted to the carapace of *D. magna*, (3) infiltrated and located in the gut, (4) attachment to the polystyrene well. (Source of *D. magna* picture: http://www.stu.hochschule-reutlingen.de/images/stoffp3.gif)

The distribution of the pathogenic organisms in the different compartments available in the batch culture were investigated and balances were calculated.

Co-cultivation of *P. aeruginosa* with *D. magna* revealed determination of the opportunistic pathogen over the experimental period of 48 h. A shift in the distribution of *P. aeruginosa* from the free-living state in the inoculation medium in the beginning of the experiments to adhesion to the carapace with preference and to the surface of the well was observed. Culturability decreased in all compartments during 24 h and afterwards increased slightly, except in the well biofilm where it increased with 100 % after 48 h. By the use of FISH the concentrations of *P. aeruginosa* were up to two log units higher compared to cultivation. Associations between *P. aeruginosa* and *D. magna* were reported recently by Qi et al., (2009) in laboratory microcosms, whereas Huq et al., (1984) described the attachment of *Pseudomonas* sp. to crustacean zooplankton organisms to be weak.

In the co-cultivation experiments with *D. magna* and *A. hydrophila*, the potential pathogen was detectable over 48 h, but culturability in all compartments decreased over time. The occurence of culturable bacteria attached to the well increased. *A. hydrophila* was found in concentrations attached to the carapace of the *Daphnia* and with less concentrations in the gut. The bacterial concentrations in all compartments determined with FISH were two to three orders of magnitude higher than with cultural methods. Dumontet et al. (1996) reported that *V. cholerae* and *A. hydrophila* able to colonize on live and dead copepods within short times, while *E. coli*, *Pseudomonas* spp. and two *Vibrio* species were not present, neither on live nor on dead copepods. These observations occurred in batch cultures with copepods collected from the Gulf of Naples (Italy). *Aeromonas salmonicida* is known to exhibit enhanced growth rates when co-cultured with the protozoan *Tetrahymena pyriformis* in batch cultures (King & Shotts, 1988).

E. faecalis co-cultivated with *D. magna* could be detected in all compartments of the system over the experimental period. The organism was generally less culturable compared to the other two tested pathogens. The culturability decreased over time, except in the well biofilm, where the cells became a little more culturable within 48 h. In the association between *D. magna* and *E. faecalis,* the highest amount of *Daphnia*-associated organisms was found in the gut and lower percentages on the carapace, whereas the situation was the other way around for *P. aeruginosa* and *A. hydrophila*. Concentrations determined with FISH were up to three orders of

magnitude higher compared to cultivation. In both lake and seawater *E. faecalis* was assumed to exist in the VBNC state, because with molecular methods the detection resulted in higher numbers than with the culure method (Signoretto et al., 2004). Mote et al. (2012) reported that the persistence of *E. faecalis* and *E. casseliflavus* was enhanced by the presence of plankton in microcosms. They suggest that plankton organisms may serve as a reservoir for growth and persistence of this faecal indicator. Zooplankton organisms may constitute an attractive environmental reservoir of enterococci. This should be disregarded in the detection and evaluation of microbiological quality of environmental samples (Signoretto et al., 2005).

In the present study associations of pathogenic bacteria with the zooplankton organism *D. magna* in co-cultivation experiments could be clearly demonstrated. It was absolutely new to observe cladoceran-pathogen associations, in particular to differentiate between the amounts of surface-attached and gut-located bacteria.
For *P. aeruginosa* and *A. hydrophila* the carapace of the daphnids were found to be the preferred attachment site (Table 4.8). The findings for *E. faecalis* were different, the organism was preferably located in the gut after 48 h.

Table 4.8 Preferential associations and attachment sites of the tested organisms (*P. aeruginosa, A. hydrophila, E. faecalis*) in the co-cultivation system with *D. magna (*intensity of association: +++ high, ++ medium, + low)

Organism	Carapace	Gut	Medium	Polystyrene well
P. aeruginosa	+++	+	+	++
A. hydrophila	+++	+	+	++
E. faecalis	+	+++	++	+

The highest culurability was determined in the biofim on the polystyrene well for *P. aeruginosa* and *A. hydrophila*. *E. faecalis* culturable cells were free living in the medium. Attachment of bacteria to nonwettable plastic surfaces, such as polystyrene, due to hydrophobic interactions has been described several times (e.g. Fletcher & Loeb, 1979; Rosenberg, 1981; McEldowny & Fletcher, 1986). Adhesion to polystyrene is dependend on nutrient availability and physical stress (Capello & Guglielmino, 2006), which can be influenced due to the interaction between *D. magna* and the bacteria in the co-culture. The adhesion of the organisms to the

polystyrene surface can be explained by (i) the low nutrient availability in the *Daphnia* medium, (ii) the stress induced by the presence of the daphnids, (iii) furthermore, in case of *P. aeruginosa,* it is known to be a primary colonizer in technical water systems and is able to develop biofilms.

E. faecalis showed low adhesion capacities, since the organism is found less attached to the carapace as well as the polystyrene well.

The quantities of investigated pathogens in co-culture with *D. magna* were higher with the FISH method than with cultural detection. With regard to the fact, that the detected rRNA could originate from already dead cells, due to the stability of rRNA. The results indicate that a fraction of the bacteria might occur in the VBNC state, particularly those associated with the *D. magna*. Associations between pathogens and plankton organisms, such as attachment to the surface of zooplankton organisms as well as accumulation inside the gut, can lead to transition of the bacteria into the VBNC state, or out of the VBNC state. Is it possible for the bacteria to return viable again? The question remains unknown and seems to be species specific. For *E. faecalis* the VBNC state was described as a surivial strategy that was induced by association with zooplankton in aquatic environments (Signoretto et al., 2004). Resuscitation for *L. pneumophila* was reported inside of amoebae (Steinert et al., 1997).

Daphnia are effective filter feeder and presumably show no selectivity between filtering small algae or large bacteria, but they are effective grazers of bacteria with typical clearance rates ranging from 0.1 – 2.8 ml/individual/hour (DeMott, 1982; Jürgens, 1994; Porter et al., 1983; Tóth et al., 2001). If it is assumed that one *D. magna* filters, at least, 0.1 mL per hour, the five daphnids in the microcosms will have filtered the amount of 10 mL inoculation medium already within 20 h. Therefore it is obvious that *D. magna* living associated to bacterial suspensions will ingest a certain amount of pathogens within 48 hours by the filtering process. It is known to take between three to 20 minutes for a particle ingested by a rotifer to move across the entire digestive tract (Wetzel, 2001), this is depending on the zooplankton species, due to their body size and on the environmental conditions, e.g. temperature. The question remains if a part of the ingested bacteria is digested in the gut, or if they are released unharmed after gut passage. Furthermore they can occur in a VBNC state after gut passage.

Another possibility in the observation of associations between hygienically relevant microorganisms and plankton is the formation of bacterial biofilms on the surface of plankton. Motility and chemotactic behavior may allow auqutic bacteria to attach and detach from particles and organisms (Grossart, 2010). The formation of biofilms on zooplankton organisms seems controlled by the natural bacterial community of the plankton organism (Kirschner 2011). It is acknowledged that biotic surfaces may represent a nutrient source for certain microorganisms (Watnick & Kolter, 2000). The chitineous exoskeleton of marine crustacean zooplankton was shown to be potentially utilised by chitinase-producing bacteria such as *Vibrio* spp. in marine environments (Carli et al, 1993; Yu et al., 1991). Both *P. aeruginosa* and *A. hydrophila* are capable of excreting chitinase enzymes which might aid the organism in nutrient acquisition (DebRoy et al., 2006; Folders et al., 2001; Wang & Chang, 1997). However, Jagmann et al. (2010) found *A. hydrophila* to grow with chitin, while *P. aeruginosa* did not. It is unknown whether growth of the organisms can be promoted by the degradation of chitin derived from living crustacean carapaces.

Despite feeding on the plankton surface itself, the pathogens may thrive on other plankton-associated compounds.

In the studies dealing with the association of pathogens and plankton organisms, different phenomenons are described. Some studies report enhancement of growth of hygienically relevant organisms in the presence of zooplankton. Kirschner et al. (2011) detected significant promotion of growth of *V. cholera* by addition of cladocerans to laboratory microcosms. Otherwise bacterial numbers of *C. jejuni* were found to be declining with up to 91 % in association with *Daphnia carrinata*, presumably they were killed during passage through the gut (Schallenberg et al. 2005). The latter case, pathogen concentrations and hence the risk of contracting waterborne diseases could be reduced by food web biomanipulations. This could be accomplished by enhancing the densities of e.g. *Daphnia* in recreational water bodies and drinking water reservoirs, which graze effectively on pathogens and lead to death during the gut passage (Schallenberg et al. 2005).

4.2 Conclusions

As a main objective of this study, the association of hygienically relevant microorganisms with freshwater plankton was clearly demonstrated. All hygienically relevant bacteria considered in this study were found associated to plankton (except for *Campylobacter* spp.) and *Elodea nuttallii* as well as in the water of Lake Baldeney. Relationships between pathogenic bacteria and plankton have been acknowledged before, mainly in marine environments (e.g. Maugeri et al., 2004), but no information was hitherto available on whether plankton may represent a potential reservoir for these pathogens.

Plankton organisms may provide large surfaces for attachment of bacteria. The calculated surface areas were enormous, especially in some seasons. In July zooplankton surfaces were about 45 cm^2/L.The fact, that plankton-associated biofilms are a site for survival and multiplication of opportunistic pathogens (Maugeri et al., 2004), highlights that plankton must be considered as a potential reservoir and vector for pathogens (Lipp et al., 2002). Bacteria attached to plankton are transported within the water body (Carli et al., 1993) and can contaminate the surrounding water column by detaching from the plankton-associated biofilm (Watnick & Kolter, 2000). Bacteria associated with zooplankton organisms migrating through the water column cover long distances during their travels and expand their habitat (Grossart 2010; van der Gucht et al., 2007). Although plankton microhabitats may account for a minor fraction of the total bacterial biomass in aquatic environments, they represent dense local populations of bacteria. In terms of bacterial processes they seem to be more active and dynamic than the bulk water. The association of plankton with bacteria can spatially enhance bacterial concentration, increase the possibility for humans to be exposed to infectious doses and therefore pose a health concern (Omar et al. 2002).

Free-living and particle-associated bacterial communities should not be perceived as separate entities, but rather as interacting assemblages. There is an active exchange of bacteria between plankton organisms and the surrounding water (Hansen & Bech, 1996; Riemann & Winding, 2005). The bacteria actively attach to the phyto- or zooplankton surface, and furthermore zooplankton organisms have active mechanisms to remove the attachted bacteria, either by active emigration or predation by bacterivorous protozoans (Simon et al., 2002; Kiørboe et al., 2002, 2003).

All of the investigated organisms are known to possibly enter the VBNC state under stress influence. Hence, it has to be considered that the concentrations determined with cultural methods underestimate the real abundance. This has been proved for *P. aeruginosa* and *L. pneumophila*, determined with orders of magnitude higher concentrations by the use of qPCR and FISH in water and plankton samples. These results should be handled with care, due to the mentioned limitations of those methods, such as overestimation by extracellular DNA, or prolonged stability of rRNA after cell death.

Co-cultivation experiments of *Daphnia magna* with selected hygienically relevant bacteria showed associations due to colonization of the zooplankton integument and of the gut. Culturablility decreased during the experimental period which accompanied the assumption of the bacteria entering the VBNC state, when associated to plankton.

Since copepods are the main dietary constitutens of many marine carnivores, including fish, bacterial attachment to the zooplankton integument can contribute to the transfer of pathogens through the food chain (Dumontet et al., 1996).

However, the association of pathogens to zooplankton organisms is preferentially compared to non-living particles. While a non-living particle contains a finite amount of organic substrates, zooplankton organisms supply bacteria continuously with organic substrates due to their own feeding processes.

Another possibility is the ingestion of bacteria by zooplankton organisms. Therefore the loss mechanism would be defecation. The bacteria can be released together with the gut flora by defecation (Tang, 2005). The faecal pellets of zooplankton organisms are known to contain high concentrations of bacteria (Hansen et al., 2001; Tang et al., 2001). The question remains if bacteria replicate inside of plankton, remain viable and if they are released unharmed and active after gut passage or in a VBNC state. There is evidence that some bacteria survive the passage through the gut, whereas others are digested or biodegraded. Colonization of the egg sac of copepods was documented by Huq et al. (1983), where multiplication of the colonizing bacteria was observed. Attachment of pathogens to plankton is documented as a vectoring mode, whereas most studies deal with *Vibrio cholerae* (Cottingham et al., 2003; Huq et al., 1983; Huq et al., 2005; Lipp et al., 2003). It is necessary to characterize the fate of pathogens digested by zooplankton. Bacteria found in contact with zooplankton or in

particular in the digestive tract were protected from chlorination and remained viable inside the digestive tract (Levy et al., 1986).

Zooplankton organisms as a habitat for pathogens might have advantages as well as disadvantages for both of the organisms. On the one hand the plankton organisms can benefit from bacterial metabolites, but on the other hand the zooplanktor can also be harmed by virulent species, as shown for *P. aeruginosa* in toxcity tests. The bacteria can benefit from metabolites excreted by plankton, or possibly degrade the chtinous carapace. If the bacteria are ingested by a zooplankton organism, they can be released viable and unharmed after gut passage, in a VBNC state or inactivated and dead. Resucitation from the VBNC state might be possible, when the bacteria are released after gut passage or by detachment of bacteria from the copepod.

Plankton organisms are ubiquitous in surface waters and some species can proliferate in granular and biological filters of water treatment plants, can be released in the filter effluent and colonize the distribution system (Castaldelli et al., 2005; Matsumoto et al., 2002; Schreiber et al., 1997). Some waterborne pathogens are known to maintain viability in amoebae or nematodes, such as *Legionella* who replicates within amoebae and benefits from the protection against disinfectents and the transport through the distribution system (e.g. Steinert et al., 1997). Most studies about zooplankton characterize their grazing activity on various species in a microbial community or their impact on other planktonic organisms. These studies do not provide information about grazing of zooplankton organisms on waterborne pathogens (Bichai et al., 2008). Furthermore the role of zooplankton organisms in pathogen transmission through drinking water, especially that pathogens are internalized in higher organisms in raw water and might be restistant to disinfection processes remains poorly understood and is less reported in literature yet (Birchai et al., 2008).

The incidence that phytoplankton might also act as a potential reservoir for pathogens was not investigated in this study. Although associations of hygienically relevant organisms with phytoplankton were observed in Lake Baldeney. Since algal exudates are known to be important nutrient sources for heterotrophic bacteria in aquatic environments, it has to be considered that there might be similar interactions than found with zooplankton. The cellular products of phytoplankton organisms can promote growth of pathogens, and there is potential for bacteria to persist and grow on these algae (Byappanahalli et al. 2003; Kaplan & Bott, 1989).

Diatoms were found to be true microbial hotspots when they are colonized by bacteria (Simon et al., 2002). They bacteria can utilize the released organic compounds of the diatoms and therefore they can display generally higher growth rates than free-living bacteria (Simon, 1987).

Znachor et al. (2012) reported several possibilities of interactions for bacteria living associated with diatoms (i) competition, bacteria compete with diatoms for inorganic nurients (Bratbak & Thingstad, 1985), (ii) commensalism, bacteria benfit from diatoms without harming them, (iii) parasitism, bacteria invade the host cell and produce metabolites which lead to cell lysis and death of the diatom (Park et al., 2010), or the diatoms can inihibit growth of bacteria by production of antibiotic compunds (Lefalive & Ten-Hage, 2009), (iv) mutualism, bacteria benefit from diatom exudates, whereas the diatoms profit from bacterial products (Pete et al., 2010; Croft et al., 2005; Droop, 2007).

Generalization of the experimental results of this study should be done with care. Because the size, abundance and physiology of plankton organisms in lakes vary spatially and temporally, this might have effects on the ambient and attached bacteria (Grossart et al., 2010). In case of eutrophy of an aquatic environment, where large quantities of plankton organisms are available, the plankton-bacteria associations might be increased dramatically. The bacterial load and proliferation of potential human pathogens within the aquatic habitat would be enhanced drastically. This is of relevance considering human health in drinking water production and recreational use of the surface water.

However, the results indicate that it is of concern to consider the issue of plankton organisms acting as vectors for human pathogens and the possibility that a single organism might carry an infectious dose through a drinking water distribution system.

When considering health risks associated with pathogens being protected by zooplankton organisms in drinking water, there are three main objectives that have to be regarded (i) the source water, (ii) the effluent water, due to colonization of granular media filters by invertebrates, (iii) the water distribution system, since *L. pneumophila* is known to proliferate within amoebae in distribution systems (Birchai et al., 2008).

5 Literature

AFNOR XP T90-471 Standard (2006): Detection and quantification of *Legionella* and *Legionella pneumophila* by concentration and PCR amplification.

Al-Bakri, A. G.; Gilbert, P.; Allison, D. G. (2004): Immigration and emigration of *Burkholderia cepacia* and *Pseudomonas aeruginosa* between and within mixed biofilm communities. *Journal of Applied Microbiology* **96**, 455-463.

Alfredsson, G.A., Xiang, J.Y., Gudmundsson, H.M., Gudmundsson, S. (1995): *Aeromonas* from marine sources in Iceland. *Proceedings Fifth International Symposium Aeromonas and Plesiomonas*, April 8-9. Heriot-Watt University, Edinburgh, Scotland.

Amann, R. I., Krumholz, L., Stahl, D. A. (1990): Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. *Journal of Bacteriology* **172**: 762–770.

Anaissie, F.J., Penzak, S.R., Dignani, M.C. (2002): The hospital water supply as a source of nosocomial infections. *Archives of International Medicine* **162**, 1483-1492.

Andersson, S., Kuttuva Rajarao, G., Land, C. J., Dalhammar, G. (2008): Biofilm formation and interactions of bacterial strains found in wastewater treatment systems. *FEMS Microbiology Letters* **283**, 83–90.

Araujo, R.M., Arribas, R.M., Lucena, F., Pares, R. (1989): Relation between *Aeromonas* and faecal coliforms in fresh waters. *Journal of Applied Microbiology* **97**: 213-217.

Araujo, R. M., Arribas, R. M., Pares, R. (1991): Distribution of *Aeromonas* species in waters with different levels of pollution. *Journal of Applied Bacteriology* **71**: 182-186.

Armisen, T. G., Servais, P. (2004): Combining direct viable count (DVC) and fluorescent in situ hybridisation (FISH) to enumaerate viable *E. coli* in rivers and wastewaters. *Water, Science and Technology* **50**: 271-275.

Armont, R., Payment, P. (1988): A modified m-CP medium for enumerating *Clostridium perfringens* from water samples. *Canadian Journal of Microbiology*. **34**: 78-79.

Arvanitidou, M., Constantinidis, T. C., Katsouyannopoulos V. (1995): A survey on *Campylobacter* and *Yersinia* spp. Occurrence in sea and river waters in Nothern Greece. *The Science of the Total Environment* **171**: 101-106.

Austin, B., Austin, D A., (1993): *Aeromonadaceae* representatives (*Aeromonas salmonicida*) In: Austin, B., Austin, D. A. (eds) Bacterial fish pathogens: disease in farmed and wild fish, 2nd edn. Ellis Horwood Ltd. Chichester, p. 86-170.

Balzer, M., Witt, N., Flemming, H.-C., Wingender, J. (2010): Faecal indicator bacteria in river biofilms. *Water Science and Technology* **61**: 1105-1111.

Banning, N., Toze, S., Mee, B.J., (2002): *Escherichia coli* survival in groundwater and effluent measured using a combination of propidium iodide and the green fluorescent protein. *Journal of Applied Microbiology* **93**: 69–76.

Behets, J., Declerck, P., Delaedt, Y., Creemers, B., Ollevier, F. (2007): Development and evaluation of a TaqMan duplex real-time PCR quantification method for reliable enumeration of *Legionella pneumophila* in water samples*. Journal of Microbiological Methods* **68**: 137-144.

Berland, B. R., Bonin, D. J., Durbec, J. P., Maestrini, S. Y. (1976): Typologie de bacteries prelevées devant le delta du Rhone. Classification hierarchique par une method d'aggregation suviant la variance. *International Review of Hydrobiology*. **61**: 359-372

Bichai, F., Payment, P., Barbeau, B. (2008): Protection of waterborne pathogens by higher organisms in drinking water: a review. *Canadian Journal of Microbiology* **54**: 509-524.

Bidle, K.D., Fletcher, M. (1995): Comparison of free-living and particle-associated bacterial communities in the Chesapeake Bay by stable low-molecular-weight RNA analysis. *Applied and Environmental Microbiology* **61**: 944-952.

Binzstein, N., Costagliola M. C., Pichel, M., Jurquiza, V., Ramirez, F. C., Akselmann, R., Vacchino, M., Huq, A., Colwell, R. (2004): Viable but nonculturable *Vibrio cholerae* O1 in the aquatic environment of Argentina. *Applied Environmental Microbiology* **70**: 7481-7486.

Birch, L., Dawson, C.E., Cornett, J.H., Keer, J.T., (2001): A comparison of nucleic acid amplification techniques for the assessment of bacterial viability. *Letters in Applied Microbiology* **33**: 296– 301.

Bisson, J. W., Cabelli, V. J. (1979): Membrane Filter Enumeration Method for *Clostridium perfringens*. *Applied and Environmental Microbiology* **37**: 55-66.

Bjergbæk, L. A., Roslev, P. (2005): Formation of nonculturable *Escherichia col*i in drinking water. *Journal of Applied Microbiology* **99**: 1090-1098.

Bomo, A.-M., Storey, M. V., Ashbolt, N. J. (2004): Detection, integration and persistence of aermonads in water distribution pipe biofilms. *Journal of Water and Health* **2**: 83-96.

Bonetta, S., Bonetta, S., Ferretti, E., Balocco, F., Carraro, E. (2010): Evaluation of *Legionella pneumophila* contamination in Italian hotel water systems by quantitative real-time PCR and culture methods. *Journal of Applied Microbiology* **108**: 1576-1583.

Bottari, B., Ercolini, D., Gatti, M., Neviani, E. (2006): Application of FISH technology for microbiological analysis: current state and prospects. *Applied Microbiology and Biotechnology* **73**: 485–494.

Boulanger, C.A., Edelstein, P.H. (1995): Precision and accuracy of recovery of *Legionella pneumophila* from seeded tap water by filtration and centrifugation. *Applied and Environmental Microbiology* **61**: 1805-1809.

Bratbak, G., Thingstad, T. F. (1985): Phytoplankton-bactria interactions: an apparent paradox? Analysis of a model system with both competition and commensalism. *Marine Ecology Progress Service* **25**: 23-30.

Brendelberger, H. (1991): Filter mesh size of cladocerans predicts retention efficiency for bacteria. *Limnology and Ocenography* **36**: 884-894.

Brennhovd, O., Kapperud, G., Langeland, G. (1992): Survey of thermotolerant *Campylobacter* spp. and *Yersinia* spp. In three surface water sources in Norway. *International Journal of Food Microbiology* **15**: 327-338.

Buchanan, R. L., Palumbo, S. A. (1985): *Aeromonas hydrophila* and *Aeromonas sobria* as potential food poisening species: a review. *Journal of Food Safety* **7**: 15-29.

Bustin, S.A., Nolan, T.: Chemistries. In: Bustin, S.A. (ed.) (2004): A-Z of Quantitative PCR. *International University Line*, La Jolla, CA, USA: 215-278.

Buswell CM, Herlihy YM, Lawrence LM, McGuiggan JTM, Marsh PD, Keevil CW, Leach SA(1998): Extended survival and persistence of *Campylobacter* spp. in water and aquatic biofilms and their detection by immunofluorescent-antibody and -rRNA staining. *Applied Environmental Microbiology* **64**:733–741.

Byappanahalli, M., Fowler, M., Shively, D., Whitman, R. (2003): Ubiquity and Persistence of *Escherichia coli* in a Midwestern Coastal Stream. *Applied and Environmental Microbiology* **69**: 4549-4555.

Byappanahalli, M.N., Shively, D.A., Nevers, M.B., Sadowsky, M.J., Whitman, R.L. (2003): Growth and survival of *Escherichia coli* and enterococci populations in the macro-alga *Cladophora* (Chlorophyta). *FEMS Microbiology Ecology **46**: 203-211.*

Byappanahalli, M.N., Whitman, R.L., Shively, D.A., Ferguson, J.F., Ishii, S., Sadowsky, M.J. (2007): Population structure of cladophora-borne *Escherichia coli* in nearshore water of Lake Michigan. *Water Research* **41**: 3649-3654.

Cabelli, V. J., Dufour, A. P., McCabe, L. J., Levin, M. A. (1983): Swimming-associated gastroenteritis and water quality. *American Journal of Epidemiology* **115**: 606-616.

Camper, A.K., Jones, W.L., Hayes, J.T. (1996): Effect of growth conditions and substratum composition on the persistence of coliforms in mixed-population biofilms. *Applied and Environmental Microbiology* **62**:4014–4018.

Cappelier, J. M., Minet, J., Magras, C., Colwell, R. R., Federighi, M. (1999): Recovery in embryonated eggs of viable but nonculturable *Campylobacter jejuni* cells and maintenance of ability to adhere to HeLa cells after resuscitation. *Applied Environmental Microbiology* **65**: 5154-5157.

Capello, S., Guglielmino, S. P. P. (2006): Effects of growth temperature on polystyrene adhesion of *Pseudomonas aeruginosa* ATCC 27853. *Brazilian Journal of Microbiology* **37**: 205-207.

Carbone, M., Maugeri, T.L., Gugliandolo, C., La Camera, E., Biondo, C., Fera, M.T. (2005): Occurrence of *Helicobacter pylori* DNA in the coastal environment of southern Italy (Straits of Messina). *Journal of Applied Microbiology* **98**: 768-774.

Carli, A., Pane, L., Casareto, L., Bertone, S., Pruzzo, C. (1993): Occurrence of *Vibrio alginolyticus* in ligurian coast rock pools (Tyrrhenian Sea, Italy) and its association with the copepod *Tigriopus filvus* (Fisher 1860). *Applied and Environmental Microbiology* **59**: 1960-1962.

Carman, K. R., Dobbs, F. C. (1997): Epibiotic microorganisms on copepods and other marine crustaceans. *Microscopy research and technique* **37**: 116-135.

Carter, A. M., Pacha, R. E., Clark, G. W., Williams, E. A. (1987): Seasonal occurrence of *Campylobacter* spp. in surface waters and their correlation with standard indicator bacteria. *Applied Environmental Microbiology* **53**: 523-526.

Carvalho, F. R. S., Vazoller, R. F., Foronda, A. S., Pellizari, V. H. (2007): Phylogenetic study of *Legionella* Species in pristine and polluted aquatic samples from a tropical Atlantic Forest ecosystem. *Current Microbiology* **55**: 288-293.

Castadelli, G., Mantovani, S., Benvenuti, M. R., Rossi, R., Fano, E. A. (2005): Invertebrate colonization of GAC filters in a potabilisation plant treating groundwater. *Journal of Water Supply, Research and Technology* **54**: 561-568.

Chang, B., Sugiyama, K., Taguri, T., Amemura-Maekawa, J., Kura, F., Watanabe, H. (2009): Specific detection of viable *Legionella* cells by combined use of photoactivated ethidium monoazide and PCR/real-time PCR. *Applied and Environmental Microbiology* **75**: 147-153.

Chan, A.B., Fox, J.D., (1999): NASBA and other transcription-based amplification methods for research and diagnostic microbiology. *Reviews in Medical Microbiology* **10**: 185–196.

Chaveerach, P., ter Huurne, A. A. H. M., Lipman, L. J. A., van Knapen, F. (2003): Survival and resuscitation of ten strains of Campylobacter jejuni and Campylobacter coli under acid conditions. *Applied and Environmental Microbiology* **69**: 711-714.

Connelly, S. J., Wolyniak, E. A., Dieter, K. L., Williamson, C. E., Jellison, K. L. (2007): Impact of zooplankton grazing on the excystation, viability, and infectivity of the protozoan pathogens *Cryptosporidium parvum* and *Giardia lamblia*. *Applied and Environmental Microbiology* **73**: 7277-7282.

Cools, I., Uyttendaele, M., Caro, C., D´Haese, E., Neils, H. D., Debevere, J. (2003): Survival of Campylobacter jejuni strains of different origin in drinking water. *Journal of Applied Microbiology* **94**: 886-892.

Costerton, J.W., Cheng, K.-J., Geesey, G.G., Ladd, T.I., Nickel, J.C., Dasgupta, M., Marrie, T.J. (1987): Bacterial biofilms in nature and disease. *Annual Review of Microbiology* **47**: 435-464.

Cottingham, K. L., Chiavelli, D. A., Taylor, R. K. (2003): Environmental microbe and human pathogen: the ecology and microbiology of *Vibrio cholerae*. *Frontiers in Ecology and the Environment* **1**: 80-86.

Craig, D., Fallowfield, H. J., Cromar, N. J. (2002): Enumeration of faecal coliforms from recreational coastal sites: evaluation of techniques for the separation of bacteria from sediments. *Journal of Applied Microbiology* **93**: 557-565.

Craig, D., Fallowfield, H. J., Cromar, N. J. (2004): Use of microcosms to determine persistence of *Escherichia coli* in recreational coastal water and sediment and validation with in situ measurements. *Journal of Applied Microbiology* **96**: 922-930.

Croft, M. T., Lawrence, A. D. Raux-Dery, E., Warren, M. J., Smith, A. G. (2005): Algae acquire vitamin B-12 through a symbiotic relationship with bacteria. *Nature* **438**: 90-93.

Declerck, P., Behets, J., van Hoef, V., Ollevier, F. (2007): Detection of *Legionella spp.* and some of their amoeba hosts in floating biofilms from anthropogenic and natural aquatic environments. *Water Research* **41**: 3159-3167.

Declerck, P., Behets, J., Margineanu, A., van Hoef, V., De Keersmaecker, B., Ollevier, F. (2009): Replication of *Legionella pneumophila* in biofilms of water distribution pipes. *Microbiological Research* **164**: 593–603.

Declerck, P. (2010): Biofilms: the environmental playground of *Legionella pneumophila*. *Environmental Microbiology* **12**: 557-566.

DebRoy, S., Dao, J., Söderberg, M., Rossier, O., Cianciotto, N.P. (2006): *Legionella pneumophila* type II secretome reveals unique exoproteins and a chitinase that promotes bacterial persistence in the lung. *Proceedings of the National Academy of Sciences of the United States of America* **103**: 19146-19151.

Delgado-Viscogliosi, P., Simonart, T., Parent, V., Marchand, G., Dobbelaere, M., Pierlot, E., Pierzo, V., Menard-Szczebara, F., Gaudard-Ferveur, E., Delabre, K., Delattre, J.M. (2005): Rapid method for enumeration of viable *Legionella pneumophila* and other *Legionella* spp. in water. *Applied and Environmental Microbiology* **71**: 4086-4096.

DeMott, W. R. (1982): Feeding selectivities and relative ingestion rates of *Daphnia* and *Bosmina*. *Limnology and Oceanography* **27**: 518-527.

DeMott, W. R. (1986): The role of taste in food selection by freswater zooplankton. *Oecologica* **69**: 334-340.

DGL (Deutsche Gesellschaft für Limnologie) (2007): Empfehlungen zu Grundlagen einheilticher Qualitätsanforderungen limnologisch tätiger Büros und Laboratorien. DGL-Arbeitskreis Qualitätssicherung, 12.09.2007, 41 S.

DIN EN ISO 7899-2 (2000): Wasserbeschaffenheit - Nachweis und Zählung von intestinalen Enterokokken - Teil 2: Verfahren durch Membranfiltration.

DIN EN 12879 (2000) Characterization of sludges – Determination of the loss of ignition of dry mass.

DIN EN 12880 (2000) Characterization of sludges – Determination of dry residue and water content.

DIN EN ISO 16266 (2008): Wasserbeschaffenheit - Nachweis und Zählung von Pseudomonas aeruginosa – Membranfiltrationsverfahren

DIN EN ISO 19456 (2006): Water quality – Sampling for microbiological analysis (Deutsche Fassung EN ISO 19458:2006).

Directive 2006/7/EC of the European Parliament and of the Council of 15 February 2006 concerning the management of bathing water quality and repealing Directive 76/160/EEC.

Donlan, R. M. (2002): Biofilms: Microbial Life on Surfaces. *Emerging Infectious Diseases* **8**: 881-890.

Droop, M. R., (2007): Vitamins, phytoplankton and bacteria: symbiosis or scavenging? *Journal of Plankton Research* **29**:107-113.

Dumontet, S., Krovacek, K., Baloda, S.B., Grottoli, R., Pasquale, V., Vanucci, S. (1996): Ecological relationship between *Aeromonas* and *Vibrio spp.* and planktonic copepods in the coastal marine environment in Southern Italy. *Comparative Immunology, Microbiology and Infectious Diseases* **19**: 245-254.

Dumontet, S., Krovacek, K., Svenson, S.B., Pasquale, V., Baloda, S.B., Figliuolo, G. (2000): Prevalence and diversity of *Aeromonas* and *Vibrio* spp. in coastal waters of Southern Italy. *Comparative Immunology, Microbiology and Infectious Diseases* **23**, 53-72.

Dussert, B.H. (1965): Les different categories de plancton. *Hydrobiologica*, **26** 72-74.

Dwidjosiswojo, Z.S.M., Richard, J., Moritz, M.M., Flemming, H.-C., Dopp, E., Wingender, J. (2011): Influence of copper ions on the viability and cytotoxicity of *Pseudomonas aeruginosa* under conditions relevant to drinking water. International *Journal of Hygiene and Environmental Health* **214**: 485-492.

Ebert, D. (2005): Ecology, Epidemiology, and evolution of parasitsm in *Daphnia* [Internet]. Bethesda (MD): National Center for Biotechnology Information (US).

Ebert, D. (2008): Host-parasite coevolution: insights from the *Daphnia*-parasite model system. *Current Opinion in Microbiology* **11**: 290-301.

EC (European Community). Richtlinie 98/83/EG des Rates vom 3. Nov. 1998 über die Qualität von Wasser für den menschlichen Gebrauch, Amtsblatt der Europ. Gemeinschaften v. 5.12.98, L330/32-54 mit Berichtigung v. 19.2.99, L45/55,1998.

Edwards, D. D., McFeters, G. A., Venkatesan, M. I. (1998): Distribution of *Clostridium perfringens* and fecal sterols in a benthic coastal marine environment influenced by the sewage outfall from McMurdo Station, Antartica. Applied *Environmental Microbiology* **64**: 2596-2600.

Eiler, A., Bertilsson, S. (2004): Composition of freshwater bacterial communities associated with cyanobacterial blooms in four Swedish lakes. *Environmental Microbiology* **6**:1228-1243.

Exner, M., Kramer, A., Kistemann, T., Gebel, J., Engelhardt, S. (2007). Wasser als Infektionsquelle in medizinischen Einrichtungen, Prävention und Kontrolle. *Bundesgesundheitsbl Gesundheitsforsch Gesundheitsschutz* **50**, 302–311.

Felföldi, T., Heeger, Z., Vargha, M., Marialigeti, K. (2010): Detection of potentially pathogenic bacteria in the drinking water distribution system of a hospital in Hungary. *Clinical Microbiology and Infection* **16**: 89–92.

Feuerpfeil, I., Rädel, U., Exner, M., 2009. Coliforme Bakterien im Trinkwasser. Empfehlung zur Risikoabschätzung und Maßnahmen bei systemischer Kontamination - Empfehlung des Umweltbundesamtes nach Anhörung der Trinkwasserkommission des Bundesministeriums für Gesundheit beim Umweltbundesamt. *Bundesgesundheitsbl. Gesundheitsforsch. Gesundheitsschutz* **52**, 474-482

Fields, B.S., Benson, R.F., Besser, R.E. (2002): *Legionella* and Legionnaires' Disease: 25 Years of Investigation. *Clinical Microbiology Reviews* **15**: 506-526.

Fisher, K., Philips, C. (2009): The ecology, epidemiology and virulence of *Enterococcus*. *Microbiology* **155**: 1749-1757.

Fiume, L., Bucca Sabattini, M.A., Poda, G. (2005): Detection of *Legionella pneumophila* in water samples by species-specific real-time and nested PCR assays. *Letters in Applied Microbiology* **41**: 470-475.

Flemming, H.-C., Szewzyk, U., Griebe, T. (eds) (2000): Biofilms. Investigative methods and applications. Technomic Publishers. Lancaster, Basel.

Flemming, H.-C., Wingender, J. (2010): The biofilm matrix. *Nature Reviews Microbiology* **8**: 623-633.

Flemming, H.-C., (eds.), Wingender, J., Szewzyk, U. (2011): Biofilm Highlights, Springer Series on Biofilms 5, Springer Verlag Berlin Heidelberg.

Fletcher, M., Loeb, G. I. (1979): Influence of substratum characteristics on the attachment of a marine pseudomonad to solid surfaces. *Applied and Environmental Microbiology* **37**: 67-72.

Fliermans, C.B., Cherry, W.B., Orrison, L.H., Smith, S.J., Tison, D.L., Pope, D.H. (1981): Ecological Distribution of *Legionella pneumophila*. *Applied and Environmental Microbiology* **41**: 9-16.

Folders J., Tommassen, J., van Loon, L.C., Bitter, W. (2000): Identification of a Chitin-Binding Protein Secreted by *Pseudomonas aeruginosa*. *Journal of Bacteriology* **182**: 1257-1263.

Folders, J., Algra, J., Roelofs, M.S., van Loon, L.C., Tommassen, J., Bitter, W. (2001): Characterization of *Pseudomonas aeruginosa* chitinase, a gradually secreted protein. *Journal of Bacteriology* **183**: 7044-7052.

Frost, J. A. (2001): Current epidemiological issues in human campylobacteriosis. *Journal of Applied Microbiology* **90**: 85S-95S.

Fujioka, R. S., Shizumura, L. K. (1985): *Clostridium perfringens* a reliable indicator of stream water quality. *Journal WPCF* **57** (10): 986-992.

Galindo, C. L., Gutierrez, C. Jr, Chopra, K. (2006): Potential involvement of galectin-3 and SNAP23 in *Aeromonas hydrophila* cytotoxic enterotoxin-induced host cell apoptosis. Microbial Pathogenesis **40**: 56-68.

Garbey, C., Murphy, K. J., Thiebaut, G., Muller, S. (2004): Variation in P-Content in aquatic plant tissue offers an efficient tool for determining plant growth strategies along a resource gradient. *Freshwater Biology* **49**: 1 – 11.

German Drinking Water Ordinance (Trinkwasserverordnung-TrinkwV 2001), 2001. Verordnung über die Qualität von Wasser für den menschlichen Gebrauch. Trinkwasserverordnung vom 21. Mai 2001 (BGBl. I S. 959), die durch Artikel 363 der Verordnung vom 31. Oktober 2006 (BGBl. I S. 2407) geändert worden ist (in German).

Gophen, M., Geller, W. (1984): Filter mesh size and food particle uptake by *Daphnia*. *Oecologica* **64**: 408-412.

Greub, G., Raoult, D. (2004): Microorganisms Resistant to Free-Living Amoebae. *Applied and Environmental Microbiology* **17**: 413-433.

Giebler, C., Mindl, B., Slezak, D. (2001): Combining DAPI and SYBR Green II for the enumeration of total bacterial numbers in aquatic sediments. *International Reviews in Hydrobiology* **86**: 453-465.

Grimm, D., Merkert, H., Ludwig, W., Schleifer, K. H., Hacker, J., Brand, B. C. (1998): Specific detection of *Legionella pneumophila*: construction of a new 16S rRNA-targeted oligonucleotide probe. *Applied and Environmental Microbiology* **64**: 2686–2690.

Grobe, S. Schaule, G. Wingender, J., Flemming, H.-C. (2010). Mikrobiologische Kontaminationen im Trinkwasser - Ursachenermittlung. *Energie-Wasser-Praxis* **61**, 18-21.

Grossart, H.-P., Riemann, L., Azam, F. (2001): Bacterial motility in the sea and its biogeochemical implications. *Aquatic Microbial Ecology* **25**: 247-258.

Grossart, H.-P. (2010): Ecological consequences of bacterioplankton lifestyles: changes in concepts are needed. *Environmental Microbiology reports* 1-9.

Grossart, H.-P., Dziallas, C., Leunert, F., Tang, K. W. (2010): Bacterial dispersal by hitchhiking on zooplankton. *Proceedings of the National Acadamy of Sciences*: 1-6.

Grossart, H.-P., Dziallas, C., Tang, K. W. (2009): Bacterial diversity associated with freshwater zooplankton. *Environmental Microbiology Reports* **1**: 50-55.

Guentzel, M. N. (1996): Escherichia, Klebsiella, Enterobacter, Serratia, Citrobacter, and Proteus. In: Baron, S., Albrecht, T., Castro, G., Couch, R. B., Davis, C. P., Dianzani, F., McGinnis, M. R., Niesel, D. W., Olson, L. J., Woods, G. L. (eds) Medical Microbiolgy, 4th Edition. The University of Texas Medical Branch, Galveston, USA, Chapter 26.

Guerrieri, E., Bondi, M., Sabia, C., de Niederhäusern, S., Borella, P., Messi, P. (2008): Effect of bacterial interference on biofilm development by *Legionella pneumophila*. *Current Microbiology* **57**: 532–536.

Guillard, R.R.L. (1975): Culture of phytoplankton for feeding marine invertebrates. pp 26-60. *In* Smith W.L. and Chanley M.H (Eds.) *Culture of Marine Invertebrate Animals*. Plenum Press, New York, USA.

Hansen, B., Bech, G. (1996): Bacteria associated with a marine planktonic copepod in culture. Bacterial genera in sea water, body surface, intestines and fecal pellet and succession during fecal pellet degradation. *Journal of Plankton Research* **18**: 257-273.

Hansen, B., Fotel, F. L., Jensen, N. J., Madsen, S. D. (1996): Bacteria associated with a marine planktonic copepod culture, II. Degradation of fecal pellets produced on a diatom, a nanoflagellate or a dinoflagellate diet. *Journal of Plankton Research* **18**: 275-288.

Hardalo, C., Edberg, S.C. (1997): *Pseudomonas aeruginosa*: Assessment of Risk from Drinking Water. *Critical Reviews in Microbiology* **23**: 47-75.

Havelaar, A. H., During, M., Versteegh, J. F. M. (1987): Ampicillin-dextrin agar medium for the enumeration of *Aeromonas* species in water by membrane filtration.*Journal of Applied Bacteriology* **62**: 297-287.

Hazen, T.C., Esch, G.W. (1983): Effect of effluent from nitrogen fertilizer factory and a pulp mill on the distribution and abundance of *Aeromonas hydrophila* in Albermarle Sound, North Carolina. *Applied and Environmental Microbiology* **45**: 31-42.

Heidelberg, J.F., Heidelberg, K.B., Colwell, R.R. (2002): Bacteria of the gamma-Subclass *Proteobacteria* Associated with Zooplankton in Chesapeake Bay. *Applied and Environmental Microbiology* **68**: 5498-5507.

Heim, S., Lleò, M.M., Bonato, B., Guzman, C.A., Canepari, P. (2002): The Viable but nonculturable state and starvation are different stress responses of *Enterococcus faecalis*, as determinate by proteome analysis. *Journal of Bacteriology* **184**: 6739-6745.

Hempel. M., Blume, M., Blindow, I., Gross, E. M. (2008): Epiphytic bacterial community composition on two common submerged macrophytes in brackish water and freshwater. *BioMed Central Microbiology* **8**.

Hervàs, A., Camaero, L., Reche, I., Casamayor E. O. (2009): Viability and potential for migration of airborne bacteria from Africa that reach high mountain lakes in Europe. *Environmental Microbiol*ogy **11**: 1612-1623.

Hoadley, A.W.: Potential health hazards associated with *Pseudomonas aeruginosa* in water. In: Hoadley, A.W., Dutka, B.J. (eds.) (1977): Bacterial indicators/health hazards associated with water. *American Society for Testing and Materials*, Philadelphia, PA, USA: 80-114.

Hobbie, J.B., Daley, E.J., Jasper, S. (1977): Use of nuclepore filters for counting bacteria by fluorescence microscopy. *Applied and Environmental Microbiology* **33**: 1225-1228.

Hoehn, E., Clasen, J., Scharf, W., Ketelaars, H. A. M., Nienhüser, A. E., Horn, H., Kersken, H., Ewig, B. (1998) Erfassung und Bewertung von Planktonorganismen. Arbeitsgemeinschaft Trinkwassertalsperren e. V., Arbeitskreis Biologie. ATT Technische Information Nr. 7, R. Oldenbourg Verlag, München

Hornemann, A. J., Ali, A., Abbott S. (2007). *Aeromonas*, p. 716-722. In. Murray, P. R., Baron, E. J., Jorgensen, J. H., Landry M. L., Pfaller, M. A. (ed.), Manual of clinical microbiology, 9th ed. ASM press, Washington, DC, USA.

Howe, G.B., Loveless, B.M., Norwood, D., Craw, P., Waag, D., England, M., Lowe, J.R., Courtney, B.C., Pitt, M.L., Kulesh, D.A. (2009): Real-time PCR for the early detection and quantification of *Coxiella burnetti* as an alternative to the murine bioassay. *Molecular and Cellular Probes* **23**: 127-131.

Huq, A., Small, E.B., West, P.A., Huq, M.I., Rahman, R., Colwell, R.R. (1983): Ecological relationships between *Vibrio cholerae* and planktonic crustacean copepods. *Applied and Environmental Microbiology* **45**: 275-283.

Huq, A., West, P.A., Small, E.B., Huq, I., Colwell, R.R. (1984): Influence of water temperature, salinity, and pH on survival and growth of toxigenic *Vibrio cholerae* serovar O1 associated with live copepods in laboratory microcosmos. *Applied and Environmental Microbiology* **48**, 420-424.

Huq, A., Sack, R. B., Nizam, A., Longini, I. M., Nair, G. B., Ali, A. (2005): Critical factors influencing the occurrence of *Vibrio cholera* in the environment of Bangladesh. *Applied and Environmental Microbiology* **71**: 4645-4653.

Ishii, S., Yan, T., Shivley, D. A., Byappanahalli, M. N., Whitman, R. L., Sadowsky, M. J. (2006): *Cladophora* (Chlorophyta) spp. harbor human bacterial pathogens in nearshore water of Lake Michigan. *Applied and Environmental Microbiology* **72**, 4545 – 4553,

Islam, M.S., Drasar, B.S., Bradley, D.J. (1989): Attachment of toxigenic *Vibrio cholera* O1 to various freshwater plants and survival with filamentous green algae Rhizoclonium fontanum. *Journal of Tropical Medicine and Hygiene* **92**: 396-401.

Islam, M.S., Drasar, B.S., Bradley, D.J. (1990): Longterm persistence of toxigenic *Vibrio cholerae* O1 in the mucilaginous sheath of a blue-green alga, *Anabaena variabilis*. Journal of Tropical Medicine and Hygiene **93**: 133-139.

Islam, M.S., Mahmuda, S., Morshed, M. G., Bakht, H. B., Khan, M. N., Sack, R. B., Sack, D. A. (2004): Role of cyanobacteria in the persistence of *Vibrio cholera* O139 in saline microcosms. *Canadian Journal of Microbiology* **50**: 127-131.

Islam, M.S., Jahid, M.I.K., Rahman, M.M., Rahman, M.Z., Islam, M.S., Kabir, M.S., Sack, D.A., Schoolnik, G.K. (2007): Biofilm acts as a microenvironment for plankton-associated *Vibrio cholerae* in the aquatic Eenvironment of Bangladesh. *Microbiology and Immunology* **51**: 369-379.

ISO 11731 (1998): Water quality – detection and enumeration of Legionella.

ISO 11731-2 (2004): Water quality – detection and enumeration of Legionella. Part 2: direct membrane filtration method for waters with low bacterial counts.

ISO 17995 (2005): Water quality -- Detection and enumeration of thermotolerant Campylobacter species.

Jagmann, N., Brachvogel, H.P., Philipp, B. (2010): Parasitic growth of *Pseudomonas aeruginosa* in co-culture with the chtiniolytic bacterium *Aeromonas hydrophila*. *Environmental Microbiology* **12**: 1787-1802.

Janda, J. M., Duffey, P. S. (1988): *Aeromonas* in human disease: Current taxonomy, laboratory identification, and infectious disease spectrum. *Reviews of Infectious Diseases* **10**: 980-997.

Janda, J. M. (1991): Recent advances in the study of the taxonomy, pathogenicity and infectious syndromes associated with genus *Aeromonas*. *Clinical Microbiology Reviews* **4**:397-410.

Jett, B. D., Huycke, M. M. & Gilmore, M. S. (1994). Virulence of enterococci. *Clinical Microbiology Reviews **7**,* 462–478.

Joly, P., Falconnet, P.-A., André, J., Weill, N., Reyrolle, M., Vandenesch, F., Maurin, M., Etienne, J., Jarraud, S. (2006): Quantitative real-time *Legionella* PCR for environmental water samples: Data Interpretation. *Applied and Environmental Microbiology* **72**: 2801-2808.

Jones, K., Betaieb, M., Telford, D. R. (1990): Thermophilic campylobacters in surface waters around Lancaster UK: negative correlation with campylobacter infections in the community. *Journal of Applied Bacteriology* **69**: 758-764.

Joseph, S. W., Carnahan, A. (1994): The isolation, identification and systematics of the motile *Aeromonas* species. Annual review of Fish diseases **4**: 315-343.

Joshua, G. W., Guthrie-Irons, C., Karlyshev, A. V., Wren, B. W. (2006): Biofilm formation in *Campylobacter jejuni*. *Microbiology* **152**:387–396.

Jürgens, K. (1994): Impact of *Daphnia* on planktonic microbial food webs – a review. *Mar. Microb. Food webs* **8**: 295-324.

Kämpfer, P., Erhart, R., Beimfohr, C., Bohringer, J., Wagner, M., Amann, R. (1996): Characterization of bacteral communities from activated sludge: Culture-dependent numerical identification versus in situ identification using group- and genus-specific rRNA-targeted oligonucleotide probes. *Microbial Ecology* **32**: 101-121.

Kalmbach, S., Manz, W., Szewzyk, U. (1997): Isolation of new bacterial species from drinking water biofilms and proof of their in situ dominance with highly specific 16S rRNA probes. *Applied and Environmental Microbiology* **63**: 4164.

Kamjunke, N., Benndorf, A., Wilbert, C., Opitz, M., Kranich, J., Bollenbach, M., Benndorf, J. (1999): Bacteria ingestion by *Daphnia galeata* in a biomanipulated reservoir: a mechanism stabilizing biomanipulation? *Hydrobiologia* **403**: 109-121.

Kaneko, T., Colwell, R.R. (1975): Adsorption of *Vibrio parahaemolyticus* onto Chitin and Copepods. *Applied Microbiology* **29**: 269-274.

Kaplan, L.A., Bott, T.L. (1989): Diel fluctuations in bacterial activity on streambed substrata during vernal algal blooms: Effects of temperature, water chemistry and habitat. *Limnology and Oceanography* **34**: 718-733.

Keer, J. T., Birch, L. (2003): Molecular methods for the assessment of bacterial viability. *Journal of Microbiological Methods* **53**: 175-183.

Kell, D. B., Kaprelyants, A. S., Weichart, D. H., Harwood, C. R., Barer, M. R. (1998). Viability and activity in readily culturable bacteria: a review and discussion of the practical issues. Antonie Van Leeuwenhoek **73**, 169–187.

Khan, N.H., Ishii, Y., Kimata-Kino, N., Esaki, H., Nishino, T., Nishimura, M., Kogure, K. (2007): Isolation of *Pseudomonas aeruginosa* from open ocean and comparison with freshwater, clinical, and animal isolates. *Microbial Ecology* **53**: 173-186.

Kiefer, F., Fryer, G. (1978): Das Zooplankton der Binnengewässer, 2. Teil, Freilebende Copepoda. Buch VIII. Schweizerbart´sche Verlagsbuchhandlung.

Kimata, N., Nishino, T., Suzuki, S., Kogure, K. (2004): *Pseudomonas aeruginosa* isolated from marine environments in Tokyo Bay. *Microbial Ecology* **47**: 41-47.

King, C. H., Sanders, R. W., Shotts, E. B. Jr., Porter, K. G. (1991): Differential survival of bacteria ingested by zooplankton from a stratified eutrophic lake. *Limnology and Oceanography* **36**: 829-845.

King, C. H., Shotts, E. B. (1988): Enhancement of *Edwardsiella tarda* and *Aeromonas salmonicida* through ingestion by the ciliated protozoan *Tetrahymena pyriformis*. Federation of European Microbiological Socities. *Microbiology Letters* **51**:95-100.

Kiørboe, T., Grossart, H. P., Plough, H., Tang, K. (2002): Bacterial colonization of sinking aggregates: mechanisms and rates. *Applied and Environmental Microbiology* **68**: 3996-4006.

Kiørboe, T., Tang, K., Grossart, H. P., Plough, H. (2003): Dynamics of microbial communities on marine snow aggregates: colonization, growth, detachment and grazing mortalitiy of attached bacteria. *Applied and Environmental Microbiology* **69**: 3036-3047.

Kirschner, A.K.T., Schauer, S., Steinberger, B., Wilhartitz, I., Grim, C.J., Huq,A., Colwell, R.R., Herzig, A., Sommer,R. (2011): Interaction of *Vibrio cholerae* non-O1/non-O139 with copepods, cladocerans and competing bacteria in the large alkaline Lake Neusiedler See, Austria. *Microbial Ecology* **61**: 496-506.

Klein,G. (2003): Taxonomy, ecology and antibiotic resistance of enterococci from food and the gastro-intestinal tract. *International Journal of Food Microbiol*ogy **88**: 123-131.

Klüttgen, B., Dulmer, U., Engels, M., Ratte, H. T. (1994): ADaM, an artificial freshwater for the culture of zooplankton. *Water Research* **28**: 743-756.

Klug, M. (2004): Einsatz von Gensonden zum Nachweis von *Pseudomonas aeruginosa* in Wasser und Biofilmen. Diploma thesis, Heinrich-Heine-University Düsseldorf, Germany.

Koenraad, P. M. F. J., Rombouts, F. M., Notermans, S. H. W. (1997): Epidemiological aspects of thermophilic *Campylobacter* in water-related environments: a review. *Water Environ. Res*. **69**: 52-63.

Kogure, K., Simidu, U., Taga, N., (1979): A tentative direct microscopic method for counting living marine bacteria. *Canadian Journal of Microbiology* **25**: 415– 420.

Krovacek, K., Faris, A., Mansson, I. (1991): Growth of and toxin production by *Aeromonas hydrophila* and *Aeromonas sobria* at low temperatures. International *Journal of Food Microbiology* **13**: 165-176.

Krovacek, K., Conte , M., Galderisis, P., Morelli, G., Postiglione, A., Dumontet, S. (1993): Fatal septicaemia caused by *Aeromonas hydrophila* in patient with cirrhosis. Comparative *Immunology, Microbiology and Infectious Diseases* **16**: 267-272.

Krovacek, K., Pasquale, V., Baloda, S.B., Soprano, V., Conte, M., Dumontet, S. (1994): Comparison of putative virulence factors in *Aeromonas hydrophila* strains isolated from marine environment and human diarrhoeal cases in Southern Italy. *Applied and Environmental Microbiology* **60**: 1379-1382.

Korhonen, L.K., Martikainen, P. J. (1991): Survival of *Escherichia coli* and *Campylobacter jejuni* in untreated and filtered lake water. *Journal of Applied Bacteriology* **71**: 379-382.

Kuiper, M.W., Wullings, B.A., Akkermans, A.D., Beumer, R.R., van der Kooij, D. (2004): Intracellular proliferation of *Legionella pneumophila* in *Hartmannella vermiformis* in aquatic biofilms grown on plasticized polyvinyl chloride. *Applied and Environmental Microbiology* **70**: 6826-6833.

Kuiper, M.W., Valster, R.M., Wullings, B.A., Boonstra, H., Smidt, H., van der Kooij, D. (2006): Quantitative detection of the free-living amoeba *Hartmannella vermiformis* in surface water by using real-time PCR. *Applied and Environmental Microbiology* **72**: 5750-5756.

Langó Z. (1999): Occurrence of *Clostridia* in the sediment of an extremely polluted Danube Reach. *Acta Hydrochimica et Hydrobiologica* **27**: 19-26.

Lampert, W. (1991): The dynamics of *Daphnia* in a shallow lake. *Verhandlungen des Internationalen Verein Limnologie* **24**: 795-798.

Lampert, W. (2006): *Daphnia*: Model herbivore, predator and prey. *Polish Journal of Ecology* **54**: 607-620.

Lampert, W. (2011): *Daphnia*: development of a model organism in ecology and evolution. Excellence in Ecology Series. Book 21. International Ecology Institute, Oldendorf/Luhe, Germany.

Långmark, J., Storey, M. V., Ashbolt, N. J., Stenstrom, T. A. (2005): Biofilms in an urban water distribution system: measurement of biofilm biomass, pathogens and pathogen persistence within the Greater Stockholm Area, Sweden. Water Science and Technology **52**: 181–189.

Lau, H. Y., Ashbolt, N. J. (2009). The role of biofilms and protozoa in *Legionella* pathogenesis: implications for drinking water. *Journal of Applied Microbiol*ogy **107**, 368–378.

Lázaro, B., Cárcamo, J., Audícana, A., Perales, I., Fernández-Astorga, A., (1999): Viability and DNA maintenance in non-culturable spiral *Campylobacter jejuni* cells after long-term exposure to low temperatures. *Applied Environmental Microbiology* **65**: 4677– 4681.

Le Chevallier, M.W., Babcock, T.M., Lee, R.G. (1987): Examination and characterization of distribution system biofilms. *Applied Environmental Microbiology* **53**: 2714–2724.

Leclerc, H., Mossel, D.A.A., Edberg, S.C., Struijk, C.B. (2001): Advances in the bacteriology of the coliform group: Their suitability as markers of microbial water safety. *Annual Review Microbiology* **55**: 201-234.

Le Codiac, M., Simon, M., Marchetti, A., Ebert, D., Cosson, P. (2012): *Daphnia magna*, a host for evaluation of bacterial virulence. *Applied and Environmental Microbiology* **78**: 593-595.

Leflaive, J., Ten-Hage, L. (2009): Chemical interactions in diatoms: role of polyunsaturated aldehydes and precursors. *New Phytologist* **184**: 794-805.

Lehtola, M. J., Torvinen, E., Kusnetsov, J., Pitkanen, T., Maunula, L., von Bonsdorff, C.-H. (2007): Survival of *Mycobacterium avium*, *Legionella pneumophila*, *Escherichia coli*, and caliciviruses in drinking water-associated biofilms grown under high-shear turbulent flow. *Applied Environmental Microbiology* **73**: 2854–2859.

Levi, K., Smedley, J., Towner, K.J. (2003): Evaluation of a real-time PCR hybridization assay for rapid detection of *Legionella pneumophila* in hospital and environmental water samples. *Clinical Microbiology and Infection* **9**: 754-758.

Levy, R. V., Hart, F. L., Cheetham, R. D. (1986): Occurrence and public health signifance of invertebrates in drinking water systems. *Journal of the American Water Works Association* 78: 105-110.

Lipp, E.K., Huq, A., Colwell, R.R. (2002): Effects of global climate on infectious disease: the cholera model. *Clinical Microbiology Reviews* **15**: 757-770.

Lipp, E. K., Rivera, I. N. G., Gil, A. I., Espeland, E. M., Choopum, N., Louis, V.R. (2003): Direct detection of Vibrio cholera and ctxA in Peruvian coastal water and plankton by PCR. *Applied and Environmental Microbiology* **69**: 3676-3680.

Lisle, J.T., Pyle, B.H., McFeters, G.A., (1999): The use of multiple indices of physiological activity to assess viability in chlorine disinfected *Escherichia coli* O157:H7. *Letters in Applied Microbiololgy* **29**: 42– 47.

Lisle, J. T., Smith, J. J., Edwards, D. D., McFeters, G. A. (2004): Occurrence of microbial indicators and *Clostridium perfringens* in wastewater, water column samples, sediments, drinking water, and Weddell seal feces collected at McMurdo Station, Antarctica. *Applied Environmental Microbiology* **70**: 7269-7276.

Lleò, M.M., Tafi, M.C., Canepari, P. (1998): Nonculturable *Enterococcus faecalis* cells are metabolically active and capable of resuming active growth. *Systematic and Applied Microbiology* **21**: 333-339.

Lleò, M. M., Pierobon, S., Tafi, M. C., Signoretto, C., Canepari, P. (2000): mRNA detection by transcription-PCR for monitoring viability over time in an *Enterococcus faecalis* viable but nonculturable population maintained in a laboratory microcosm. *Applied Environmental Microbiology*. **66**: 4564-4567.

Lowder, M., Unge, A., Maraha, N., Jansson, J.K., Swiggett, J., Oliver, J.D., (2000): Effect of starvation on the viable-but-nonculturable state on green fluorescent protein (GFP) fluorescence in GFP-tagged *Pseudomonas fluorescens* A506. *Applied Environmental Microbiology* **66**: 3160– 3165.

Mackay, I.M., Mackay, J.F., Nissen, M.D., Sloots, T.P.: Real-time PCR: History and Fluorogenic Chemistries. In: Mackay, I.M. (ed.) (2007): Real-time PCR in microbiology – from diagnosis to characterization. *Caister Academic Press*, Norfolk, UK: 1-39.

Madigan M.T. & Martinko J.M. *Brock: Biology of microorganisms.* 11th edition. 2006. Pearson Prentice Hall. New Jersey, USA.

Manz, W., Amann, R., Szewzyk, R., Szewzyk, U., Stenstrom, T. A., Hutzler, P., et al. (1995): In situ identification of *Legionella*ceae using 16S rRNA-targeted oligonucleotide probes and confocal laser scanning microscopy. Microbiology **141**: 29–39.

Marino, R. P., Gannon, J. J. (1991): Survival of fecal coliforms and fecal streptococci in storm drain sediment. *Water Research* **25**: 1089-1098.

Mary, P., Chihib, N.E., Charafeddine, O., Defives, C., Hornez, J. P. (2002): Starvation survival and viable but nonculturable states in *Aeromonas hydrophila*. *Microbial Ecology* **43**: 250-258.

Matsumoto, N., Aizawa, T., Ohgaki, S., Hirata, T., Toyooka, K., Kanbayashi, T., Tsutsumi, Y., Hasegawa, T. (2002): Removal methods of nematode contained in the effluent of activated carbon. *Journal of Water Supply, Water Science and Technology* **2**: 183-190.

Maugeri, T.L., Carbone, M., Fera, M.T., Irrera, G.P., Gugliandolo, C. (2004): Distribution of potentially pathogenic bacteria as free living and plankton associated in a marine coastal zone. *Journal of Applied Microbiology* **97**: 354-361.

McEldowny, S., Fletcher, M. (1986): Variability of the influence of physicochemical factors affecting bacterial adehesion to polystyrene substrata. *Applied and Environmental Microbiology* **52**: 460-465.

McFeters, G. A., LeChevallier, M. W., Singh, A., Kippin, J. S. (1986): Health significance and occurrence of injured bacteria in drinking water. *Water Science and Technology* **18**: 227-231.

McKay (1992): Viable but non-culturable forms of potentially pathogenic bacteria in water. *Letters in Applied Microbiology* **97**: 354-361.

McMahon, J. W., Rigler, F. H. (1965): Feeding rate of *Daphnia magna* Straus in different foods labeled with radioactive phosphorus. *Limnololgy and Oceanography* **10**: 105-113.

Medema, G. J., Bahar, M., Schets, F. M. (1997): Survival of *Crytosporidium parvum*, *Escherichia coli*, faecal enterococci and *Clostridium perfringens* in river water. Influence of temperature and autochthonous microorganisms. *Water, Science and Technology* **35**: 549-252.

Meier, H., Koob, C., Ludwig, W., Amann, R., Frahm, E., Hoffmann, S., Obst, U., Schleifer, K.H. (1997): Detection of Enterococci with rRNA targeted DNA probes and their use for hygienic drinking water control. *Water Science and Technology* **35**: 437-444.

Messi, P., Guerrieri, E., Bondi, M. (2002): Survival of an *Aeromonas hydrophila* in an artificial mineral water microcosm. *Water Research* **36**: 3410-3415.

Mischke, U., Behrendt, H. (2007): Handbuch zum Bewertungsverfahren von Fließgewässern mittels Phytoplankton zur Umsetzung der EU-WRRL in Deutschland, Weißensee Verlag.

Møller, E.F., Riemann, L., Søndergaard, M. (2007): Bacteria associated with copepods: abundance, activity and community composition. *Aquatic Microbial Ecology* **47**: 99-106.

Moore, J.E., Crower, M., Heaney, N., Crothers, E. (2001): Antibiotic resistance in *Campylobacter* spp. isolated from human faeces (1980-2000) and foods (1997-2000) in Northern Ireland: An update. *Journal of Antimicrobial Chemotherapy* **48**: 455-457.

Moritz, M.M., Flemming, H.-C., Wingender, J. (2010): Integration of *Pseudomonas aeruginosa* and *Legionella pneumophila* in drinking water biofilms grown on domestic plumbing materials. *International Journal of Hygiene and Environmental Health* **213**: 190-197.

Mote, B. L., Turner J. W., Lipp E. K. (2012): Persistence and growth of the fecal indicator bacteria enterococci in detritus and natural estuarine plankton communities. *Applied Environmental Microbiology* **78**: 2569-2577.

Moter, A., Göbel, U. B. (2000). Fluorescence in situ hybridization (FISH) for direct visualization of microorganisms. *Journal of Microbiological Methods* **41**: 85–112.

Mueller, R.S., McDougald, D., Cusumano, D., Sodhi, N., Kjelleberg, St., Azam, F., Bartlett, D.H. (2007): *Vibrio cholerae s*trains possess multiple strategies for abiotic and biotic surface colonization. *Journal of Bacteriology* **189**: 5348-5360.

Mueller-Spitz, S. R., Stewart, L. B., Val Klump, J., McLellan, S. (2010): Freshwater suspended sediments and sewage are reservoirs for enterotoxin-positive *Clostridium perfringens*. *Applied and Environmental Microbiology* **76**: 5556-5562.

Nagasawa, S. (1988): Copepod-bacteria associations in Zielony Lake, Poland. *Journal of Plankton Research* **10**: 551-554.

Ng, D.L.K., Koh, B.B., Tay, L., Heng, B.H. (1997): Comparison of polymerase chain reaction and conventional culture for the detection of legionellae in cooling tower waters in Singapore. *Letters in Applied Microbiology* **24**: 214-216.

Niewolak, S., Opieka, A. (2000): Potentially pathogenic microorganisms in water and bottom sediments in the Czarna Hancza River. *Polish Journal of Environmental Studies* **9**: 183-194.

Nocker, A., Sossa-Fernandez, P., Burr, M. D., Camper, A. K. (2007). Use of propidium monoazide for live/dead distinction in microbial ecology. *Applied Environmental Microbiology* **73**, 5111–5117.

Nwachuku, N., Gerba, C.P. (2008): Occurrence and persistence of *Escherichia coli* O157:H7 in water. *Reviews in Environmental Science and Biotechnology* **7**: 267-273.

Nwoguh, C.E., Harwood, C.R., Barer, M.R., (1995): Detection of induced beta-galactosidase activity in individual non-culturable cells of pathogenic bacteria by quantitative cytological assay. *Molecular Microbiology* **17**: 545– 554.

Obiri-Danso, K., Jones, K. (1999): Distribution and seasonality of microbial indicators and thermophilic campylobacters in two freshwater bathing sites on the River Lune in northwest England. *Journal of Applied Microbiology* **87**: 822-832.

Olapade, O. A., Depas, M. M., Jensen, E. T., Mc Lellan, S. L. (2006): Microbial communities and fecal indicator bacteria associated with *Cladophora* mats on beach sites along Lake Michigan shores. *Applied and Environmental Microbi*ology **72**, 1932-1938

Oliver, J.D. (2000): The public health significance of viable but nonculturable bacteria, p. 277-300. *In* R.R. Colwell and D.J. Grimes (ed.), Nonculturable microorganisms in the environment. ASM Press, Washington, D.C.

Oliver, J.D. (2005): The viable but nonculturable state in bacteria. *The Journal of Microbiology* **43**: 93-100.

Oliver, J. D. (2009): Recent findings on the viable but nonculturable state in pathogenic bacteria. *FEMS Microbiology Reviews*: 1-11.

Oliver, J. D. (2010). Recent findings on the viable but nonculturable state in pathogenic bacteria. *FEMS Microbiology Reviews* **34**: 415–425.

Omar, R., Ali Rahman, Z., Latif, M.T., Lihan, T.m Adam, J.H. (Eds.) (2002): The occurrence of aerolysin-positive *Aeromonas hydrophila* strains in seawater and associated with marine copepods (Asmat, A., Gires, U.). In Proceedings of the Regional Symposium on Environment and Natural Ressources, Hotel Renaissance Kuala Lumpur, Malaysia. Vol.: **1**: 495-502.

Padmanabhan, V., Prabakaran, G., Paily, K.P., Balaraman, K. (2005): Toxicity of a mosquitocidal metabolite of *Pseudomonas fluorescence* on larvae & pupae of the house fly, *Musca dometica*. *Indian Journal of Medical Research* **121**: 116-119.

Palmer, C.J., Bonilla, G.F., Roll, B., Paszko-Kolva, C., Sangermano, L.R., Fujioka, R.S. (1995): Detection of *Legionella* species in reclaimed water and air with the EnviroAmp *Legionella* PCR Kit and direct fluorescent antibody staining. *Applied and Environmental Microbiology* **61**: 407-412.

Park, J. H., Yoshinaga, I., Nishikawa, T., Imai, I. (2010): Algicidal bacteria in particle-associated form and in free-living form during a diatom bloom in the Seto Inland Sea, Japan. *Aquatic Microbial Ecology* **60**: 151-161.

Parthuisot, N., West, N.J., Lebaron, P., Baudart, J. (2010): High Diversity and Abundance of *Legionella* spp. in a Pristine River and impact of seasonal and anthropogenic effects. *Applied and Environmental Microbiology* **76**: 8201-8210.

Pearce, D.A., van der Gast, C.J., Woodward, K., Newsham K.K. (2005): Significant changes in the bacterioplankton community structure of maritime Antarctic freshwater lake following nutrient enrichment. *Microbiology* **151**:3237-3248.

Pearson, A.D., Greenwood, M.H., Feltham, R.K.A., Healing, T.D., Donaldson, J., Jones, D.M., Colwell, R.R. (1996): Microbial ecology of *Campylobacter jejuni* in a United Kingdom chicken supply chain: Intermittent common source, vertical transmission, and amplification by flock propagation. *Applied and Environmental Microbiology* **62**: 4614-4620.

Pellett, S., Bigley, D.V., Grimes, D.J. (1983): Distribution of *Pseudomonas aeruginosa* in a Riverine Ecosystem. *Applied and Environmental Microbiology* **45**: 328-332.

Prescott, A. M., Fricker, C. R., (1999): Use of PNA oligonucleotides for the in situ detection of *Escherichia coli* in water. *Molecular Cell probes* **13**: 261-268.

Pete, R., Davidson, K., Hart, M.C. Gutierrez, T., Miller, A. E. J. (2010): Diatom derived dissolved organic matter as a driver of bacterial productivity: the role of nutrient limitation. *Journal of Experimental Marine Biology and Ecology* **391**:20-26.

Petit, L., Gibert, M., Popoff, M. R. (1999): *Clostridium perfringens*: toxinotype and genotype. *Trends in Microbiology* **7**: 104-110.

Pinto, B., Pierotti, R., Canale, G., Reali, D. (1999): Characterization of 'faecal streptococci' as indicator of faecal pollution and distribution in the environment. *Letters in Applied Microbiology* **29**: 258-263.

Piqueres, P., Moreno, Y., Alonso, J. L., Ferrus, M.A. (2006): A combination of direct viable count and fluorescent in situ hybridization for estimating Helicobacter pylori cell viability. *Research in Microbiology* **157**: 345-349.

Pirnay, J.-P., Matthijs, S., Colak, H., Chablain, P., Bilocq, F., Van Eldere, J., De Vos, D., Zizi, M., Triest, L., Cornelis, P. (2005): Global *Pseudomonas aeruginosa* biodiversity as reflected in a Belgian river. *Environmental Microbiology* **7**: 969-980.

Plough, H., Grossart, P. (2000): Bacterial growth and grazing on diatom aggregates: Respiratory carbon turnover as a function of aggregate size and sinking velocity. *Limnology and Oceanography* **45**: 1467-1475.

Porter, K. G., Feig, Y. S., Vetter, E. F. (1983): Morphology, flow regimes, and filtering rates of *Daphnia*, *Ceriodapnia* and *Bosmina* fed natural bacteria. *Oecologia* **58**: 156-163.

Pruzzo, C., Tarsi, R., Lleò, M. M., Signoretto C., Zampini, M., Colwell, R. R., Canepari, P. (2002): In vitro adhesion to human cells by viable but nonculturable *Enterococcus faecalis*. *Current Microbiology* **45**: 105-110.

Qi, W. Nong, G., Preston, J.F., Ben-Ami, F., Ebert, D. (2009): Comparative metagenomics of *Daphnia* symbionts. *BMC Genomics* **10**: 172.

Qin, X., Emerson, J., Stapp, J., Stapp, L., Abe, P., Burns, J.L. (2003): Use of real-time PCR with multiple targets to identify *Pseudomonas aeruginosa* and other nonfermenting Gram-negative Bacilli from patients with Cystic Fibrosis. *Journal of Clinical Microbiology* **41**: 4312-4317.

Rainey, F. A., Hollen, B. J., Small, A. (2005): Family I. Clostridiaceae. In: Parte, A. C. (ed. in chief), de Vos, P., Garrity, G. M. Jones, D., Krieg, N. R., Ludiwg, W., Rainey, F. A., Schleifer, K.-H., Whitman, W. B. (eds). Bergey`s Manual of Systematic Bacteriology, Second Edition. Volume three, The Firmicutes. Springer, New York. 738-858.

Rawlings, T.K., Ruiz, G.M., Colwell, R.R. (2007): Association of *Vibrio cholerae* O1 E1 Tor and O139 Bengal with the copepods *Acartia tonsa* and *Eurytemora* affinis. *Applied and Environmental Microbiology* **73**: 7926-7933.

Reasoner, D. J., Geldreich, E. E. (1985): A new medium for the enumeration and subculture of bacteria from potable water. *Applied and Environmental Microbiology* **49**:1-7.

Reche, I., Carillo, P., Cruz-Pizarro, L. (1997): Influence of metazooplankton on interactions of bacteria and phytoplankton in an oligotrophic lake. *Journal of Plankton Research* **19**: 631-646.

Regnault, B., Martin-Delautre, S., Grimont, P. A. (2000): Problems associated with the direct viable count procedure applied to Gram-positive bacteria. International *Journal of Food Microbiology* **55**: 1-3.

Reuter, S., Sigge, A., Wiedeck, H., Trautmann, M. (2002). Analysis of transmission pathways of *Pseudomonas aeruginosa* between patients and tap water outlets. *Critical Care Medicine* **30**: 2222–2228.

Riemann, L., Steward, G. F., Azam, F. (2000): Dynamics of bacterial community composition and activity during a mesocosm diatom bloom. *Applied and Environmental Microbiology* **66**: 578-587.

Riemann, L., Winding, A. (2005): Community dynamics of free-living and particle-associated bacterial assemblages during a freshwater phytoplankton bloom. *Microbial Ecology* **42**: 274-285.

Rodriguez, S., Araujo, R. (2010): Occurrence of thermotolerant Campylobacter species in surface waters of Mediterranenan area and its prevailing pollution sources. Journal of Applied Microbiology 109: 1027-1034.

Rodriguez GG, Phipps D, Ishiguro K, Ridgway HF (1992): Use of a fluorescent redox probe for direct visualization of actively respiring bacteria. *Applied and Environmental Microbiology* **58**: 1801–1808.

Rogers, J., Dowsett, A.B., Dennis, P.J., Lee, J.V., Keevil, C.W. (1994): Influence of temperature and plumbing material selection on biofilm formation and growth of *Legionella pneumophila* in a model potable water system containing complex microbial flora. *Applied and Environmental Microbiology* **60**: 1585-1592.

Rollins, D. M., Colwell, R. R. (1986): Viable but nonculturable stage *Campylobacter jejuni* and its role in survival in the natural aquatic environment. *Applied and Environmental Microbiology* **52**: 531-538.

Rompré, A., Servais, P., Baudart, J., de-Roubin, M.-R., Laurent, P. (2002): Detection and enumeration of coliforms in drinking water: current methods and emerging approaches. *Journal of Microbiological Methods* **49**: 31-54.

Rosenberg, M. (1981): Bacterial adherence to polystyrene: a replica method of screening for bacterial hydrophobicity. *Applied and Environmental Microbiology* **42**: 375-377.

Roszak, D. B., Colwell, R. R. (1987): Metabolic activity of bacterial cells enumerated by direct viable count. *Applied Environmental Microbiology* **53**: 2889.

Routtu, J., Jansen, B., Colson, I., De Meester, L., Ebert, D. (2010): The first-generation *Daphnia magna* linkage map. *BMC Genomics* **11**: 508.

Rowe, M. T. (2000): *Campylobacter*. In Encyclopedia of Food Microbiology ed. Robinson, R. K., Batt, C. A., Patel, P. D. Vol. **1**: 335-352. London: Academic press.

Ruhrverband (2009): Ruhrgütebericht 2009.

Ruhrverband (2010): Ruhrgütebericht 2010.

Ryzinska-Paier, G., Sommer, R., Haider, J. M., Knetsch, S., Frick, C., Kirschner, A. K. T., Farnleitner, A. H. (2011): Acid phosphatase test proves superior to standard phenotyic identification procedure for *Clostridum perfringens* strains isolated from water. *Journal of Microbiological Methods* **87**: 189-194.

Sanders, W. E. Jr., Sanders, C. C. (1997): *Enterobacter* spp.: pathogens poised to flourish at the turn of the century. *Clinical Microbiology Reviews* **10**: 220-241.

Sapp, M., Wichels, A., Wiltshire, K. H., Gerdts, G. (2007): Bacterial community dynamics during the winter-spring transition in the North Sea. *FEMS Microbiological Ecology* **59**: 622-637.

Scalbert, A. (1991): Antimicrobial properties of tannins. *Phytochemistry* **30**: 3875-3883.

Schaffer, J.C. (1755): Die grünen Armpolypen, die geschwänzten und ungeschwänzten zackigen Wasserflöhe und eine besondere Art kleiner Wasseraale Germany: Regensburg.

Schallenberg, M., Bremer, P. J., Henkel, S., Launhardt, A., Burns, C. W. (2005): Survival of *Campylobacter jejuni* in Water: Effect of Grazing by the Freshwater Crustacean *Daphnia carrinata* (Cladocera). *Applied and Environmental Microbiology* **71**: 5085-5088.

Scheffer, M. (1989): Alternative stable states in eutrophic freshwater systems. A minimal model. *Hydrobiological Bulletin* **23**: 73-83.

Scheffer, M. (1998): Ecology of shallow lakes. Chapmann & Hall, London.

Scheffer, M., Hosper, S. H., Meijer, M. L., Moss, B., Jeppesen, E. (1993): Alternative equilibria in shallow lakes. *Trends Ecology Evolution* **8**: 275-279.

Scheutz, S., Strockbine, N.A. (2005): Family I. Enterobacteriaceae. In: Garrity GM, (ed. in chief), Brenner DJ, Krieg NR, Stale JT. (eds). Bergey`s Manual of Systematic Bacteriology, Second Edition. Volume two, The Proteobacteria, Part B: The Gammaproteobacteria. Springer, New York. 607-623.

Schreiber, H., Schoenen, D., Traunspurger, W. (1997): Invertebrate colonization of granular activated carbon filters. *Water Research* **31**: 743-748.

Schwartz, T., Volkmann, H., Kirchen, S., Kohnen, W., Schön-Hölz, K., Jansen, B., Obst, U. (2006): Real-time PCR detection of *Pseudomonas aeruginosa* in clinical and municipal wastewater and genotyping of the ciprofloxacin-resistant isolates. *FEMS Microbiology Ecology* **57**: 158-167.

Seyfried, P.L., Cook, R.J. (1984): Otitis externa infections related to *Pseudomonas aeruginosa* levels in five Ontario lakes. *Canadian Journal of Public Health* **75**:83–91.

Shin, J. E., Jang, M. J., Koo, J. Y., Ahn, S. K., Yu, M. J. (2007). Variation of biofilm formation on distribution pipe depending on water treatment processes and pipe materials. *Water Science and Technology: Water Supply* **7**, 25–32.

Shipley, G.L.: An introduction to real-time PCR. In: Dorak, M.T. (ed.) (2006): Real-time PCR. *Taylor & Francis Group*, Milton Park, UK: 1-31.

Sibille I, Sime-Ngando T, Mathieu L, Block JC (1998): Protozoan bacterivory and *Escherichia coli* survival in drinking water distribution systems. *Applied and Environmental Microbiology* **64**:197–202.

Signoretto, C., Burlacchini, G., Lleò, M., Pruzzo, C., Zampini, M., Pane, L., Franzini, G., Canepari, P. (2004): Adhesion of *Enterococcus faecalis* in the nonculturable State to plankton is the main mechanism responsible for persistence of this bacterium in both lake and seawater. *Applied and Environmental Microbiology* **70**: 6892-6896.

Signoretto, C., Burlacchini, G., Pruzzo, C., Canepari, P. (2005): Persistence of *Enterococcus faecalis* in aquatic environments via surface interactions with copepods. *Applied and Environmental Microbiology* **71**: 2756-2761.

Simon, M. (1987): Biomass and production of small and large free-living and attached bacteria in Lake Constance. *Limnology and Oceanography* **32**: 591-607.

Simon, M., Grossart, H. P., Schweitzer, B., Plough, H. (2002): Microbial ecology of organic aggregates in aquatic ecosystems. *Aquatic Microbial Ecology* **28**: 175-211.

Simpson, D. A. (1990): Displacement of *Elodea canadensis* by *Elodea nuttallii* (Planch.) St. John in the British Isles. *Watsonia* **18**: 173-177.

Sørensen, D. L., Eberl, S. G., Dicksa, R. T. (1989): *Clostridium perfringens* as a point source indicator in non-point polluted streams. *Water Research* **23**, 191–197.

Steinert, M., Emody, L., Amann, R., Hacker, J. (1997): Resuscitation of viable but nonculturable *Legionella pneumophila* Philadelphia JR32 by *Acanthamoeba castellanii. Applied Environmental Microbiology* **63**: 2047-2053.

Stewart, P. S., Camper, A. K., Handran, S. D., Huang, C.-T., Warnecke, M. (1997). Spatial distribution and coexistence of *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* in biofilms. *Microbial Ecology* **33**, 2–10.

Stocker, R., Seymour, J. R., Samadani, A., Hunt, D. F., Polz, M. (2008): Rapid chemotactic response enables marine bacteria to exploit ephemeral microscale nutrient patches. *Proceedings of the National Acadamy of Science USA* **105**: 4209-4214.

Stover, C.K., Pham, X.Q., Erwin, A.L., Mizoguchi, S.D., Warrener, P., Hickey, M.J., Brinkman, F.S.L., Hufnagle, W.O., Kowalik, D.J., Lagrou, M., Garber, R.L., Goltry, L., Tolentino, E., Westbrock-Wadman, S., Yuan, Y., Brody, L.L., Coulter, S.N., Folger, K.R., Kas, A., Larbig, K., Lim, R., Smith., K., Spencer, D., Wong, G.K.-S., Wu, Z., Paulsen, I.T., Reizer, J., Saier, M.H., Hancock, R.E.W., Lory, S., Olson, M.V. (2000): Complete genome sequence of *Pseudomonas aeruginosa* PA01, an opportunistic pathogen. *Nature* **406**: 959-964.

St. Pierre, K., Lévesque, S., Frost, E., Carrier, N., Arbeit, R.D., Michaud, S. (2009): Thermotolerant coliforms are not a good surrogate for *Campylobacter* spp. in environmental water. *Applied and Environmental Microbiology* **75**: 6736-6744.

Struwe, C., Krogfeldt, K. A. (2004): Pathogenic potential of environmental *Klebsiella pneumoniae* isolates. *Environmental Microbiology* **6**: 584-590.

Swift, S., Lynch, M.J., Fish, L., Kirke, D.F., Tomás, J.M.,Stewart, G.S., and Williams, P. (1999) Quorum sensing dependent regulation and blockade of exoprotease production in *Aeromonas hydrophila*. *Infection and Immunity* **67**: 5192–5199.

Szabo, J.G., Rice, E.W., Bishop, P.L. (2006): Persistence of *Klebsiella pneumoniae* on simulated biofilm in a model drinking water system. *Environmental Science and Technology* **40**:4996–5002.

Szewzyk, U., Szewzyk, R., Manz, W., Schleifer, K. H. (2000). Microbiological safety of drinking water. *Annual Review of Microbiology* **54**, 81–127.

Tan, M. W., Mahajan-Miklos, S., Ausubel, F. M. (1999): Killing of *Caenorhabditis elegans* by *Pseudomonas aeruginosa* used to model mammalian bacterial pathogenesis. *Proceedings of the National Acadamy of Science* USA **96**: 715-720.

Tang, K. W., Visscher, P. T., Dam, H. G., (2001): DMSP-consuming bacteria associated with the calanoid copepod *Acartia tonsa* (Dana). *Journal of Experminetal Marine Biology and Ecology* **256**:185-198.

Tang, K.W. (2005): Copepods as microbial hot spots in the ocean: effects of host feeding activities on attached bacteria. *Aquatic Microbial Ecology* **38**: 31-40.

Tang, K. W., Hutalle, K. M. L., Grossart, H. P. (2006): Microbial abundance, composition and enzymaticactivity during decomposition of copepod carcasses. *Aquatic Microbial Ecology* **45**: 219-227.

Tarsi, R., Pruzzo, C. (1999): Role of surface proteins in *Vibrio cholerae* attachment to chitin. *Applied and Environmental Microbiology* **65**: 1348-1351.

Temmerman, R., Vervaeren, H., Noseda, B., Boon, N., and Verstraete, W. (2006) Necrotrophic growth of *Legionella pneumophila*. *Applied Environmental Microbiology* **72**: 4323–4328.

Terzieva, S. I., McFerters, G. A. (1991): Survival and injury of *Escherichia coli*, *Campylobacter jejuni*, and *Yersinia enterocolitica* in stream water. *Canadian Journal of Microbiology* **37**: 785-790.

Tholozan, J. L., Cappelier, J. M., Tissier, J. P., Delattre, G., Federighi, M. (1999): Physiological characterization of viable-but-nonculturable *Campylobacter jejuni* cells. *Applied Environmental Microbiology* **65**: 1110-1116.

Tison, D.L., Pope, D.H., Cherry, W.B., Fliermans, C.B. (1980): Growth of *Legionella pneumophila* in association with blue-green algae (Cyanobacteria). *Applied and Environmental Microbiology* **39**: 456-459.

Tolker-Nielsen, T., Larsen, M. H., Kyed, H., Molin, S. (1997): Effects of stress treatments on the detection of *Salmonella typhimurum* by in situ hybridization. *International Journal of Food Microbiology* **35**: 251-258.

Tóth, L. G., Kato, K., Abe, D. (2001): Grazing of *Daphnia galeata* (Crustacea, Cladocera) on epi- and hypolimnetic bacteria in the mesotrophic Lake Kizaki, Japan. *Verhandlungen des Internationalen Verein Limnol*ogie **27**: 3712-3717.

Toze, S., Sly, L.I., Macrae, I.C., and Fuerst, J.A. (1990): Inhibition of growth of *Legionella* species by heterotrophic plate-count bacteria isolated from chlorinated drinking water. *Current Microbiology* **21**, 139–143.

Trautmann, M., Lepper, P.M., Haller, M. (2005): Ecology of *Pseudomonas aeruginosa* in the intensive care unit and the evolving role of water outlets as a reservoir of the organism. *American Journal of Infection Control* **33**: 41-49.

Trautmann, M., Halder, S., Lepper, P. M., Exner, M. (2009): Reservoirs of *Pseudomonas aeruginosa* in the intensive care unit. The role of tap water as a source of infection. *Bundesgesundheitsblatt Gesundheitsforschung Gesundheitsschutz* **52**: 339–344.

Turley, C.M., Mackie, P. J. (1995): Bacterial and cyanobacterial flux to the deep. NE Atlantic on sedimenting particles. *Deep Sea Research* **42**: 1453-1474.

Valentino, L., Torregrossa, M.V. (1995): Risk of *Bacillus cereus* and *Pseudomonas aeruginosa* nosocomial infections in a burns centre: The microbiological monitoring of water supplies for a preventive strategy. *Water Science and Technology* **31**, No. 5-6: 37-40.

Valiela, I., Alber, M., LaMontagne, M. (1991): Fecal coliform loadings and stocks in Buttermilk Bay, Massachusetts, USA, and management implications. *Environmental Managament* **15**: 659-674.

Van der Gucht, K., Cottenie, K., Muylart, K., Vloemans, N., Cousin, S., Declerck, S., Jeppesen, E., Conde-Porcuna, J.-M., Schwenk, K., Zwart, G., Degans, H., Vyverman, W., De Meester, L. (2007): The power of species sorting: Local factors drive bacterial community composition over a wide range of spatial scales. *Proceedings of the National Acadamy of Science USA* **104:** 20404-20409.

Vandamme. P., De Ley, J. (1991): Proposal for a new family *Campylobacteriaceae*. *International Journal of Systematic Bacteriology* **41**: 451-455.

Vila, J., Ruiz, J., Gallardo, F., Vargas, M., Soler, L., Figueras, M. J., Gascon, J. (2003): *Aeromonas* spp. and traveler´s diarrhea: clinical features and antimicrobial resistance. Emerging Infectious Diseases **9**: 552-555.

Villari, P., Motti, E., Farullo, C., Torre, I. (1998): Comparison of conventional culture and PCR methods for the detection of *Legionella pneumophila* in water. *Letters in Applied Microbiology* **27**: 106-110.

Vöge, M. (1995): Langzeitbeobachtungen an *Elodea nuttallii* (Planch.) St. John in norddeutschen Seen. *Floristische Rundbriefe* **29**: 189-193.

Volkmann, H., Schwartz, T., Kirchen, S., Stofer, C., Obst, U. (2007): Evaluation of inhibition and cross-reaction effects on real-time PCR applied to the total DNA of wastewater samples for the quantification of bacterial antibiotic resistance genes and taxon-specific targets. *Molecular and Cellular Probes* **21**: 125-133.

Wallner, G., R. Amann, and W. Beisker (1993): Optimizing fluorescent in situ hybridization with rRNA-targeted oligonucleotide probes for flow cytometric identification of microorganisms. *Cytometry* **14**: 136-143.

Wang, S.-L., Chang, W.-T. (1997): Purification and characterization of two bifunctional chitinases/lysozymes extracellularly produced by *Pseudomonas aeruginosa* K-187 in a shrimp and crab shell powder medium. *Applied and Environmental Microbiology* **63**: 380-386.

Watnick, P., Kolter, R. (2000): Biofilm, City of Microbes. *Journal of Bacteriology* **182**: 2675-2679.

Weichart, D., McDougald, D., Jacobs, D., Kjelleberg, S., (1997): In situ analysis of nucleic acids in cold-induced nonculturable *Vibrio vulnificus*. *Applied Environmental Microbiology* **63**: 2754– 2758.

Weisburg, W.G., Barns, S.M., Pelletier, D.A., Lane, D.J. (1991): 16S ribosomal DNA Amplification for Phylogenetic Study. *Journal of Bacteriology* **173**: 697-703.

Wellinghausen, N., Frost, C., Marre, R. (2001): Detection of legionellae in hospital water samples by quantitative real-time LightCycler PCR. *Applied and Environmental Microbiology* **67**: 3985-3993.

Wellinghausen, N., Kothe, J., Wirths, B., Sigge, A., Poppert, S. (2005): Superiority of molecular techniques for identification of gram-negative, oxidase-positive rods, including morphologically nontypical *Pseudomonas aeruginosa*, from patients with cystic fibrosis. *Journal of Clinical Microbiology* **43**: 4070–4075.

Wéry, N., Bru-Adan, V., Minervini, C., Delgénes, J.-P., Garrelly, L., Godon, J.-J. (2008): Dynamics of *Legionella* spp. and bacterial populations during the proliferation of *L. pneumophila* in a cooling tower facility. *Applied and Environmental Microbiology* **74**: 3030-3037.

Wetzel, R. G. (2001): Limnology, lakes and river ecosystems. *Elsevier Academic Press*, New York.

Whiteside, M. D., Oliver, J. D. (1997). Resuscitation of *Vibrio vulnificus* from the Viable but Nonculturable State. *Applied Environmental Microbiology* **63**: 1002–1005.

Whitman, R.L., Shively, D.A., Pawlik, H., Nevers, M.B., Byappanahalli, M.N. (2003): Occurrence of *Escherichia coli* and Enterococci in *Cladophora* (Chlorophyta) in nearshore water and beach sand of Lake Michigan. *Applied and Environmental Microbiology* **69**: 4714-4719.

Whitman, R.L., Nevers, M.B., Byappanahalli, M.B. (2006): Examination of the watershed-wide distrbution of *Escherichia coli* along southern Lake Michigan: an integrated approach. *Applied and Environmental Microbiology* **72**: 7301-7301.

Wiedenmann, A., Krüger, P., Dietz, K., López-Pila, J. M., Szewzyk, R., Botzenhart, K. (2006): A randomized controlled trial assessing infectious disease risks from bathing in fresh recreational waters in relation to the concentration of *Escherichia coli*, intestinal Enterococci, *Clostridium perfringens*, and somatic coliphages. *Environmental Health Perspectives* **114**: 228-236.

Wilson, I.G. (1997): Inhibition and facilitation of nucleic acid amplification. *Applied and Environmental Microbiology* **63**: 3741-3751.

Wingender, J., Hambsch, B., Schneider, S. (2009): Mikrobiologisch-hygienische Aspekte des Vorkommens von *Pseudomonas aeruginosa* im Trinkwasser. *Energie Wasser-Praxis* **60**: 60-66.

Wingender, J., Flemming, H.-C. (2011): Biofilms in drinking water and their role as reservoir for pathogens. *International Journal of Hygiene and Environmental Health*. *Article* in *press*.

Wullings, B.A., van der Kooij, D. (2006): Occurence and genetic diversity of uncultured *Legionella* spp. in drinking water treated at temperatures below 15°C. *Applied and Environmental Microbiology* **72**: 157-166.

Yáñez, M.A., Carrasco-Serrano, C., Barberá, V.M., Catalán, V. (2005): Quantitative detection of *Legionella pneumophila* in water samples by immunomagnetic purification and real-time PCR Amplification of the *dotA* gene. *Applied and Environmental Microbiology* **71**: 3433-3441.

Yaradou, D.F., Hallier-Soulier, S., Moreau, S., Poty, F., Hillion, Y., Reyrolle, M., André, J., Festoc, G., Delabre, K., Vandenesch, F., Etienne, J., Jarraud, S. (2007): Integrated real-time PCR for detection and monitoring of *Legionella pneumophila* in water systems. *Applied and Environmental Microbiology* **73**: 1452-1456.

Yu, C., Lee, A.M., Bassler, B.L., Roseman, S. (1991): Chitin utilization by marine bacteria. *Journal of Biological Chemistry* **266**: 24260-24267.

Yu, V.L., Plouffe, J.F., Castellani Pastoris, M., Stout, J.E., Schousboe, M., Widmer, A., Summersgill, J., File, T., Heath, C.M., Paterson, D.L., Chereshsky, A. (2002): Distribution of *Legionella* species and serogroups isolated by culture in patients with sporadic community-acquired legionellosis: An International Collaborative Survey. *Journal of Infectious Diseases* **186**: 127-128.

Ziegert, E., Stelzer, W. (1986): Comparative study of the detection of *Pseudomonas aeruginosa* in water. *Zentralblatt für Mikrobiologie* **141**: 121-28.

Ziglio, G., Andreottola, G., Barbesti, S., Boschetti, G., Bruni, L., Foladori, P., et al. (2002): Assessment of activated sludge viability with flow cytometry. *Water Research* **36**: 460–468.

Znachor, P., Simek, K., Nedoma, J. (2012): Bacterial colonization of the freshwater planktonic diatom *Fragilaria crotonensis*. *Aquatic Microbial Ecology* **66**: 87-94.

Appendix

Sources of pictures used in Figure 1.1

Phytoplankton, *Daphnia magna* and *Elodea nuttallii*: source: Miriam Tewes, Biofilm Centre, University of Duisburg-Essen

Lake Baldeney:

http://www.google.de/imgres?num=10&um=1&hl=de&client=firefox-a&rls=org.mozilla:de:official&channel=np&biw=1366&bih=574&tbm=isch&tbnid=MNiQDPM0zOCeAM:&imgrefurl=http://www.luftbild-archiv.de/page-g51.htm&docid=ulFWL2uGAseEPM&imgurl=http://www.luftbild-archiv.de/d090831_027u_Luftbild-Baldeneysee.jpg&w=980&h=715&ei=l9irUKWPJIyVswaI5IBQ&zoom=1&iact=rc&dur=351&sig=109059784839555650694&sqi=2&page=1&tbnh=136&tbnw=209&start=0&ndsp=17&ved=1t:429,r:14,s:0,i:161&tx=102&ty=50

Cormorant:

http://www.google.de/imgres?num=10&um=1&hl=de&client=firefox-a&rls=org.mozilla:de:official&channel=np&biw=1366&bih=574&tbm=isch&tbnid=izhq5xzYd7CLtM:&imgrefurl=http://www.naturfoto-cz.de/kormoran-foto-15.html&docid=tkZm-8Vp8E_kmM&imgurl=http://www.naturfoto-cz.de/bilder/vogel/kormoran-575.jpg&w=600&h=410&ei=VsyrUOapG4zLtAaM04DoDQ&zoom=1&iact=hc&vpx=867&vpy=196&dur=777&hovh=185&hovw=272&tx=137&ty=88&sig=109059784839555650694&sqi=2&page=1&tbnh=147&tbnw=201&start=0&ndsp=19&ved=1t:429,r:5,s:0,i:134

Sewage plant:

http://www.google.de/imgres?num=10&um=1&hl=de&client=firefox-a&rls=org.mozilla:de:official&channel=np&biw=1366&bih=574&tbm=isch&tbnid=NL0hdaNzHbSqDM:&imgrefurl=http://www.beilngries.de/index.php%3Fcontent_id%3D101&docid=lYobGHcxw3Y71M&imgurl=http://www.beilngries.de/timm_images/kommunales/klaeranlage.jpg&w=500&h=375&ei=IsyrUPGLJ9DgtQbr4YGoAw&zoom=1&iact=hc&vpx=398&vpy=240&dur=782&hovh=194&hovw=259&tx=119&ty=83&sig=109059784839555650694&sqi=2&page=1&tbnh=139&tbnw=198&start=0&ndsp=17&ved=1t:429,r:7,s:0,i:153

Tractor:

http://www.google.de/imgres?um=1&hl=de&client=firefox-a&rls=org.mozilla:de:official&channel=np&biw=1366&bih=574&tbm=isch&tbnid=nkLDSR9MwdiCnM:&imgrefurl=http://www.schuette-wulkotte.de/allcontent.php&docid=5d0NVvnv_XntHM&imgurl=http://www.schuette-wulkotte.de/images/22CIMG0119_Guellefass1.jpg&w=410&h=308&ei=4cyrUKa0D8TZtAbbmIHYDg&zoom=1&iact=hc&vpx=315&vpy=159&dur=434&hovh=140&hovw=193&tx=127&ty=91&sig=109059784839555650694&page=1&tbnh=138&tbnw=190&start=0&ndsp=18&ved=1t:429,r:1,s:0,i:69

Village:

http://www.google.de/imgres?um=1&hl=de&client=firefox-a&rls=org.mozilla:de:official&channel=np&biw=1366&bih=574&tbm=isch&tbnid=a-w6XfJWYSXDwM:&imgrefurl=http://www.zum.de/Faecher/G/BW/Landeskunde/rhein/staedte/kl2/wimpfen/stadt1.htm&docid=Brs_rAe3m0ek5M&imgurl=http://www.zum.de/Faecher/G/BW/Landeskunde/rhein/staedte/kl2/wimpfen/stadt1.jpg&w=435&h=326&ei=p86rUIjzNonxsgbY94CwAg&zoom=1&iact=hc&vpx=537&vpy=109&dur=4596&hovh=194&hovw=259&tx=136&ty=131&sig=109059784839555650694&page=1&tbnh=147&tbnw=201&start=0&ndsp=18&ved=1t:429,r:14,s:0,i:174

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