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Microbiology in swimming pools
UV-based treatment versus chlorination

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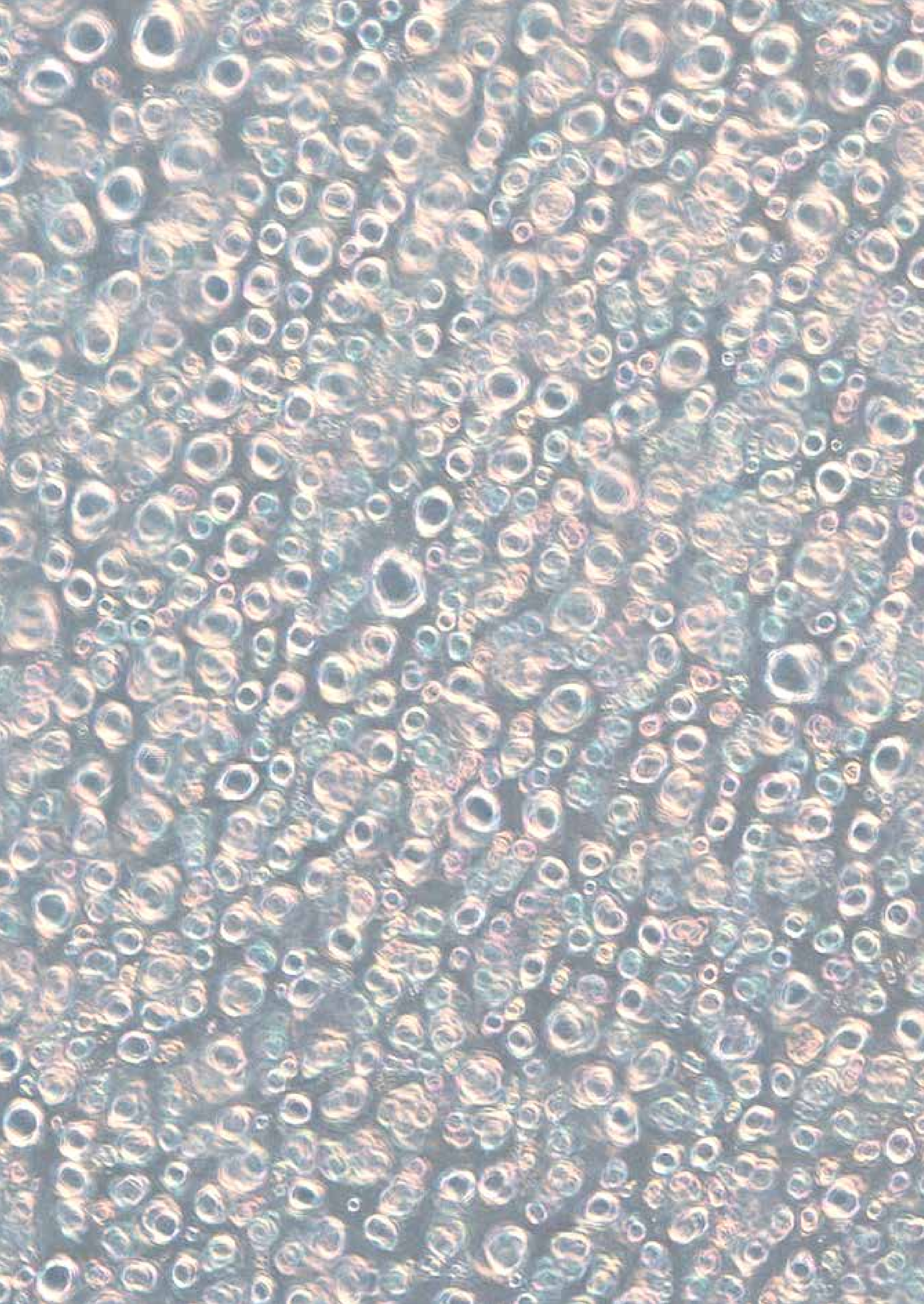
UV-based treatment versus chlorination



Microbiology in swimming pools

UV-based treatment versus chlorination

Marjolein Peters

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Microbiology in swimming pools

UV-based treatment versus chlorination

Proefschrift

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aan de Technische Universiteit Delft,
op gezag van de Rector Magnificus prof. ir. K.C.A.M. Luyben,
voorzitter van het College voor Promoties,
in het openbaar te verdedigen op
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ingenieur Life Science and Technology
geboren te Leidschendam, Nederland

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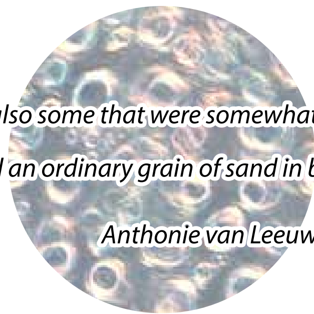
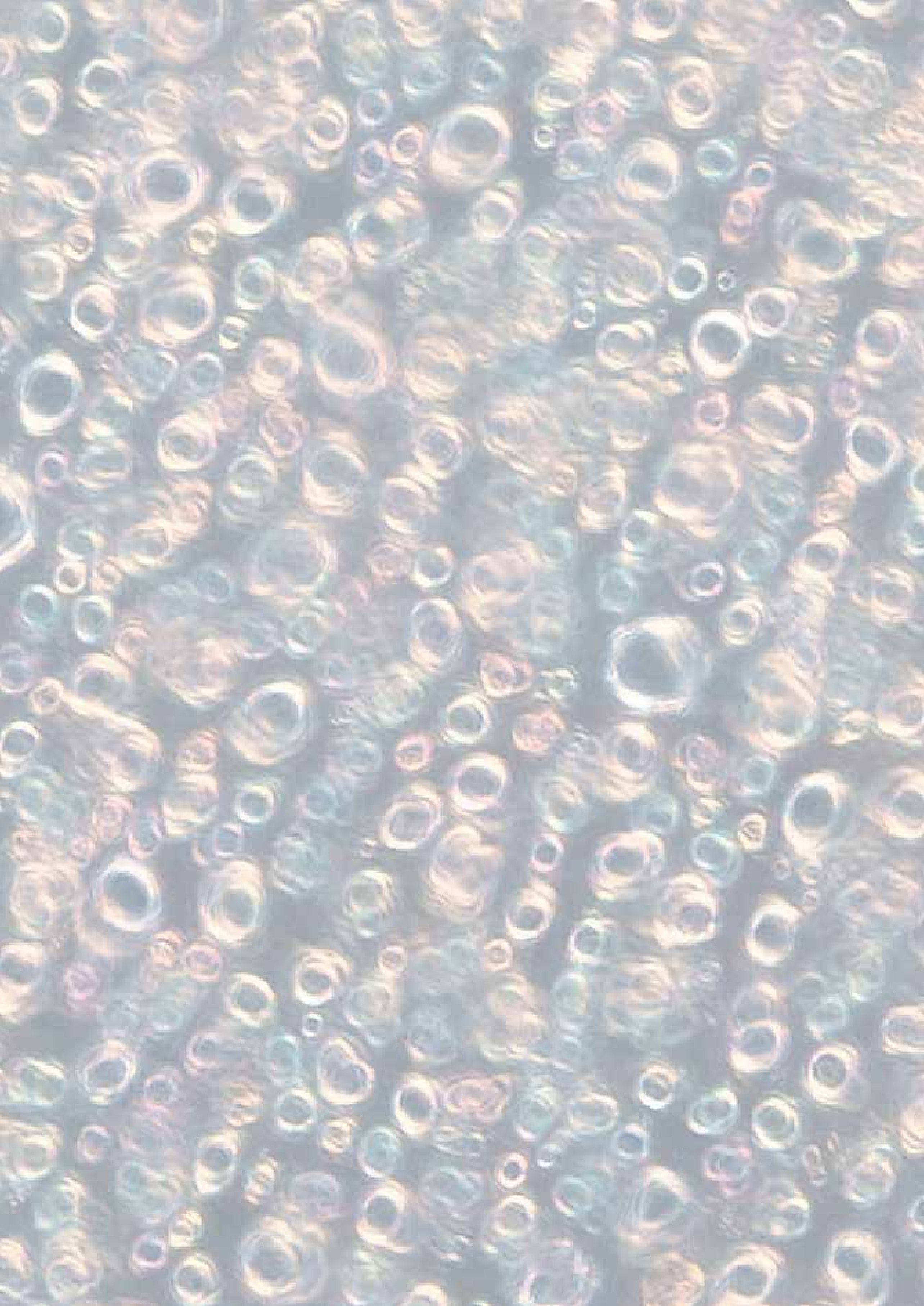


"I perceived in the same water more of those animals, as also some that were somewhat bigger.

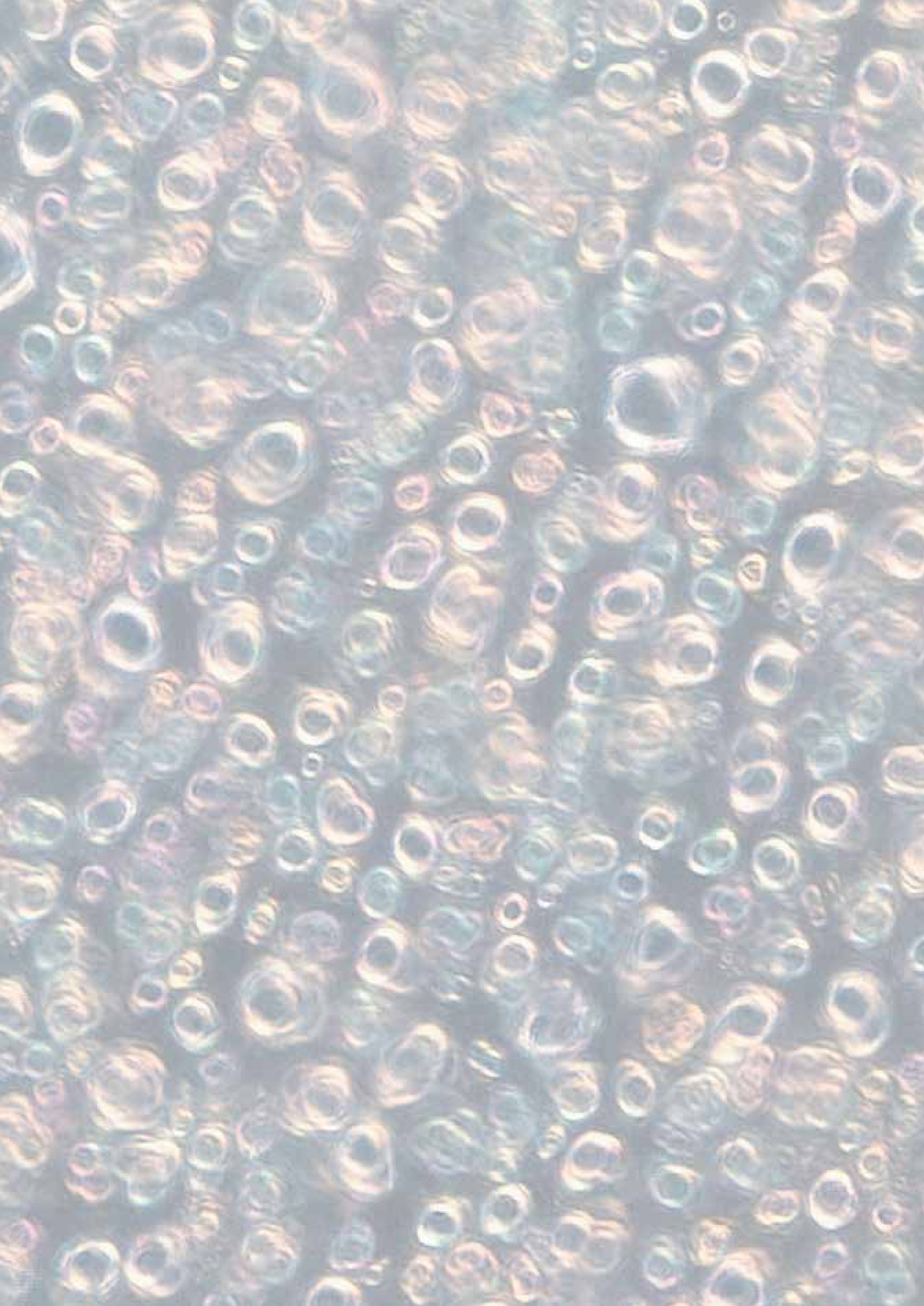
And I imagine, that 10,000 of these little creatures do not equal an ordinary grain of sand in bigness"

Anthonie van Leeuwenhoek



Contents

Summary		9
Samenvatting		11
Chapter 1	Introduction	15
Chapter 2	Quantification of continual anthropogenic pollutants released in swimming pools	23
Chapter 3	Characterization of the bacterial community in shower water before and after chlorination – Chlorine resistant bacteria entering swimming pools	49
Chapter 4	Impact of chlorination and UV irradiation on an anthropogenic microbial community from bathers, *Escherichia coli* and *Pseudomonas fluorescens*	65
Chapter 5	Biofouling in swimming pools: role of material properties and nutrients	83
Chapter 6	Biofilm disinfection by UV irradiation and removal by brushing	99
Chapter 7	Quantitative microbial risk assessment for an indoor swimming pool with chlorination and UV-based treatment	113
Chapter 8	General conclusions	127
References		135
List of abbreviations		153
Curriculum Vitae		155
List of publications		157
Dankwoord		161



Summary

Swimming is a popular activity for a variety of people who visit (indoor) swimming pools for reasons such as recreation, education, exercising and rehabilitation therapy. Disinfection of swimming pool water is often performed by chlorination. However, the chlorine-based products react, besides with microorganisms, also with other anthropogenic (human) pollutants released by bathers. The disinfection by-products (DBPs) formed are unwanted from a health point of view; some are irritating, while others might be carcinogenic. This thesis contains a reflection of the research performed to reduce the level of DBPs by characterisation of the anthropogenic pollutant release, including microorganisms, as well as the usage of an alternative, UV-based swimming pool treatment without residual disinfectant, but with biological sand filtration, coagulation, ultrafiltration and UV irradiation.

In order to reduce the anthropogenic pollutants, firstly the continual anthropogenic pollutant release during swimming was characterised. The continual anthropogenic pollutant release has been found to mainly consist of sweat, but also consists of particles (mainly skin fragments and hair), microorganisms and sebum (skin lipids). The sweat rate of bathers per skin area was found to be 0.1-0.2 L m^{-2} h^{-1} at water temperatures below 29 °C and increased linearly with increasing water temperatures to 0.8 L m^{-2} h^{-1} at 35 °C. The chemical pollutants, like nitrogen compounds, were continuously released during a swim visit, while the release of particles and microorganisms decreased in time. Although water temperature and level of exercise had important roles in anthropogenic release, the duration of the swim visit was the main parameter determining the continual anthropogenic pollutant release. Nevertheless, the pool water temperature is the only parameter that can be controlled by pool operators and is therefore the main parameter to restrain the continual anthropogenic pollutant release.

Characterisation of the bacteria released in swimming pool water indicated that the main bacterial families of an anthropogenic microbial community of bathers (AMCB) were *Flavobacteriaceae*, *Xanthomonadaceae*, *Moraxellaceae* and *Pseudomonadaceae*. By subjecting the AMCB to chlorination with 1 mg Cl_2 L^{-1} for 30 s, the relative abundance of *Pseudomonadaceae* increased in the total bacterial community, while the abundance of *Moraxellaceae* increased in the intact bacterial cell community. Both *Pseudomonadaceae* and *Moraxellaceae* were therefore suggested to be, relatively, more chlorine resistant than the other identified bacteria.

However, microbial safety in swimming pools is not assessed by the presence of AMCB in the pool, but by the presence of indicator organisms. Therefore, the disinfection efficiency of an AMCB was compared to the disinfection efficiency of indicator organisms *Pseudomonas fluorescens* and *Escherichia coli*. In chlorinated water, *P. fluorescens* and the AMCB showed similar log reductions at similar free available chlorine doses, while *E. coli* showed higher log reductions. Furthermore, the disinfection efficiency after UV irradiation was tested to determine the disinfection efficiency of the alternative UV-based treatment concept without residual disinfectant. Disinfection by UV irradiation showed a similar log reduction at similar UV doses for the *E. coli* culture and the AMCB, while irradiation of the *P. fluorescens* culture showed higher log reductions. These studies therefore indicate that the choice of swimming pool indicator organisms should be dependent on the disinfection method used.

In addition, microorganisms which survived in the disinfected pool water might start growing in biofilms, which is unwanted in swimming pools and should therefore be prevented and controlled. A strategy to prevent biofouling may be the use of materials with low biofouling properties. The biofilm development was studied on 11 swimming pool materials (PVC on stainless steel, PVC, stainless steel, rough foil, smooth foil, concrete, rough tiles, smooth tiles, pultrusion polyester, polyester and polypropylene). Tap water was used as inoculum, and nutrients were dosed (simulating highly occupied swimming pool conditions) to determine the influence of nutrient release by bathers. It was observed that polypropylene had the lowest biofouling potential under high bathing load, while concrete and stainless steel had the highest biofouling potential. While the surface roughness seemed to have no influence, the surface hydrophobicity had some, although this effect diminished in time. Overall, the materials characteristics had less influence on the biofilm formation than the presence of nutrients, used to simulate a high bathing load.

Furthermore, tests were performed to control biofilm development with UV irradiation and/or brushing. In order to determine whether UV irradiation would also inactivate the microorganisms in the lower regions of the biofilm, the passage of UV light through biofilms of different thicknesses was measured. UV light was found to be able to pass through a biofilm of 104 µm thick. Varying the UV irradiation dose indicated that from doses ≥ 50 J m^{-2} the biofilm development was less on concrete plates, while 250 J m^{-2} stabilised the biofilm growth and 1500 J m^{-2} reduced the active biofilm amount. Finally, the biofilm reduction of the different treatments, determined with total and cellular adenosine_-tri-phosphate (ATP, measure for active biomass), was for (i) only brushing 9-7%, (ii) only 50 J m^{-2} UV irradiation 36-48% and (iii) brushing followed by 50 J m^{-2} UV irradiation 45-57% respectively, indicating that the combination of subsequent brushing and UV irradiation was the most effective strategy for biofilm control.

Finally, these results were used in a quantitative microbial risk assessment to determine the yearly risk of infection of gastroenteritis from swimming in an indoor UV-based treated and chlorinated swimming pool. The risk assessment was based on the presence of the bacterial enteric reference pathogens *Campylobacter jejuni*, *E. coli* O157:H7 and *Salmonella enterica*. The average pathogen concentrations in a UV-based swimming pool over the day were the highest for *C. jejuni* (3.1×10^{-3} cells L^{-1}) > *S. enterica* (9.5×10^{-4} cells L^{-1}) > *E. coli* (7.2×10^{-4} cells L^{-1}) and about a factor of 180 times higher for *E. coli* in chlorinated swimming pools (4.0×10^{-6} cells L^{-1}). However, the yearly risks of infection of these pathogens in a UV-based swimming pool were for *C. jejuni* 1.7×10^{-3}, *E. coli* 1.8×10^{-5} and *S. enterica* 3.5×10^{-7}, and therefore for *E. coli* and *S. enterica* lower than the drinking water guidelines. This suggests that UV-based treatment is a promising concept for swimming pools without residual disinfectants.

Samenvatting

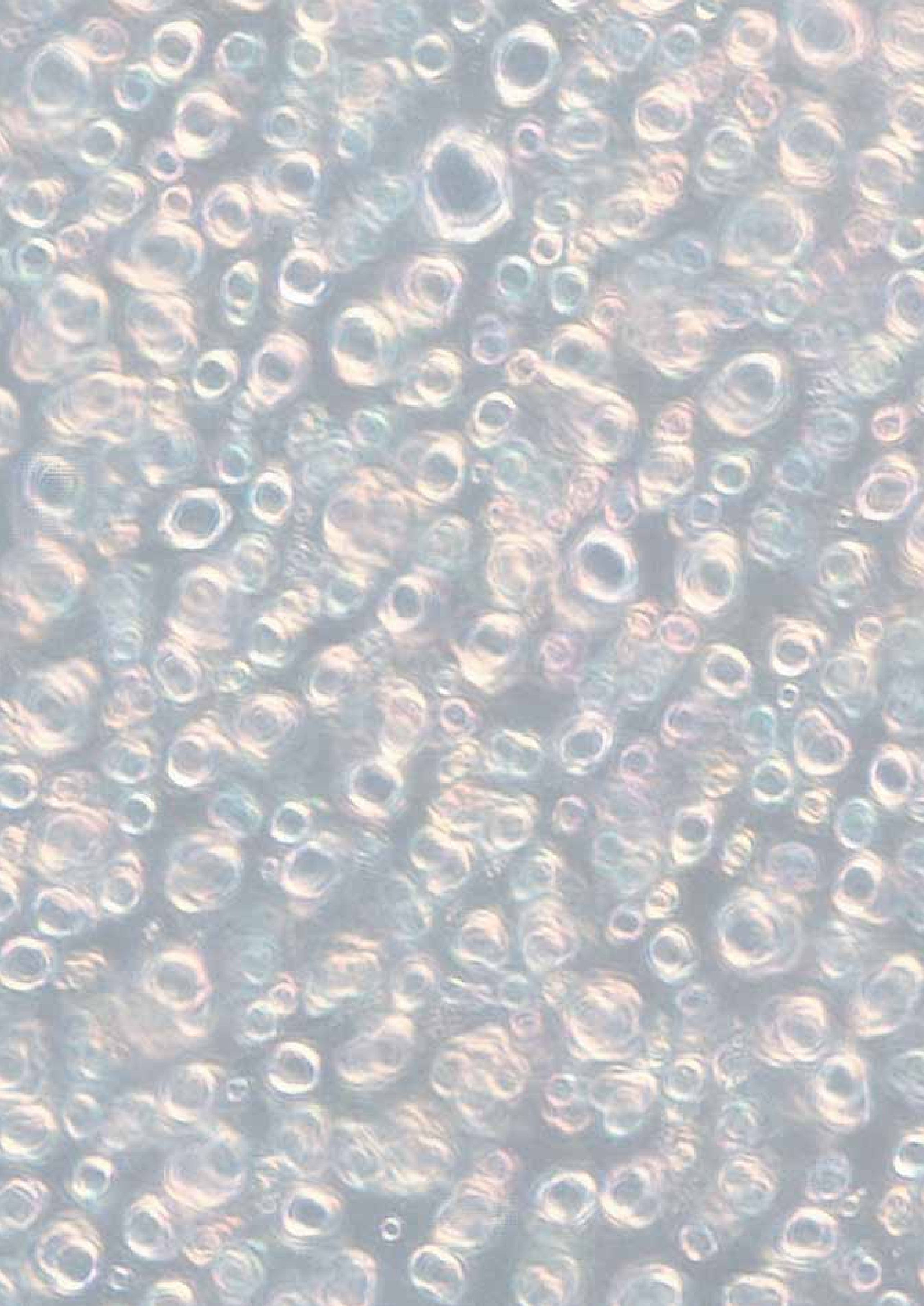
Zwemmen is een populaire activiteit voor veel mensen en zwembaden worden dan ook om verschillende redenen zoals recreatie, educatie, sport en/of therapie bezocht. Het badwater wordt in de meeste zwembaden gedesinfecteerd met hypochloriet. Echter, zwemmers geven verschillende antropogene (mens eigen) stoffen af aan het water, welke met het chloor reageren. Deze desinfectie bij-producten (DBPs) kunnen slecht zijn voor de gezondheid, aangezien sommige stoffen irriteren en andere mogelijk zelfs kankerverwekkend zijn. Om de hoeveelheid en aantal soorten DBPs te verminderen, is er onderzoek gedaan naar de afgifte van antropogene stoffen (inclusief de microbiële populatie) en alternatieve desinfectie methoden zonder het residueel hypochloriet, maar met biologische zand filtratie, coagulatie, ultrafiltratie en UV irradiatie. In dit proefschrift wordt het uitgevoerde onderzoek beschreven. Om te bepalen hoe de antropogene stoffen afgifte verminderd kan worden, is eerst de continue afgifte van antropogene stoffen tijdens het zwemmen bepaald. De continue afgifte van antropogene stoffen blijkt voornamelijk uit zweet te bestaan, maar ook uit deeltjes zoals huidschilfers en haren, micro-organismen en huidvetten zoals talg. Actieve zwemmers bleken 0.1-0.2 L m^{-2} u^{-1} zweet per huid oppervlak te creëren wanneer de zwembadwater temperatuur onder de 29 °C is. Hogere watertemperaturen zorgen voor een lineaire stijging van het geproduceerde zweet, naar 0.8 L m^{-2} u^{-1} bij 35 °C. De afgifte van chemische componenten, zoals stikstof, was continue tijdens het zwemmen, terwijl de afgifte van deeltjes en micro-organismen minder werd in de tijd. Ondanks dat de watertemperatuur en het inspanningsniveau van de zwemmer een belangrijke invloed heeft op de mate van het zweten tijdens het zwemmen, is de tijdsduur van het zwemmen nog belangrijker voor de continue afgifte van antropogene stoffen. Echter, omdat de watertemperatuur de enige parameter is die ingesteld kan worden door zwembad personeel, is dit de belangrijkste parameter om de continue afgifte van antropogene stoffen in zwembaden te verminderen. Naast de kwantificatie van antropogene micro-organismen afgifte tijdens het zwemmen, is de bacterie populatie ook gekarakteriseerd. De relatief dominante bacteriefamilies van een antropogene microbiologische populatie van zwemmers (AMCB) zijn *Flavobacteriaceae*, *Xanthomonadaceae*, *Moraxellaceae* en *Pseudomonadaceae*. Na chlorering van de AMCB met 1 mg Cl_2 L^{-1} voor 30 s werden er relatief meer *Pseudomonadaceae* gevonden in de totale (intact en niet-intacte cellen) AMCB, terwijl meer *Moraxellaceae* werden gevonden in de intacte cel populatie. Omdat beide, *Pseudomonadaceae* en *Moraxellaceae*, relatief meer werden gevonden na chlorering, worden beide chloor-resistenter geacht dan de overige aanwezige bacteriën.

Echter, de microbiologische veiligheid in zwembaden wordt niet beoordeeld aan de hand van de aan- of afwezigheid van micro-organismen van de AMCB, maar door indicator organismen. Om te bepalen of indicator organismen vergelijkbaar reageren als een AMCB na chlorering of UV irradiatie, is de desinfectie efficiëntie van een AMCB vergeleken met die van de indicator organismen *Pseudomonas fluorescens* en *Escherichia coli*. Hieruit bleek dat het desinfectie patroon bij verschillende chloor concentraties en incubatietijden van *P. fluorescens* vergelijkbaar is met dat van een AMCB, terwijl de *E. coli* concentratie gemakkelijker te reduceren was (hogere log reducties). Echter, wanneer het desinfectie patroon na verschillende UV dosissen werd vergeleken kwam het desinfectie patroon van *E. coli* overeen met die van de AMCB, terwijl de concentratie *P. fluorescens* gemakkelijker gedesinfecteerd was (hogere log reductie). Hieruit blijkt dat de keuze van het (zwembad) indicator organisme afhankelijk van de desinfectie methode moeten zijn.

Bovendien kunnen micro-organismen die desinfectie van het zwembadwater overleven, op de bodem en muren terecht komen en een biofilm vormen. Biofilms zijn ongewenst in zwembaden en moeten daarom worden voorkomen en verwijderd. Een strategie om biofilmvorming te voorkomen is het gebruik van materialen met lage biofilmvorming eigenschappen. Daarom werd op 11 zwembad materialen (PVC op roestvast staal, PVC, roestvast staal, ruw folie, glad folie, beton, ruwe tegels, gladde tegels, pultrusie polyester, polyester en polypropyleen) de biofilm ontwikkeling bestudeerd. Niet-gechloreerde zwembad condities werden nagebootst door kraanwater te gebruiken waaraan nutriënten werden toegevoegd om een hoge badbelasting (veel zwemmers) te simuleren en de invloed van nutriënten afgifte door zwemmers op biofilmgroei te bepalen. Uit de resultaten bleek dat op polypropeen de minste biofilm was gevormd onder hoge badbelasting, terwijl op beton en roestvast staal de meeste biofilm was gevormd. Daarnaast bleek de ruwheid van het materiaal weinig invloed te hebben op de mate van biofilmgroei. Hydrofobiciteit had wel enig effect, hoewel dit effect in de tijd verminderde. Hoewel het effect van verschillende materiaaleigenschappen niet direct een duidelijke invloed had op de biofilmvorming, had de aan- of afwezigheid van de toegevoegde nutriënten dit wel.

Naast het voorkomen van biofilmgroei door gebruik van materialen met een lage biofilmvorming potentie, is ook de verwijdering van biofilm met UV irradiatie en/of borstelen bestudeerd. Bij het gebruik van UV licht is het belangrijk dat de gehele biofilm, dus ook de micro-organismen in de lagere regio's van de biofilm, bestraald kan worden. Door de UV penetratie van biofilms met verschillende biofilmdikte te bepalen, bleek dat UV licht een biofilm van 104 µm dik geheel kan bestralen. Na het variëren van de UV dosis bleek dat een dosis van ≥ 50 J m^{-2} de biofilmontwikkeling vermindert, terwijl 250 J m^{-2} de biofilmgroei stabiliseert en 1500 J m^{-2} de actieve biofilm reduceert. Vergelijking van UV irradiatie met 50 J m^{-2}, borstelen en de combinatie van de twee heeft laten zien dat de biofilmvermindering, bepaald totaal en cellulaire adenosine_-tri-fosfaat (ATP, maat voor actieve biomassa), door borstelen gelijk is aan 9-7%, terwijl 50 J m^{-2} UV irradiatie 36-48% verwijdert en de combinatie van borstelen gevolgd door 50 J m^{-2} UV irradiatie respectievelijk 45-57% verwijdert. Dit geeft aan dat onder deze condities de combinatie behandeling van borstelen en UV irradiatie de meest effectieve strategie is voor biofilmverwijdering.

Tenslotte zijn deze resultaten gebruikt in een kwantitatieve microbiële risicoanalyse waarin het jaarlijkse risico op infectie van bacteriën die gastro-enteritis veroorzaken door het zwemmen in een binnen zwembad gedesinfecteerd met hypochloriet en met biologische zand filtratie, coagulatie, ultrafiltratie en UV irradiatie. De risicoanalyse is gebaseerd op de aanwezigheid van de bacteriële referentie pathogenen *Campylobacter jejuni*, *E. coli* O157: H7 en *Salmonella enterica*. De gemiddelde pathogeen concentraties tijdens openingstijden in een zwembad met onder andere UV irradiatie waren het hoogste berekend voor *C. jejuni* ($3{,}1\times10^{-3}$ cellen L^{-1}) > *S. enterica* ($9{,}5\times10^{-4}$ cellen L^{-1}) > *E. coli* ($7{,}2\times10^{-4}$ cellen L^{-1}). Met deze berekende pathogen concentraties is het jaarlijkse risico op infectie van deze pathogenen in een zwembad met onder andere UV irradiatie berekend: *C. jejuni* $1{,}7\times10^{-3}$, *E. coli* $1{,}8\times10^{-5}$ en *S. enterica* $3{,}5\times10^{-7}$. In vergelijking met een gechloreerd zwembad was de *E. coli* concentratie ongeveer een factor 180 maal hoger ($4{,}0\times10^{-6}$ cellen L^{-1}), alsook het jaarlijkse risico op infectie ($9{,}8\times10^{-8}$). Uit deze risicoanalyse blijkt dat ondanks dat het jaarlijkse risico op infectie in gechloreerde zwembaden lager is, het jaarlijkse risico op infectie door *E. coli* en *S. enterica* beide lager zijn dan de jaarlijkse risico op infectie richtlijnen voor drinkwater (10^{-4}). Dit suggereert dat een zwembad met biologisch zandfilter, coagulatie, ultrafiltratie en UV irradiatie een veelbelovend concept is voor zwembaden zonder chemische desinfectiemiddelen.



1 Introduction

1.1 Swimming pools

Swimming is a popular activity for a variety of people who visit (indoor) swimming pools for reasons such as recreation, education, exercising and rehabilitation therapy. During swimming, bathers release different microorganisms, soluble substances and particles. The shared use of swimming pool water by different individuals requires pool water to be treated. Disinfection of the pool water is needed to inactivate present microorganisms. The most frequently used disinfectants are chlorine based products because of their effectiveness and low costs (Shannon et al. 2008) and their mandatory use in many countries. Disadvantages of chlorination are (i) the chlorine resistance of some microorganisms and waterborne pathogens, such as *Cryptosporidium* and *Giardia* (Hijnen et al. 2006), and (ii) the formation of a variety of disinfection by-products (DBPs) due to the oxidation of organic matter from human and/or natural origin (Aggazzotti et al. 1995, Florentin et al. 2011, Richardson et al. 2010, Zwiener et al. 2007). Some DBPs may be carcinogenic (Font-Ribera et al. 2010, Glauner et al. 2005, LaKind et al. 2010), associated with potential genotoxic effects (Kogevinas et al. 2010b), or irritating to the skin, eyes or respiratory tract (Eichelsdörfer et al. 1975a, Erdinger et al. 1998a). In order to overcome the disadvantages of chlorination, alternative methods were researched within the DIPool project. The developed UV-based treatment consists of biological sand filtration to remove soluble substances, ultrafiltration for enhanced particle removal, including microorganisms, and UV irradiation for final disinfection. The advantage of this treatment is that there is no chemical residual disinfectant present in the swimming pool and thus no chemical DBPs are formed. However, microorganisms are only inactivated when they pass through the treatment, meaning that they could be present in the pool and possible infect other bathers, giving rise to additional research. Within the DIPool project, different sub-projects were studied; (i) performance of the proposed treatment scheme, (ii) contamination of swimming pools by bathers, (iii) biofouling and (iv) determination of the microbiological risks. Within this thesis, the focus was on the microbiological aspects of the last three sub-projects.

1.2 Contamination of swimming pools by bathers

1.2.1 Pollutant release by bathers

Microorganisms can be released at different stages of swimming activity as initial, continuous, and incidental anthropogenic pollutant release (Keuten et al. 2012). The initial anthropogenic pollutant release is introduced into the pool water during the first minutes of body contact with the water and consists of the residue of evaporated sweat, microorganisms and pollutants on the swimmer's skin, as well as any cosmetics on the skin. The initial anthropogenic pollutant release has been quantified by the use of standardized shower experiments. The continuous anthropogenic pollutant release is produced during swimming activities and was assumed to mainly consist of sweat and skin cells. The incidental anthropogenic pollutant release, however, is the result of human excreta such as urine, vomit or faecal material entering the pool water, either accidentally or on purpose.

During these different stages of anthropogenic pollutant release, microorganisms may enter the pool water through different routes. Microorganisms of non-faecal origin, such as *Pseudomonas* spp., *Staphylococcus aureus* and adenoviruses, may enter while being washed from the skin or from released saliva, mucus or vomit (WHO 2006). Faecally-derived microorganisms, such as *Escherichia coli*, *Cryptosporidium* and enteric viruses however, may be washed from swimmers bodies or enter the water when a person has an (accidental) faecal release (WHO 2006). Some of these microorganisms are (opportunistic) pathogens and are related to e.g. skin infections (Al-Tatari et al. 2007, Chang et al. 2008, Jurado et al. 2002, Mashouf et al. 2008a, Wade et al. 1991). To determine how often these pathogens are shed by bathers, and thus introducing a health risk, an anthropogenic microbiological community should be characterized.

1.2.2 Disinfection of an anthropogenic community

The microorganisms shed by bathers will become harmless in chlorinated swimming pools as soon as they enter the pool water due to the interaction of hypochlorite with the cell membrane (Venkobachar et al. 1977). Furthermore, different intercellular molecules are oxidised, resulting in complete disruption of bacterial adenosine triphosphate (ATP) production as a consequence of inhibition of the inner membrane systems (Barrette et al. 1989). The free available chlorine concentration in Dutch swimming pools is required to be between 0.5 and 1.5 mg Cl_2 L^{-1}, which is based on a 4-log removal of *Pseudomonas aeruginosa* within 30 seconds (Anonymous 2011). In order to ensure microbial safety, the swimming pool water is regularly analysed on the presence of different indicator organisms. *P. aeruginosa* is used as an indicator organism for disinfection, whereas *Escherichia coli* is often used as faecal indicator (WHO 2006). Although these indicator organisms have been used for many years, a study comparing their response after chlorination to the response of an anthropogenic microbial community released by bathers (AMCB) after chlorination has not, to the best of our knowledge, been previously reported.

Besides chlorination, alternative disinfection without the use of a residual disinfectant, like UV-based treatment, is commonly used for disinfection of drinking water in the Netherlands. Disinfection with UV light at 254 nm is based on the formation of pyrimidine dimers, which distort the deoxyribonucleic acid (DNA) helix and blocks cell replication (Lado and Yousef 2002). Furthermore, cross-linking of aromatic amino acids occur at their carbon–carbon double bonds, resulting in denaturation of proteins, which contributes to membrane depolarization and abnormal ionic flow (Lado and Yousef 2002). Besides DNA damage, UV irradiation also independently damages other cell components, such as the cell membrane and cytoplasm (Schwarz 1998). Although some swimming pools are operated with UV, the response of swimming pool indicator organisms and an AMCB after UV irradiation have not yet clearly been compared.

1.2.3 Measurement techniques

Effectiveness of disinfection is often measured with heterotrophic plate count (HPC) (Pernitsky et al. 1995). One major disadvantage of any cultivation method is the long period of time before results are known. In addition, only a very small fraction of the microbial population is culturable. Even if bacteria could be cultivated, bacterial cells can, under certain conditions, reach a viable but non-culturable (VBNC) state after chlorination (Oliver 2005)

and UV irradiation (Zhang et al. 2015). Therefore, even if no colony forming units (CFU) were detected with HPC, VBNC microorganisms could still be present and pose a potential threat upon human contact. With new techniques, such as flow cytometry (FCM), cell counts can be performed faster and without constraints towards microorganisms in the VBNC state (Hoefel et al. 2005b). One way to determine viable microbial cells with FCM is based on cell membrane integrity by means of live/dead staining; when the cytoplasmic cell membrane is not intact, propidium iodide (PI) can enter the cell and stain its DNA (Novo et al. 2000).

Besides HPC and FCM, also ATP measurements can be used to determine the active biomass (Velten et al. 2007). E.g. Berney et al. (2008), Hammes et al. (2010) and Siebel et al. (2008) have investigated the correlations between HPC, FCM and ATP measurements and found that while the ATP concentrations were found to be related with cell counts, no relation was observed between CFU and cell counts. Furthermore, not all active cells are culturable (Hammes et al. 2008, Hoefel et al. 2003), indicating that a relation between ATP and CFU does not exist (Hammes et al. 2010, Siebel et al. 2008, Venkateswaran et al. 2003). Although no relation has been found before, it was hypothesised that after disinfection the dead cells will be destructed, while the cells that survived will repair themselves. In time, these methods should thus show similar results. To investigate this hypothesis, the response of the cells present in the chlorinated or UV irradiated samples were studied up to 29 h after disinfection.

1.3 Biofouling

1.3.1 Biofouling in swimming pools

In water, microorganisms can be found as planktonic cells, flocs or attached to a surface. When microorganisms are attached to a surface, in time, the attachment becomes irreversible (not removed by gentle rinsing) as the microorganisms excrete extracellular polymeric substances (EPS) (Donlan 2002, Dufrêne et al. 1996) and form a biofilm. While swimming, bathers release nutrients, promoting the formation of biofilms on swimming pool surfaces. Also pathogens have been found in swimming pools (Papadopoulou et al. 2008), which could shelter in those biofilms. This introduces a health risk, and therefore extensive biofilms are unwanted in swimming pools.

Various methods have been used to prevent biofouling such as disinfection of water containing microorganisms and the use of materials with biofouling limiting characteristics. Although most microorganisms become harmless by disinfection of swimming pool water with a residual disinfectant, biofilms have been found in chlorinated swimming pools (Davis et al. 2009). The extent of biofilm growth has been suggested to depend on various material characteristics like surface charge (Kerr et al. 1998), surface roughness (Kerr et al. 1999) and hydrophobicity. An increase of biofouling has been observed on material with an increased surface roughness (Donlan 2002). Besides, it has been observed that biofouling occurs more rapidly to hydrophobic and nonpolar surfaces than to hydrophilic materials (Bendinger et al. 1993, Fletcher and Loeb 1979, Pringle and Fletcher 1983). Although the influence of both, surface roughness and hydrophobicity, on biofouling has been studied before (Bikerman 1950, Bundy et al. 1993, Bundy et al. 1992, Hallab et al. 2001), the formation of biofilms on submerged swimming pool materials has not been researched, and the extent to which these characteristics influence the growth compared to the pool water quality is unknown.

1.3.2 Biofilm disinfection and removal

In the case that a biofilm has been formed, the biofilm should be disinfected and removed. Similar disinfection techniques as for planktonic cells could be used to disinfect biofilms, although lower log reductions have been observed with chlorination (Flemming 2002, Nett et al. 2008, Smith et al. 2008, Wong et al. 2010). The rate limiting factor for disinfection is the diffusion of chlorine into the biofilm (Chen and Stewart 1996), which is related to physicochemical interactions between the disinfectant and the EPS (Bridier et al. 2011). Therefore, microorganisms in the deeper regions of the biofilm might be exposed to a lower concentration of disinfectant, resulting in adaptive response to sub-lethal concentrations of the disinfectant (Bridier et al. 2011), which could lead to disinfectant resistance. Also, UV irradiation could be applied as an alternative disinfection method, although minimal literature on its effectiveness exists.

In addition, to remove biofilms, the mechanical stability of the EPS matrix should be broken in the cleaning process (Flemming 2002; Brigidier 2011; Maukonen 2003), which could be done by creating shear stress by brushing, for example. The biofilm removal efficiency by brushing a submerged biofilm grown under UV-based swimming pool treatment conditions was investigated.

1.4 Microbiological risks

Because bathers release microorganisms, they might also introduce pathogens in the pool water, which may pose a health risk for other bathers due to shared use of the swimming pool. Quantitative microbial risk assessment can be used to determine this health risk for both chlorinated swimming pools and pools with a UV-based treatment. The quantitative microbial risk assessment calculations are based on the concentration of pathogenic cells and the exposure to pathogens. The key is to determine which pathogens are shed in which numbers by bathers during a swimming event. Although most shed microorganisms are harmless, some bacteria, viruses or parasites of faecal origin may cause illnesses such as gastroenteritis (WHO 2003). Gastroenteritis is one of the most common diseases throughout the world (Bern et al. 1992, Guerrant et al. 1990), and the relation between the presence of faecal indicator organisms and swimming-associated gastroenteritis has been demonstrated before (Prüss 1998, Wade et al. 2003, Zmirou et al. 2003). Bacterial faecal indicator organisms that could enhance gastroenteritis are *Campylobacter jejuni*, *Escherichia coli* O157:H7 and *Salmonella enterica*. To determine whether it is microbiological safe to swim in a UV-based treated swimming pool, the yearly risk of infection by one of these pathogens should be investigated.

1.5 This thesis

1.5.1 Objective and research questions

This thesis focused on the microbiology in a swimming pool, studying how many microorganisms and which bacteria are released by bathers and how such an anthropogenic community coming from bathers respond to chlorination and UV irradiation. Besides, microorganisms that survived the water disinfection treatment might attach to the pool surface and start growing a biofilm, while biofilms are unwanted in swimming pools. To prevent any suspended or attached pathogens remaining in the pool, the probability of biofilm formation should be as low as possible and controlled. Therefore, it should be investigated which swimming pool materials have a low biofouling potential as well as how biofilms can be removed without chemical disinfectants. Finally, taking all this into account, a quantitative microbial risk assessment should be made to determine what the yearly risks of infections are when bathers swim in an swimming pool with a UV-based treatment and a chlorinated pool.

Specific research questions were:

- How many microorganisms are released during swimming (continual anthropogenic pollution)?
- Which bacteria might initially be released in swimming pools (initial anthropogenic community)?
- Can the disinfection response of an anthropogenic microbial community released by bathers (AMCB) in swimming pool water be evaluated by the response of indicator organisms present in swimming pool water?
- Which swimming pool materials have a low biofouling potential when not subjected to chlorination?
- How can biofilm growth be controlled in swimming pools with UV irradiation?
- What are the microbial health risks in a swimming pool with a UV-based treatment, compared to a chlorinated swimming pool?

These research questions are depicted in Figure 1.

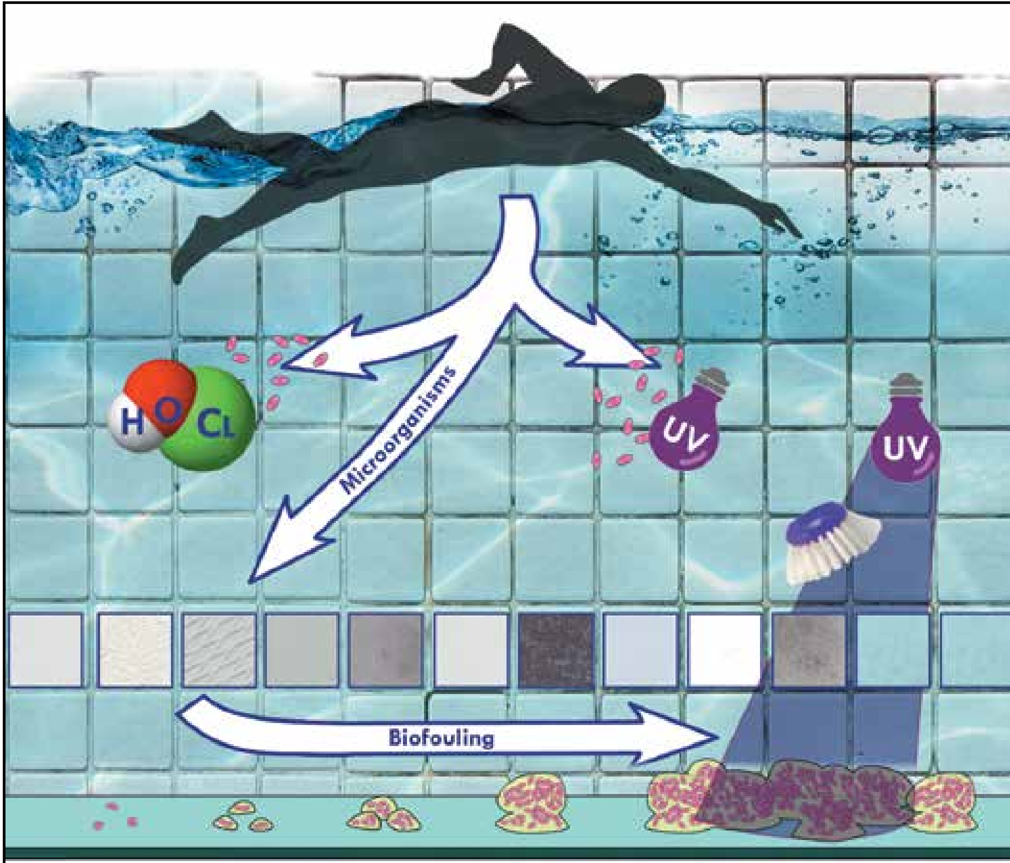
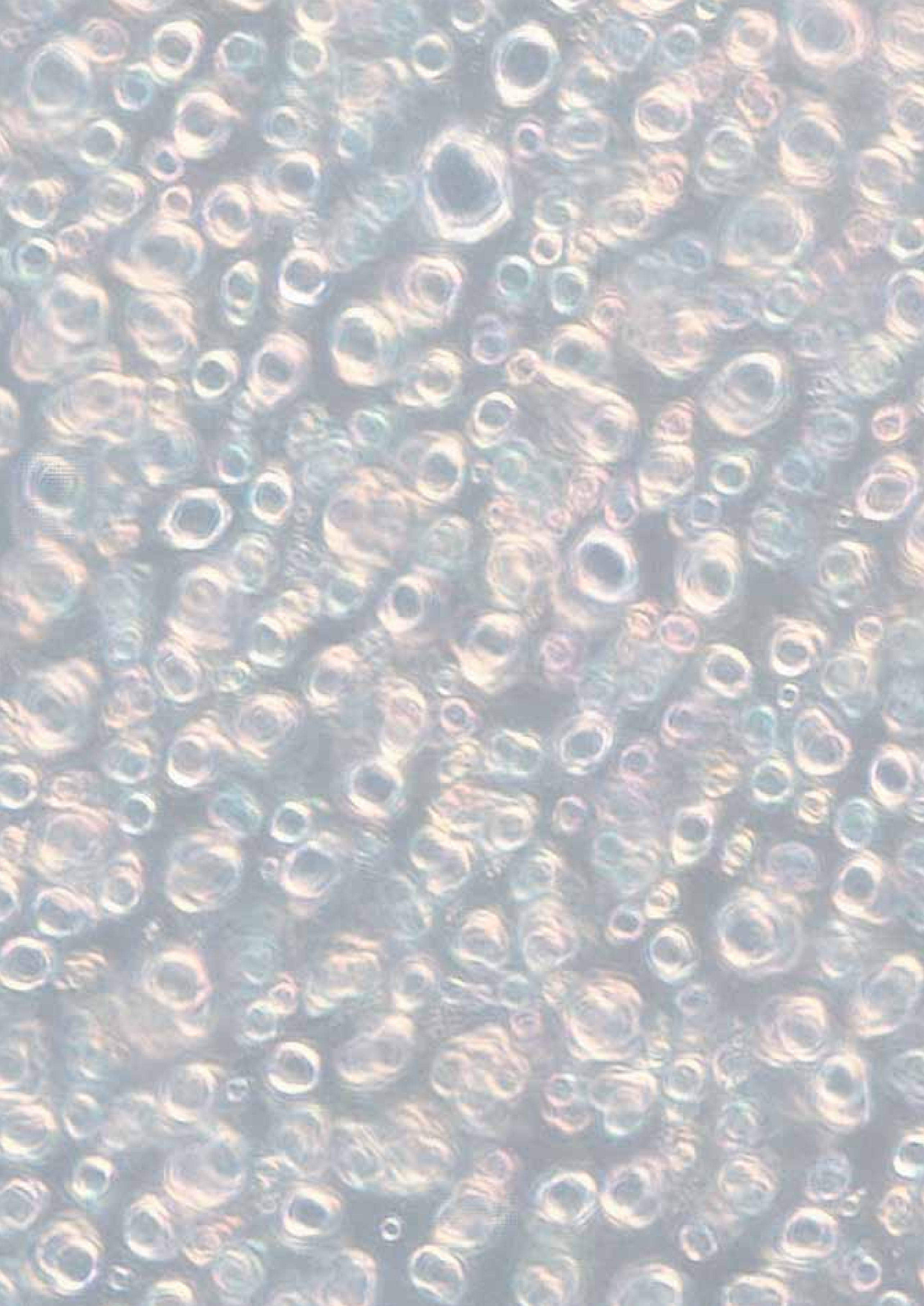


Figure 1 *When bathers enter the pool, different microorganisms are shed that can form a biofilm on the different materials present in swimming pools. In this research the number of microorganisms released was studied and the anthropogenic community characterized. Furthermore, the response of an anthropogenic microbial community released by bathers (AMCB) in swimming pool water after chlorination and UV irradiation was researched, biofilm development on different materials investigated and how biofouling could be controlled with UV irradiation and brushing studied.*

1.5.2 Thesis outline

In **Chapter 2** – *Quantification of continual anthropogenic pollutants released in swimming pools*, the continual pollution of different water quality parameters from bathers during swimming was determined.
In **Chapter 3** – Ch*aracterization of the bacterial community in shower water before and after chlorination*, the bacteria which might end up in swimming pool water were identified as well as their response to chlorination. The bacteria were obtained from shower experiments representing an initial anthropogenic community.
In **Chapter 4** – *Impact of chlorination and UV irradiation on an anthropogenic microbial community from bathers, Escherichia coli and Pseudomonas fluorescens,* the response of the initial anthropogenic community coming from bathers to both chlorination and UV irradiation was researched and compared to the response of swimming pool microbial water quality indicators *P. fluorescens* and *E. coli*. The response was monitored in time with heterotrophic plate count (HPC) and intact cell count with flow cytometry (FCM).
In **Chapter 5** – *Biofouling on swimming pool materials: role of material properties and pool water nutrients*, biofouling under UV-based swimming pool treatment conditions without residual disinfectant was studied in time on 11 different swimming pool materials. The materials had different characteristics of which surface roughness and hydrophobicity were characterised.
In **Chapter 6** – *Biofilm disinfection by UV irradiation and removal by brushing*, the effect of directly disinfection of a biofilm by UV irradiation was studied and/or combined with brushing to control biofouling.
In **Chapter 7** – *Quantitative microbial risk assessment for an indoor swimming pool with chlorination and UV-based treatment*, modelled the risks of gastroenteritis caused by the bacterial indicator organisms *Campylobacter jejuni*, *Escherichia coli* O157:H7 and *Salmonella enterica*.
In **Chapter 8**, the general conclusions and recommendations based on insights acquired during the studies were presented.

This thesis is structured as a paper dissertation, except for the introduction and the outlook. Repetitions between chapters are therefore unavoidable. Besides, while this thesis focused on the microbiological aspects of a swimming pool with UV-based treatment, treatment design and optimization were studied by, and in collaboration with, Maarten Keuten. Both theses and publications will give the whole picture of the advantages, disadvantages and possible risks of the UV-based treatment concept. This close collaboration during the whole research project led to a publication included in this thesis.



2 Quantification of continual anthropogenic pollutants released in swimming pools

Abstract

Disinfection of swimming pool water is often performed by chlorination. However, anthropogenic pollutants from swimmers will react with chlorine and form disinfection by-products (DBPs). DBPs are unwanted from a health point of view, because some are irritating, while others might be carcinogenic. The reduction of anthropogenic pollutants will lead to a reduction in DBPs. This paper investigates the continual release of anthropogenic pollutants by means of controlled sweat experiments in a pool tank during laboratory time-series experiments and also during on-site experiments in a swimming pool. The sweat released during the laboratory time-series and on-site experiments was very similar. The sweat rate found was 0.1-0.2 L m^{-2} h^{-1} at water temperatures below 29 °C and increased linearly with increasing water temperatures to 0.8 L m^{-2} h^{-1} at 35 °C.
The continual anthropogenic pollutant release not only consisted of sweat, particles (mainly skin fragments and hair) and microorganisms, but also sebum (skin lipids) has to be considered. The release of most components can be explained by the composition of sweat. The average release during 30 min of exercise is 250 mg per bather non-purgeable organic carbon (NPOC), 77.3 mg per bather total nitrogen (TN), 37.1 mg per bather urea and 10.1 mg per bather ammonium. The release of NPOC cannot be explained by the composition of sweat and is most probably a result of sebum release. The average release of other components were 1.3×10^9 particles per bather (2-50µm), 5.2 µg per bather cellular adenosine triphosphate (cATP) and 9.3×10^6 intact cell counts per bather. The pool water temperature was the main parameter to restrain the continual anthropogenic pollutant release. This study showed that a significant amount of the total anthropogenic pollutants release is due to unhygienic behaviour of bathers.

This chapter is based on: M.G.A. Keuten, M.C.F.M. Peters, H.A.M. Daanen, M.K. de Kreuk, L.C. Rietveld, J.C. van Dijk. (2014) Quantification of continual anthropogenic pollutants released in swimming pools. Water Res 53, 259-270.

2.1 Introduction

Swimming is a popular activity all over the world for all age and social classes. The provision of safe and hygienic swimming water is an important health issue. Anthropogenic pollutants, that are introduced into swimming pool water by bathers, can be divided into suspended and colloidal matter, microorganisms and soluble substances (Powick 1989). Suspended and colloidal matter include particles such as organic and inorganic substances that float, suspend or settle in the swimming pool water and include hair, skin cells, dust and fibres from clothes and swimwear. Microorganisms enter the pool water through different routes. Microorganisms of non-faecal origin, like *Pseudomonas* spp., *Staphylococcus aureus* and adenoviruses enter the pool water while being washed from the skin or from released saliva, mucus or vomit, whereas faecally-derived microorganisms like *Escherichia coli*, *Cryptosporidium* and enteric viruses are washed from swimmers bodies or enter the water when a person has an (accidental) faecal release (WHO 2006). Soluble substances can be organic or inorganic. Soluble organic substances include urea, creatinine, lactic acid and amino acids. Soluble inorganic material includes ions such as ammonium, chloride, sodium, potassium, calcium and sulphate (Kuno 1956).

The shared use of swimming pool water by different individuals requires pool water treatment to remove pollutants and disinfect the water to inactivate possible anthropogenic pathogenic microorganisms. Swimming pool water is generally disinfected with chlorine-based products. However, the anthropogenic pollutants, introduced in the pool water by swimmers, react with chlorine, leading to the formation of a variety of disinfection by-products (DBPs) (Aggazzotti et al. 1995, Florentin et al. 2011, Richardson et al. 2010, Zwiener et al. 2007).
Some of these DBPs are associated with impaired respiratory health and possibly asthma, while others may be carcinogenic (Font-Ribera et al. 2010, Glauner et al. 2005, LaKind et al. 2010). Other DBPs are associated with potential genotoxic effects (Kogevinas et al. 2010b), whereas other DBPs are irritating to the skin, eyes or respiratory tract (Eichelsdörfer et al. 1975a, Erdinger et al. 1998a). The overall health effects of swimming might be increasingly positive when the potential negative health risks from DBPs in pool water are reduced (Kogevinas et al. 2010b). Expected is that a reduction in the amount of anthropogenic pollutants in the pool water will result in reduced concentrations of DBPs and chlorine demand.

Many papers emphasise the importance of reducing the anthropogenic pollutants released to decrease the formation of DBPs formed (Borgmann-Strahsen 2003, Eichelsdörfer et al. 1980, Hansen et al. 2013, Hery et al. 1995, Keuten et al. 2012, Lahl et al. 1981, LaKind et al. 2010, WHO 2006). Although it is obvious that reduction of anthropogenic pollutants will lead to reduction of DBPs, there are no recent scientific reports or studies known to the authors, that have demonstrated the actual effect of anthropogenic pollutant reduction on the level of DBPs.

To establish whether anthropogenic pollutant reduction results in decreased DBP formation, information is required about anthropogenic pollutant release. The anthropogenic pollutants release can be divided into three parts (Keuten et al. 2012). The first part is the initial anthropogenic pollutant release, defined as the amount of anthropogenic pollutants that are rinsed off from a subject's body during a 60-second shower. The second part is the continual anthropogenic pollutants release during the subsequent swimming exercise. The continual anthropogenic pollutant release is assumed to consist mainly of sweat, microorganisms and skin cells. The third part is the incidental anthropogenic pollutant release which is the result of human excreta such as urine, vomit or faecal material entering the pool water, either accidentally or on purpose.

The continual anthropogenic pollutant release was the focus for this study and assumed was that sweat is its main component. Several studies reporting sweat rates for swimmers focussed on the temperature regulation during swimming (Kounalakis et al. 2010, McMurray and Horvath 1979, Robinson and Somers 1971, Taimura et al. 1998), while other studies focussed on the water and/or swimmers' salt balance (Cox et al. 2002, Henkin et al. 2010, Macaluso et al. 2011, Maughan et al. 2009, Taimura and Sugahara 1996), or even muscle damage during swimming (Cade et al. 1991). Few studies have been found on anthropogenic pollutant release (De Laat et al. 2011, Gunkel and Jessen 1986, Weng and Blatchley 2011): one study focussed on blood plasma urea concentration (Lemon et al. 1989) and one study focussed on sebum (skin lipids) released during swimming (Gardinier et al. 2009). Previous scientific publications report sweat rates of 0.08-1.62 L h^{-1} at various swimming pool conditions (20-35 °C) and at different exercise levels (Lemon et al. 1989, Macaluso et al. 2011, Maughan et al. 2009, McMurray and Horvath 1979, Nielsen et al. 1984, Robinson and Somers 1971). The normalised sweat release rate, calculated from the sweat rates reported in these publications, was 0.04-0.91 L m^{-2} h^{-1}. Urea and NPOC were the two reported parameters of which the released urea varied from 0.40-1.20 g urea per bather (Gunkel and Jessen 1986, Weng and Blatchley 2011) to 11.1 g urea per bather (De Laat et al. 2011) and the released NPOC was 12.4 g NPOC per bather. In addition to the continual anthropogenic pollutant release, these previous urea and NPOC results might also include initial and incidental anthropogenic pollutants release. Before policies can be developed to restrain anthropogenic pollutants release, more information is needed on the continual anthropogenic pollutant release and its main influencing parameters.

Anthropogenic pollutant release can be determined through basin-studies, bath-tub and shower experiments. It was expected that the continual anthropogenic pollutant release was mainly determined by the pool water temperature and the level of exercise. Because the heat-balance is an important parameter for sweat release, a pool tank study was chosen as the experimental setup. Preliminary trials showed that the dilution in a 3 m^3 pool tank was too much to observe differences with a 5-min sampling interval, therefore, experiments were conducted in a water-filled suit inside the pool tank (Figure 2.1) with laboratory time-series experiments. On-site experiments were used to validate the laboratory findings.

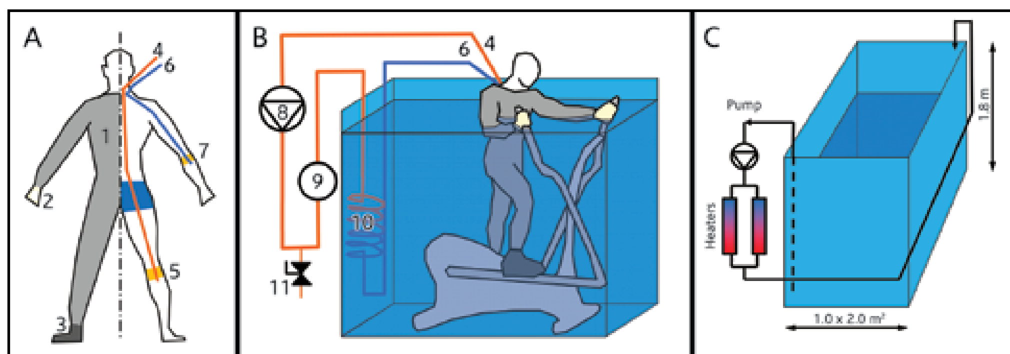


Figure 2.1 *A – Subjects were wearing a rain overall (1) connected to gloves (2) and plastic bags (3). Suit water was extracted with a tube (4) connected just below the subjects knees (5). Suit water was re-injected with a tube (6) connected just below the elbow (7). B – Water inside suit extracted from suit legs with tubes (4) using circulation pumps (8), led through flow indicators (9) and heat exchanging coils (10) to the suit sleeves with tubes (6). Regular samples were taken from a sampling valve (11). C – Schematic diagram of the pool tank, tank circulation and tank heating (Fabr. Hellebrekers Technieken, Nunspeet, the Netherlands).*

2.2 Materials and methods

2.2.1 Subjects

2.2.1.1 Subjects in laboratory time-series experiments

Four subjects joined the laboratory time-series experiments, two male and two female. General information of the subjects is shown in Table 2.1.

Table 2.1 *Age and height of participants during laboratory time-series experiments.*

	Participant A	Participant B	Participant C	Participant D
Gender	female	female	male	male
Age (y)	29	24	43	42
Height (m)	1.70	1.79	1.91	1.86
Weight (kg)	52.4	64.3	87.5	74.0
VO_{2max} (L min^{-1})	2.5	2.6	4.2	3.4

1 Shvartz and Reibold (1990)

2.2.1.2 Subjects in on-site experiments

The different subject groups for the on-site experiments and experiment conditions are described in Table 2.2.

Table 2.2 *Subject groups, gender, pool water temperature and level of exercise during on-site experiments.*

Subject groups	Males	Females	Water temp. (°C)	Level of exercise
First lane swim group	16	12	28	light or moderate effort
Second lane swim group	2	1	28	light or moderate effort
First triathlete group	12	3	28	vigorous swimming
Second triathlete group	12	1	28	vigorous swimming
Laboratory time-series experiments group	2	2	32	sitting, leisure swimming and aqua spinning

2.2.2 Experimental set-up: tank, shower and suit

To determine the continual anthropogenic pollutant release, standardised experiments were performed in a laboratory setting in a specially constructed pool tank (Figure 2.1). The water in the pool tank was circulated and heated with electric heaters and controlled to maintain a constant water temperature. A standardised shower cabin (Keuten et al. 2012) was used for showering before and after the experiment. During the experiment, the subjects wore

a polyester rain overall with a polyurethane coating (M Wear 5400 Warona) (Figure 2.1A). The suit was prepared for recirculation by connecting tubes to subjects' arms and legs (Figure 2.1B). The subjects' feet were wrapped in plastic bags and connected to the trouser legs of the rain overall. To prevent the plastic feet bags from damage, diving shoes were worn over them. The subjects wore lab gloves connected to the sleeves of the rain overall. The zipper of the rain overall was closed with waterproof tape. The neck part of the suit was closed but not sealed, for easy passage of the four tubes. Because the suit had a good fit at the neck, it was assumed that the evaporation of water and volatile components during the experiment was negligible. The suit water was recirculated inside the suit to ensure proper mixing and to control the water temperature. The suit water was circulated with two pumps at a rate of 150 L h^{-1} each and entered at the arms and left at the legs (Figure 2.1A). Heat exchanging coils, made from copper piping and situated in the pool tank, were used to ensure a constant water temperature of the suit water (Figure 2.1B).

2.2.3 Tank, shower and suit water

The water used for the pool tank, the pre-swim shower and for filling the suit was standard Dutch drinking water (tap water) which is distributed without a chlorine residual (Anonymous 2009). The water used for the pre-swim shower was additionally pasteurised (70 °C during $\geq$ 5 min) to inactivate all micro-organisms. Cold and hot tap water was used for the shower and was mixed using a thermostatic valve to ensure a constant water temperature of 37 ± 0.5 °C. The suit was filled with 25-30 L preheated tap water, using the heat exchanging coils from the suit circulation. A volumetric water meter (Sensus 620C, QN1.5) was used to measure the amount of tap water added to the suit.

2.2.4 Equipment preparation

The suit was rinsed with tap water. The suit recirculation loop was disinfected by recirculating a 10 ppm chlorine solution for 10 min. After disinfection, the recirculation loop was thoroughly rinsed with tap water and drained. The shower cabin was thermally disinfected with tap water at 70 °C for $\geq$ 5 min, rinsed with shower water and drained. Between the pre-shower and the after-shower, the shower cabin was rinsed with shower water and drained. The pool tank was filled with approximately 3 m^3 of water. The pool tank water was recirculated and heated at the selected temperature (25, 30 or 35 °C).

2.2.5 Pool site during on-site experiments

The on-site experiments were performed in an indoor swimming pool 'Het Sterrenbad' in Wassenaar, the Netherlands. General pool characteristics, pool water quality and environmental conditions are shown in Table 2.3. The experiments were performed in different pool basins with temperatures of 28, 32 and 34 °C.

Table 2.3 *General characteristics of pool basins, water quality and environmental conditions at test location; Sterrenbad in Wassenaar, the Netherlands.*

Parameter	Competition pool	Recreational pool	Therapeutic pool
Pool water surface (m^2)	375	167	355
Pool basin content (m^3)	1,111	210	169
Pool recirculation ($m^3\ h^{-1}$)	225	200	175
Set point water temperature (°C)	28	32	34
Set point air temperature (°C)	30	34	36
Set point humidity (%RH)	50	50	50
Set point chlorine (mg L^{-1})	0.9	0.8	0.9
Set point acidity (pH)	7.2	7.2	7.2
Average combined chlorine (mg L^{-1})	0.3	0.3	0.3

2.2.6 Experimental procedures

2.2.6.1 Laboratory time-series experiments

Each subject had a pre-swim shower (60 s) to remove all initial anthropogenic pollutants. After the pre-swim shower, the subjects dried themselves with a clean dry towel and their before-weight was determined (±5 g) on a scale (JBS loadcell, fabr. BWT, Boxtel, the Netherlands). Subsequently, the subjects were dressed in the suit, as described in Section 2.2.1 and shown in Figure 2.1A. They entered the pool tank and the filling of the suit was started. After approximately 5 min, the suit recirculation was started. During the first 15 min, the subjects rested in the tank. During the subsequent 30 min, the subjects performed an exercise on a submerged cross-trainer, an Aqua Nordic Walker (Kodin, Gundelsheim, Germany). The energy consumption during the exercise was similar in all laboratory time-series experiments and was estimated at 60-70% VO_{2max} with the use of the Compendium of Physical Activities (Ainsworth et al. 1993, Ainsworth et al. 2000). Each subject did the experiment at three different temperatures (25, 30 and 35 °C ± 0.5 °C) and they were asked not to drink during the experiment. During the experiment, samples were taken from the suit water every 5 min, starting simultaneously with the filling of the suit. The volume of all samples was determined by weighing, assuming a density of 1,000 g L^{-1}. After the experiment, all suit water was removed and collected to complete a mass balance as a check for leakages. After emptying the suit, the subjects had an after-shower, followed by drying with a clean dry towel and stepping on a scale to measure their after-weight.

2.2.6.2 On-site experiments in a swimming pool

Continual anthropogenic pollutant release was determined on-site by weighing the subjects on a scale (JBS loadcell, fabr. BWT, ±5 g) before and after their swimming activity and by measuring their height. After full submersion in pool water, subjects dried themselves with a clean dry towel before the weights were determined. They were asked not to drink during the experiment. The initial weight was also used to calculate the subjects' body surface area.

The subjects from the laboratory time-series experiments also participated in on-site experiments. During the on-site experiments, the continual anthropogenic pollutant release for the four subjects was determined in a 32 °C pool, at three different exercise levels: 30 min at rest, 30 min of leisure swimming and 30 min of aqua spinning. The resistance of the aqua spin bike (Waterfly, Regalbuto Italy) could be adjusted using rotatable perpendicular paddles on the crank axle that lead to water displacement. This resistance was set to the maximum.

2.2.7 Analytical methods

Samples were analysed for chemical and microbiological parameters. A wide range of parameters was examined to describe the continual anthropogenic pollutant release. These parameters included non-purgeable organic carbon (NPOC), total nitrogen (TN), cellular adenosine triphosphate (cATP), ammonium, urea, phosphate, nitrate, ultraviolet spectrophotometry, particle distribution, total and intact cells. The parameters that were found most relevant to describe the anthropogenic pollutant release were NPOC, TN, urea, ammonium, cATP, particle distribution and intact cell count, of which a short description of the methods is given below. Besides, each subject's heart rate was measured by counting the pulse.

NPOC was determined according to NEN-EN-1484 (1997) using a Shimadzu TOC-Vcph analyser. After acidifying and purging, the samples were injected into the combustion chamber at 680°C to oxidise all carbon into CO_2, which was subsequently detected by using infrared spectrometry.

TN was determined according to NEN-EN-12260 (2003) using a Shimadzu TNM-1 analyser connected to the Shimadzu TOC-Vcph analyser. The samples were injected into the combustion chamber at 720°C where nitrogen compounds were converted into nitric oxide and subsequently exposed to ozone to induce emission of light, which was detected by a chemiluminescent detector.

Ammonium was analysed according to ISO-7150/1 (2002) with an ammonium test kit (Merck, Darmstadt, Germany), which can be used for non-chlorinated water. For ammonium analysis, samples were alkalinised with sodium hydroxide to transform all ammonium nitrogen into ammonia. After chlorination and formation of monochloramine, thymol was added to form a blue indophenol derivative that was determined photometrically (Spectroquant Nova 60, Merck, Darmstadt, Germany).

Urea was analysed with a test kit (Merck, Darmstadt, Germany). After adding urease, urea was cleaved into carbon dioxide and ammonia. The subsequent ammonia analysis was similar to the ammonia analysis described above. A deviation from the test kit manual was the semi- quantitative measurement done with a visual comparator; samples were determined photometrically (Spectroquant Nova 60, Merck, Darmstadt, Germany). A 4-point calibration curve was made to calibrate the method.

Determination of cATP was based on bioluminescence (van der Wielen and van der Kooij 2010). Water samples were filtered through a glass fibre filter, 0.7µm, to remove all extracellular ATP. Subsequently, the cATP was extracted from the filter with a trisodiumphosphate solution (UltraLyse 7) and collected in a 15mL cuvette. The extracted cATP was diluted with Ultralute (ATP dilution buffer), added to a luciferine/luciferase complex to induce the emission of light,

and then placed directly into a Luminometer (Junior LB 9509, fabr. Aqua-tools) to measure the generated light signal (Relative Light Units, RLU). The concentration of cATP was calculated from the RLU values using a conversion factor determined from calibration measurements.

Particle distribution was determined with a Pacific scientific particle counter using a syringe-operated sampler Hiac Royco Model 3000 with a sensor Hiac HRCD-400 HC (2-400μm) and sizing counter Hiac Royco Model 9064. Highly concentrated samples (> 18×10^3particles/mL^{-1}) were diluted with demineralised water.

The number of total and intact cells was measured with a flow cytometer (FCM) as described previously (Prest et al. 2013). Two types of staining solutions were used to highlight either all cells with SYBR® Green I, or only intact cells with SYBR® Green Propidium Iodide. Where necessary, samples were diluted just before measurement with filtered (0.22μm; Millex-GP, Millipore) bottled mineral water (EVIAN, France). Measurements were performed using a BD Accuri C6® flow cytometer (BD Accuri cytometers, Belgium). Equipment settings and protocol were all according to Prest et al. (2013).

2.2.8 Calculations used in this study

The level of exercise during the exercises was estimated using the Compendium of Physical Activities (Ainsworth et al. 1993, Ainsworth et al. 2000). To estimate the individual energy consumption (VO_2), the level of exercise was multiplied by the individual VO_{2max}. The individual VO_{2max} was determined from fitness norms for males and females (Shvartz and Reibold 1990). The average energy consumption (VO_2) during the different experiments is shown in Table 2.4.

Table 2.4 *Average fractions of weight loss during 30 min of laboratory time-series and on-site experiments.*

Parameter	**Laboratory time-series experiments**	**On-site experiments**				
		Sitting	**Leisure swim**	**Aqua spin**	**Lane swim**	**Vigorous swim**
VO_2 ($L\ min^{-1}$)	2.1[(1)]	0.3[(2)]	1.3[(2)]	2.5[(2)]	2.1[(2)]	3.3[(2)]
Water temperature (°C)	25-30-35	32	32	32	28	28
P_a (mm Hg)	17	37	34	34	30	30
% BSA submerged	77%	94%	94%	52%	94%	94%
Total weight loss (g)	279	7	58	349	133	337
Ingested water (g)[3]	0	0	3.1	0	3.2	3.2
Skin hydration (g)[4]	2.1	1.8	1.8	1.0	1.9	2.0
Substrate oxidation (g)[5]	13.9	1.3	6.9	16.9	19.2	38.1
Respiratory water (g)[6]	44.1	1.4	6.3	9.7	16.3	25.4
Data points (subjects)	12 (4)*	12 (4)*	12 (4)*	12 (4)*	3 (3)	13 (13)

[1] Ainsworth et al. (1993), Ainsworth et al. (2000), Shvartz and Reibold (1990)
[2] calculated from actual heart rate
[3] Suppes et al. (2014)
[4] Scheuplein and Blank (1971)
[5] Maughan et al. (2007), Mitchell et al. (1972)
[6] Mitchell et al. (1972)
* the same 4 subjects

The sweat release was calculated from the body mass loss, corrected for substrate oxidation and respiratory water loss, unless noted otherwise (Maughan et al. 2007). Other parameters like ingested fluid, skin hydration and urine and faecal losses were found to be smaller than 5 g and therefore not included, unless noted otherwise. This resulted in a simplified equation:

$$\text{Sweat release} = \Delta M_b - M_{so} - M_{rw} \qquad \text{(EQ 2-1)}$$

Where
ΔM_b = body mass loss = before-weight – after-weight
M_{so} = mass substrate oxidation
M_{rw} = respiratory water loss

The body mass loss was determined on a scale and presented as kg weight loss. During the oxidation of substrates, O_2 and substrates are consumed and CO_2 and water are produced. Substrate oxidation is primarily determined by the exercise intensity, the aerobic fitness of the individual, the preceding exercise and diet regimen (Maughan et al. 2007). The production of CO_2 depends on the type of substrate that is used. At a high level of exercise, carbohydrates will be used as substrate; at a low level of exercise, fat will also be used. During the laboratory time-series experiments, estimated at 60-70% VO_{2max} (Ainsworth et al. 1993, Ainsworth et al. 2000) carbohydrates contribute to 67-75% in the energy consumption (Maughan et al. 2007).
The respiratory water was calculated according to Mitchell et al. (1972):

$$M_{rw} = 0.019 \times VO_2 \times (44 - P_a) \qquad \text{(EQ 2-2)}$$

Where
VO_2 = oxygen uptake in L min^{-1}
P_a = ambient water vapour pressure in mm Hg

Table 2.4 shows the calculated weight loss of the different fractions during 30 min of laboratory time-series and on-site experiments.
For a comparison between different subjects, all sweat release results are given as normalised sweat release per body surface area in L m^{-2} h^{-1}. An approximation of the body surface area of the subjects was determined using the empirical equation of Mosteller (1987):

$$\text{BSA} = \frac{\sqrt{\text{W} \times \text{H}}}{6} \qquad \text{(EQ 2-3)}$$

Where
BSA = body surface area (m^2)
W = body weight (kg)
H = body height (m)

2.2.9 Excluded data

The calculated sweat release for subject B during the 25 °C laboratory time-series experiment resulted in a negative normalised sweat release. Although it was not clear what caused this negative result, negative sweating is not possible, and the weight data of this experiment was therefore excluded from this paper. The analytical data of the water quality parameters of this experiment were not excluded.
During some trial experiments, some subjects also had a negative weight loss. These negative values could have been caused by drinking during the experiment, skin hydration or inaccurate wetting procedures at the start of the experiment, resulting in a too low before-weight. All subjects with negative weight loss were excluded from this study, 24 subjects in total.

Within a group of elderly swimmers there were three subjects that had a weight loss > 450 g. It was assumed that besides sweating also some incidental urine release occurred. These three subjects were therefore excluded from this study.

2.3 Results

2.3.1 Sweat release rate

During the laboratory time-series experiments, the continual anthropogenic pollutant release data from four subjects (two men, two women) were collected. The sweat release rate was calculated from the total weight loss (Table 2.4). The amount of ingested water, skin hydration, substrate oxidation and respiratory water is also shown in Table 2.4. All subjects had an increased sweat release at increasing experiment temperatures (Figure 2.2). Starting at 25 °C, the average sweat release of 0.1 L m^{-2} increased to 0.22 L m^{-2} and 0.46 L m^{-2} at 5 and 10 °C temperature increase, respectively. One subject did a resting experiment at a temperature of 35 °C, resulting in a normalised sweat release of 0.02 L m^{-2} h^{-1} (Figure 2.2).

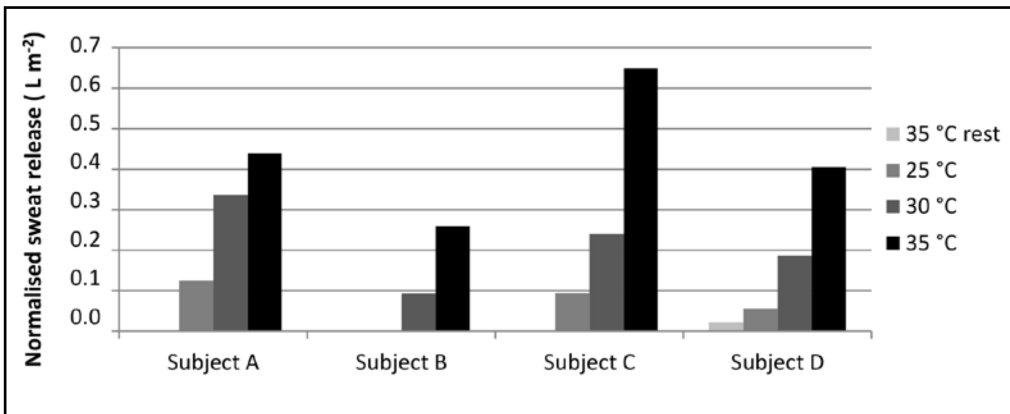


Figure 2.2 *Normalised sweat release during laboratory time-series experiments after 30 min of exercise at 60-70% VO_{2max} at different temperatures.*

Similar sweat releases are reported in literature, see Figure 2.3. To calculate the normalised sweat release from the data of Robinson and Somers (1971), the body surface area for the Olympic and World Champion medal winners was estimated at 2.0 m^2. Except for the subjects in this study, all subjects in literature were well-trained swimmers. Although the level of exercise was not the same for all experiments, the level was described as > 60% VO_{2max}. The subjects in the Macaluso et al. (2011) experiment had a high level of exercise, estimated at 90% VO_{2max} which resulted in higher normalised sweat releases.

The time-series subjects also participated in the on-site experiments at different levels of exercise. Figure 2.4 shows an increasing sweat release at increasing levels of exercise in a pool with a constant water temperature (32 °C). The sweat release was low (< 0.1 L m^{-2} h^{-1}) at an exercise level < 40% VO_{2max} and it increased linearly to 0.37 L m^{-2} h^{-1} at increasing exercise rates > 40% VO_{2max}. During aqua spinning, the subjects were only partially submerged due to the limited pool depth. The level of submersion for all experiments is shown in Table 2.4. During on-site experiments, two groups of lane swimmers did a moderate lap swimming exercise in a 28 °C competition pool. The first exercise, estimated at 60-70% VO_{2max} (Ainsworth et al. 1993, Ainsworth et al. 2000), had an average normalised sweat release of 0.12 L m^{-2} h^{-1}, not corrected for substrate oxidation and respiratory water. The second lane swim group, with a measured level of exercise by taking their pulse, had an average normalised sweat release of 0.18 L m^{-2} h^{-1}, corrected for substrate oxidation and respiratory water (Figure 2.5).

During other on-site experiments in a 28 °C competition pool, two groups of triathletes performed a heavy exercise. During the first experiment, estimated at 70-90% VO_{2max} (Ainsworth et al. 1993, Ainsworth et al. 2000), the normalised sweat release was determined at 0.04-0.39 L m^{-2} h^{-1} (including correction for ingested water, substrate oxidation and respiratory water). For the second triathlete group, with a measured level of exercise by taking their pulse, the normalised sweat release was determined at 0.07-0.83 L m^{-2} h^{-1}, including correction for ingested water, substrate oxidation and respiratory water (Figure 2.5). The actual VO_2 was calculated from the maximum heart rate percentage and the estimated VO_{2max} (Shvartz and Reibold 1990).

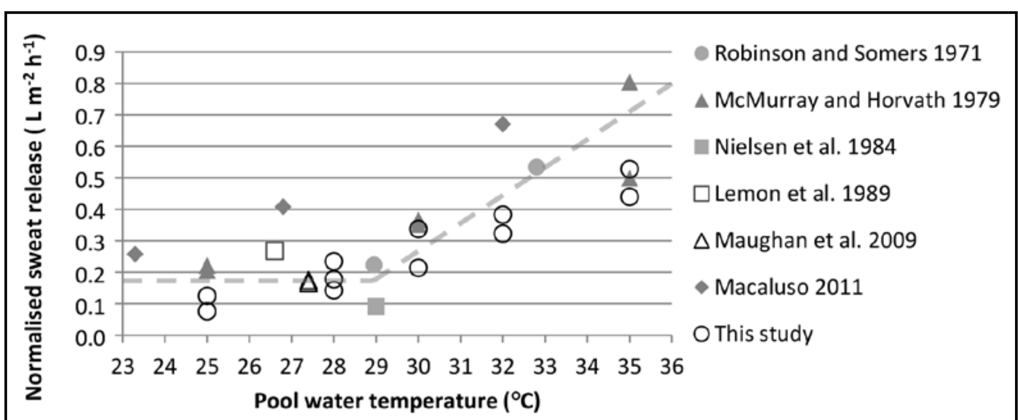


Figure 2.3 *Normalised sweat release in scientific literature at exercise levels > 60%* VO_{2max}.

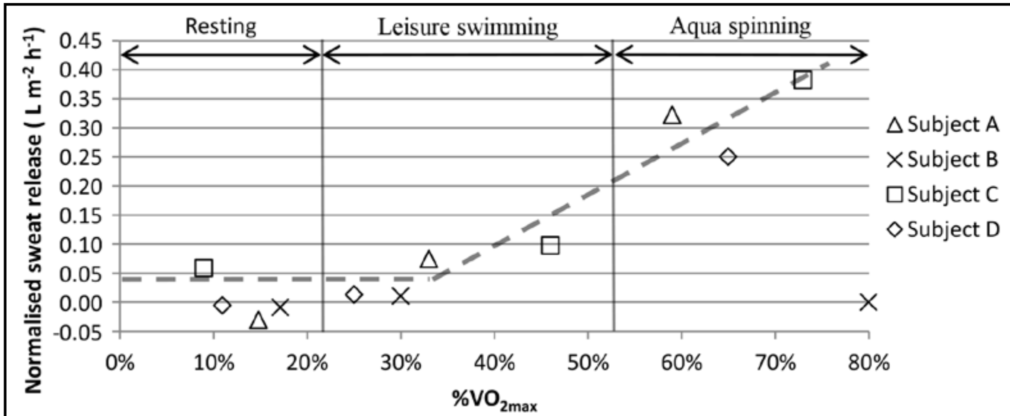


Figure 2-4 *Individual normalised sweat release during on-site experiments in 32 °C pool water at different exercise levels.*

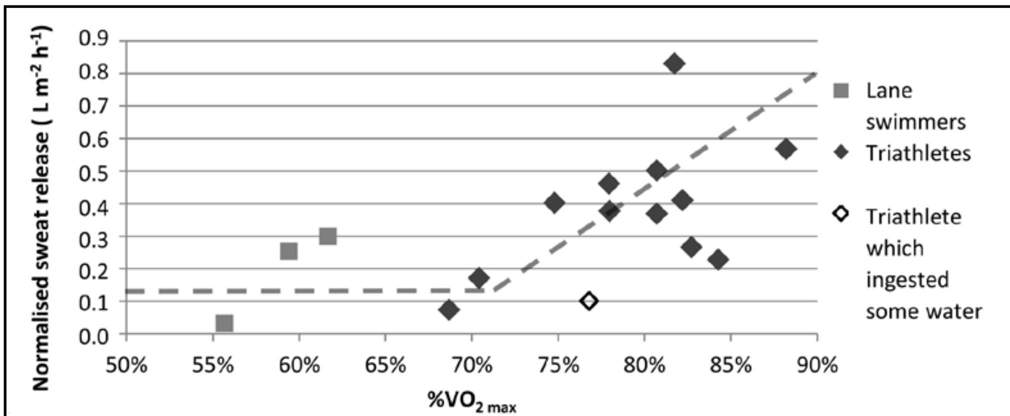


Figure 2-5 *Normalised sweat release for on-site experiments in a 28 °C competition pool with lane swimmers and triathletes. The subject with the open marker admitted ingestion of an unknown amount of pool water during the experiment.*

2.3.2 Continual anthropogenic pollutant composition

During the laboratory time-series experiments, four subjects (two females and two males) were studied in water-proof suits to determine the anthropogenic pollutants composition at each three different water temperatures (25-30-35 °C). The level of exercise was similar during all experiments. Descriptive parameters of the anthropogenic pollutants released during 30 min of exercise are shown in Table 2.5. The chemical components (NPOC, TN, urea and ammonium) showed a more-or-less steady release over time (Figure 2.6). The release of the particle-related components (particle count, cATP and intact cells) dropped over time (Figure 2.7).

There was a clear difference in the release of chemical anthropogenic pollutants at 25 °C compared to 35 °C, both during exercise and rest (Figure 2.8). At 35 °C during rest the release was 20-40% of that at 25 °C during exercise while at 35 °C during exercise the release was 170% of that at 25 °C for NPOC, TN and urea. The release of ammonium decreased at higher temperatures (Figure 2.8). There was no clear relation between the release of particle-related components and different temperatures (Figure 2.8).

Although the release of most chemical anthropogenic pollutants increased at higher temperatures, the increase was much lower compared to the sweat release during exercise at elevated water temperatures. The sweat release increased 2.3 and 4.6 times at 5 and 10 °C temperature increase, respectively, (Figure 2.2) while the pollutants release increased by a factor 1.1 to 1.7 (Figure 2.8).

Table 2.5 *Release of anthropogenic pollutants during 30 min of exercise for each of 4 subjects at three different water temperatures during laboratory time-series experiments.*

Parameter	Range	Average	St.deviation	n
NPOC (mg)	30-503	250	91.6	12
TN (mg)	44-161	77.3	31.5	12
Urea (mg)	14-76	37.1	16.7	11
Ammonium (mg)	4.5-17	10.1	4.1	11
Particles 2-50 µm (×10^9#)	0.2-2.3	1.3	0.6	12
cATP (µg)	1.2-20.6	5.2	5.2	12
Intact cell count (×10^6#)	1.0-21.9	9.3	6.5	12

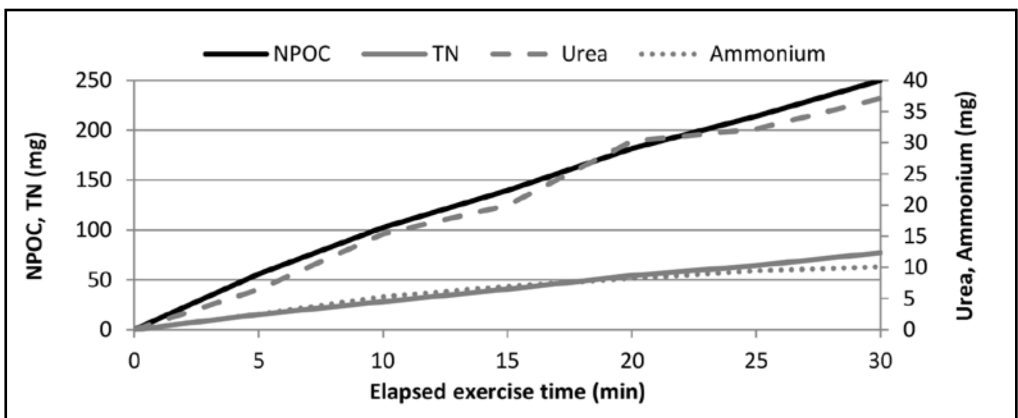


Figure 2-6 *Average release of anthropogenic pollutants during all laboratory time-series experiments (genders and temperature levels combined).*

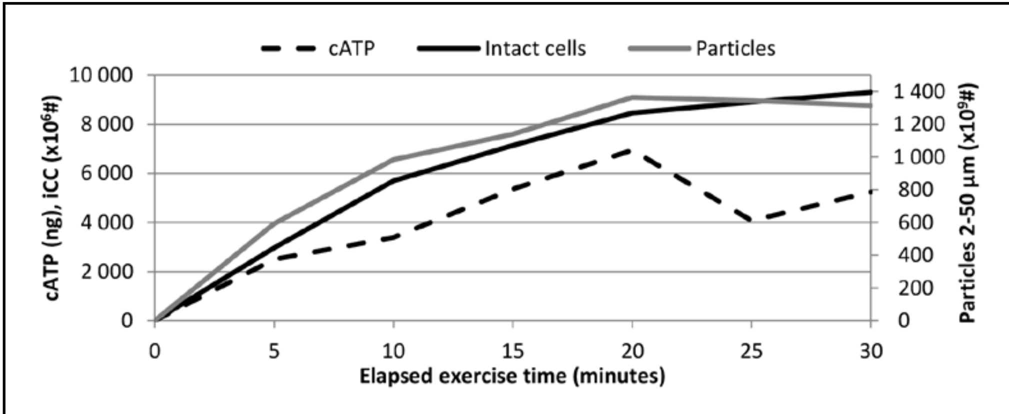


Figure 2-7 *Average microbiological and particle release of all laboratory time-series experiments (genders and temperature levels combined).*

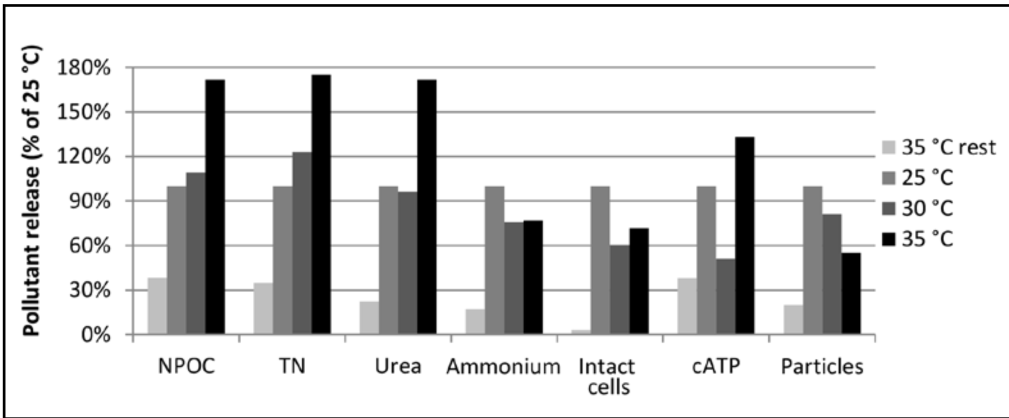


Figure 2.8 *Release of anthropogenic pollutants during laboratory time-series experiments compared to the release at 25°C.*

2.4 Discussion

This experiment was designed to be a static (in position) submerged exercise. Some of the conditions during the test were equal to swimming and some were not. For heat transfer during submerged exercise, important aspects are: the water temperature, the hydraulics, the type of exercise (which muscles are used), the level of exercise and the level of submersion. The water temperature was equal to swimming conditions and the water temperature in the suit was controlled and frequently measured. The hydraulics were not equal to swimming conditions, but the flow inside the suit was turbulent which is important for an optimal heat transfer. Most probably the heat transfer from the skin to the water was similar to swimming because the direction of the flow near the skin is not important for heat transfer and the water temperature in the suit was kept at a constant level. The type of muscles used was not similar to swimming, but the level of exercise was chosen to be similar to swimming. The level of submersion was limited due to the limitations of the suite and was therefore also not exactly the same as during swimming. However, the level of submersion was estimated (Table 2.4) and the results were calculated and presented as sweat released per surface area submerged skin.

2.4.1 Sweat rate

Assumed was that sweat is the main contributor to the continual anthropogenic pollutant release. Heat production from the physical exercise was assumed to be the same during all laboratory time-series experiments, while the cooling efficiency of the water differed at the three temperature settings. As the cooling efficiency of the pool water was reduced at higher temperatures, the sweat release was increased (Figure 2.2). Being submerged, the cooling mechanism from evaporating sweat appeared to be not effective, except for the unsubmerged body parts, in this case the head. A growing increase of sweat release is a logical result of the ineffective sweat mechanisms during submerged physical exercise in heated pool water (Kuno 1956).

The sweat release shown in Figure 2.3 can be explained by the sweat mechanism which is triggered by an increase of a subject's core temperature (Kuno 1956, McMurray and Horvath 1979, Robinson and Somers 1971). The body temperature is the net result of the difference between heat production and heat loss. Colder water enhances heat loss and thus blunts the increase in body core temperature. At a pool water temperature of 23 °C, and a medium exercise level, the sweat release will be low and will not increase at water temperatures slightly higher than 23 °C due to the high cooling efficiency of the water. If the pool water temperature rises, there will be a temperature threshold at which the muscular heat production and pool water cooling are in equilibrium. Above this threshold, the subject's core temperature will rise at increasing water temperatures (Nadel 1979). The type of swim clothing and percentage of body fat also influences the sweat release because insulation from swim wear or body fat will interfere with the heat exchange between the pool water and the human body (McMurray and Horvath 1979). A high percentage of body fat and insulated full-body swim wear will reduce the threshold and increase sweat release at lower water temperatures. The threshold is a function of the level of exercise and the pool water temperature. Due to training, a swimmer's threshold may become lower (Ichinose et al. 2009) and the sweat rate above the threshold may increase (Nadel 1979). Figure 2.3 shows the influence of pool water temperature. At pool water temperatures below 29 °C combined with a ≥ 60% VO_{2max} exercise level, the sweat release is more or less stable at 0.1-0.2 L m^{-2} h^{-1}.

At pool water temperatures above 29 °C, there is a linear increase in the normalised sweat release to 0.5-0.8 L m^{-2} h^{-1} at 35 °C. Figure 2.5 shows the influence of exercise level. The sweat release is 0.1-0.2 L m^{-2} h^{-1} at an exercise level < 70% VO_{2max} and increases with increasing exercise levels to 0.8 L m^{-2} h^{-1} (Figure 2.5).

Most swimming pool activities in recreational pool water (≥ 30 °C) will remain ≤ 60% VO_{2max} and most activities in competition pool water (≤ 29 °C) will be ≥ 70% VO_{2max} (Table 2.6). The low-sweating threshold is shown in Figure 2.3, Figure 2.4 and Figure 2.5. The sweat rate is 0.1-0.2 L m^{-2} h^{-1} at water temperatures < 29 °C and < 70% VO_{2max} level of exercise and increases linearly with increasing water temperatures and exercise level to 0.8 L m^{-2} h^{-1} at 35 °C and > 60-70% VO_{2max} level of exercise (Figure 2.3, Figure 2.4 and Figure 2.5). Sweat release in swimming pools cannot be avoided. To reduce the sweat release to a minimum, it is recommended that pool operators maintain a low pool water temperature in pools with high exercise levels, preferably ≤ 27 °C for competition pools. It is also recommended to avoid a high level of exercise activities in pools with elevated water temperatures (≥ 30 °C).

Table 2.6 *Specific swimming pool activities and their energy consumption (Ainsworth et al. 1993, Ainsworth et al. 2000).*

Activity	**%VO_{2max}**
Whirlpool sitting	10%
Standing still	18%
Walking lifeguard	23%
Water volleyball	30%
Swimming, treading water, moderate effort, general	40%
Water aerobics, water calisthenics	40%
Swimming - lake, ocean, river	60%
Swimming, leisurely, not lap swimming, general	60%
Lap swimming, freestyle, slow, moderate or light effort	70%
Swimming, backstroke, general	70%
Swimming, crawl, slow (25m in 32.8 seconds), moderate or light effort	80%
Swimming, sidestroke, general	80%
Swimming, synchronised	80%
Water jogging	80%
Lap swimming, freestyle, fast, vigorous effort	100%
Swimming, breaststroke, general	100%
Swimming, treading water, fast vigorous effort	100%
Water polo	110%
Swimming, butterfly, general	110%
Swimming, crawl, fast (25m in 21.9 seconds), vigorous effort	110%

2.4.2 Continual anthropogenic pollutants

The water temperature showed a strong influence on the release of some anthropogenic pollutants as well as the level of exercise (Figure 2.8). The release of chemical components (NPOC, TN and urea) is increased by a factor 1.7 at 10 °C temperature increase and increased by a factor 5-7 if resting is compared with the laboratory time-series exercise. If all anthropogenic pollutants from the laboratory time-series experiments originate from sweat, the sweat composition could be calculated by dividing the amount of anthropogenic pollutants by the corresponding sweat release. In Table 2.7, this sweat composition for the chemical components is compared with sweat compositions from other studies. For ammonium, urea and TN, the calculated sweat concentrations were within the same range as the results from previous studies. Sweat is therefore very likely to be the main source for the measured TN, urea and ammonium. The release of ammonium at elevated temperatures during the laboratory time-series experiment was different compared to the release of TN and urea (Figure 2.8). It is not clear why ammonium was reduced at higher temperatures while TN and urea increased. This could be due to the fact that ammonium is a volatile component and the experiment was done during an exercise in a suit, where the suit water was constantly circulating and evaporation of ammonium may have occurred, especially at elevated temperatures that reduce the solubility of ammonium.

For NPOC, the results in Table 2.7 were not comparable with previous studies on sweat composition. Very little information has been published on the NPOC content of sweat because sweat samples from human skin are easily contaminated with skin lipids, thereby disturbing the NPOC measurement (Kuno 1956). The calculated NPOC value from reported sweat components is much lower than the maximum value found in this study (Table 2.7). The release of sebum (skin lipids) is an explanation for the elevated NPOC concentrations, because sebum mainly contains carbon-strains (Downing et al. 1983). The sebum on human skin is washed off during swimming at a rate of 0.24 mg m^{-2} h^{-1} (Gardinier et al. 2009). When the skin surface is defatted, fresh sebum is rapidly secreted from the sebum reservoir in an attempt to restore the surface lipid film. This sebum will not stay on the skin surface, but will be released into the water. Secretion from the sebum reservoir appears at a greater rate than sebum is actually being produced by the sebaceous glands (Downing et al. 1983). The average sustainable sebum secretion rate is 0.27 mg m^{-2} h^{-1} in healthy subjects and can be 0.84 mg m^{-2} h^{-1} for subjects with acne (Harris et al. 1983). This means that most TN compounds originated from sweat whereas most NPOC compounds originated from sebum.

Although the laboratory time-series experiments only focussed on a 30 min exercise period, the release of sweat will most probably continue at prolonged exercise periods. Therefore, it is assumed that the chemical related continual anthropogenic pollutant release will also continue after 30 min of exercise at a similar rate.

Table 2.7 *Reported sweat composition compared to the results of laboratory time-series experiments in this study.*

Publication	Concentrations (mg L^{-1} sweat)			
	NPOC	TN	Urea	Ammonia
Craig et al. (2010)			655	105
Stefaniak and Harvey (2006)	965*	493*	601	102
Eichelsdörfer et al. (1975a)		992	1,447	220
Kuno (1956)		170-1,960	456	30-100
Mosher (1933)			240-1,120	40-200
This study	444-4,402	119-1,281	80-445	20-184

* calculated from the sweat composition without vitamins and ionic components

The particle-related anthropogenic pollutants (intact cells, cATP and particles) seemed to be less temperature related, but there was an influence from the level of exercise (Figure 2.8). During exercise, the microbiological components (intact cells and cATP) fluctuated at different temperatures; this might be due to individual (hygienic) differences. This also explains the large variance within the microbiological data (Table 2.5). Although the average data show a temperature influence on the particle release (Figure 2.8), the individual data show that the temperature influence was not consistent during all laboratory time-series experiments (Figure S2.7).

While the chemical components of the continual anthropogenic pollutant release were more-or-less constantly released over time, the release of the microbiological components (cATP and intact cells) dropped over time. This can be explained by the fact that chemical components originate from sweat and sebum glands and are constantly produced by the human skin, while the microbiological components are attached to the human skin or hair. Nevertheless, the release of microbiological components was assumed to continue after 30 min of exercise, but at a reduced rate.

The release of particles dropped earlier in time compared to the microbiological components. Besides microorganisms, the particles released by bathers also contain skin cells, hair, textile fibres, dust/sand and other particles attached to the human skin. The individual graphs show that the release of 2-50 µm particles became more-or-less negligible after 30 min of exercise (Figure S2.7).

Although the water temperature had an influence on the anthropogenic pollutants released from bathers (Figure 2.8), individual differences and duration of the exercise had a more dominant influence (Figure S2.1 till S2.7). Nevertheless, the pool water temperature was the only parameter that can be controlled by pool operators and is, therefore, the main parameter to restrain the continual anthropogenic pollutant release.
A study with more subjects is needed to investigate the effects of temperature and exercise on the continual anthropogenic pollutant release. During future laboratory time-series experiments, the level of exercise should be closely monitored by measuring VO_2 and the water temperature and the level of exercise can easily be varied.

Other publications did not report what part of the reported anthropogenic pollutant releases is related to the continual anthropogenic pollutant release. There is no information on whether subjects in these studies had a pre-swim shower or, if they did, what the duration of the shower was. Furthermore, it is not clear what part of the reported data is due to incidental anthropogenic pollutants released. The existing literature data, therefore, cannot be used for comparison with this study.

Combining the results from this study with a previous study on the initial anthropogenic pollutant release (Keuten et al. 2012), the overall picture of anthropogenic pollutants released by bathers becomes clearer. Table 2.8 shows the NPOC, TN and cATP for the initial, continual and incidental pollutants released. At this exercise level, the continual anthropogenic pollutant release for NPOC and TN, equalled 37% of the total anthropogenic pollutant release. The remaining part of the total anthropogenic pollutant release, 63%, is a result of unhygienic behaviour, meaning not having a pre-swim shower, 31%, and not using a toilet "when nature calls", 32% (Figure 2.9). While the continual release of NPOC and TN was reduced at lower exercise levels and lower water temperatures, at the same time the remaining unhygienic portion of the anthropogenic pollutants released increased from 55% at high temperatures to 68% at low temperatures for NPOC and TN (Figure 2.9). Reduction of the unhygienic part of the released anthropogenic pollutants has the potential to reduce the DBP formation by 55% in 35 °C water and 68% in 25 °C water, both at high exercise levels. The reduction potential is assumed to be > 68% for recreational and leisure pools.

Although the level of exercise in competition pools is similar to the level of exercise investigated in this study, the level of exercise in other pool types (recreational pools, therapeutic pools, toddler pools, whirlpools etc.) is not expected to exceed the 60% VO_{2max} level. The continual anthropogenic pollutant release is assumed to be smaller at lower exercise levels. A smaller portion of the continual pollutants means a larger share of the unhygienic pollutants in these pool types. Therefore, more future research on the continual anthropogenic pollutant release at these conditions should be done.

Table 2.8 *Total anthropogenic pollutants released during a 30-min swimming pool visit at 60-70%* VO_{2max} *for bather that did not have a pre-swim shower.*

Parameter	**Anthropogenic pollutant release**			
	Initial[1,2]	**Continual**	**Incidental[3]**	**Total**
NPOC (mg)	217	250	192	659
TN (mg)	57	77.3	70	204
cATP (µg)	1.7	5.2	n.a.	6.9*
Particles 2-50 µm ($\times 10^9$#)	0.2	1.3	n.a.	14.6*

n.a.; not available data
[1],[2]) Keuten et al. (2012) and corrigendum
[3]) based on a 30 mL urine release per bather (Gunkel and Jessen 1986)
* incidental fraction not included

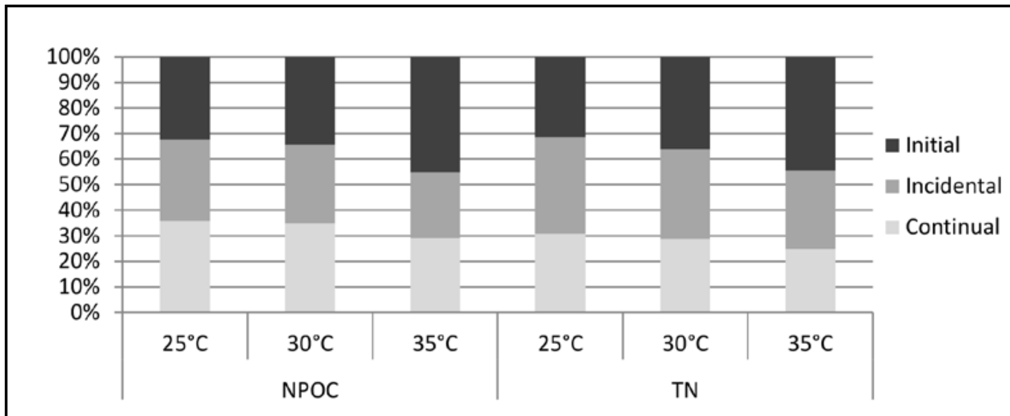


Figure 2.9 *Fractions of initial, continual and incidental anthropogenic pollutants release at different temperatures during laboratory time-series experiments at 60-70%* VO_{2max} .

2.5 Conclusion

Laboratory time-series experiments with controlled exercise conditions (level of exercise and water temperature) showed to be a good way to determine the continual anthropogenic pollutant release. The continual anthropogenic pollutant release consisted of sweat, sebum, particles and microorganisms. By weight, sweat is the main component of the continual anthropogenic pollutant release. The net weight loss caused by a submerged exercise was strongly related to the water temperature and the level of exercise. At low water temperatures (< 29 °C), the cooling effect of the water was large and only a vigorous level of exercise induced a sizable the continual anthropogenic pollutant release. The sweat rate was 0.1-0.2 L m^{-2} h^{-1} at water temperatures below 29 °C and increased linearly with increasing water temperatures to 0.8 L m^{-2} h^{-1} at 35 °C. The sweat rates found in this study were comparable to the results from recent scientific publications.

Although water temperature and level of exercise had important roles in anthropogenic release, the duration of the swim visit is, logically, the main parameter determining the continual anthropogenic pollutant release. Nevertheless, the pool water temperature is the only parameter that can be controlled by pool operators and is therefore the main parameter to restrain the continual anthropogenic pollutant release.

Chemical pollutants were continuously released during a swim visit, while the release of particles seemed to become negligible after 30 min of swimming. The release of most components could be explained with the reported composition of sweat. The average releases during 30 min of exercise for the different components are 77.3 mg per bather TN, 37.1 mg per bather urea and 10.1 mg per bather ammonium. The release of NPOC could not be explained by the composition of sweat and was, most probably, a result of sebum release and was determined at 250 mg per bather NPOC per 30 min. The release of particles (2-50 µm) was measured at an average of 1.3×10^{9} particles per bather. The average release of cATP and intact cells was measured at 5.2 µg cATP per bather and 9.3×10^{6} intact cells per bather.

The continual anthropogenic pollutant release is a significant part of the total anthropogenic pollutants released and therefore also plays a role in the production of DBPs. At a 60-70% maximum exercise level, 37% of the total released pollutants were released as the continual anthropogenic pollutant release. This means that 63% of the total released pollutants are due to unhygienic behaviour such as no pre-swim shower and no use of toilets "when nature calls". At lower exercise levels, the percentage unhygienic release is expected to be even larger. It is recommended that future studies focus on the continual anthropogenic pollutant release at lower exercise levels.

2.6 Supplement

Individual release of pollutants during laboratory time-series experiments

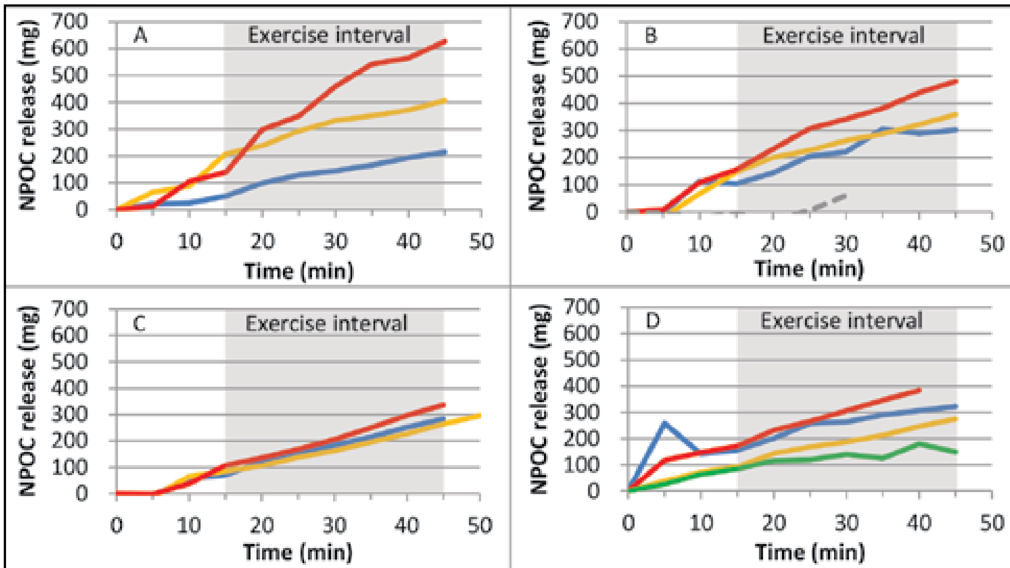


Figure S2.1 *Individual (subject A-D) NPOC release (mg) during laboratory time-series experiments at different temperatures (—25°C, — 30°C, — 35°C, — 35°C rest, — 35°C suit only).*

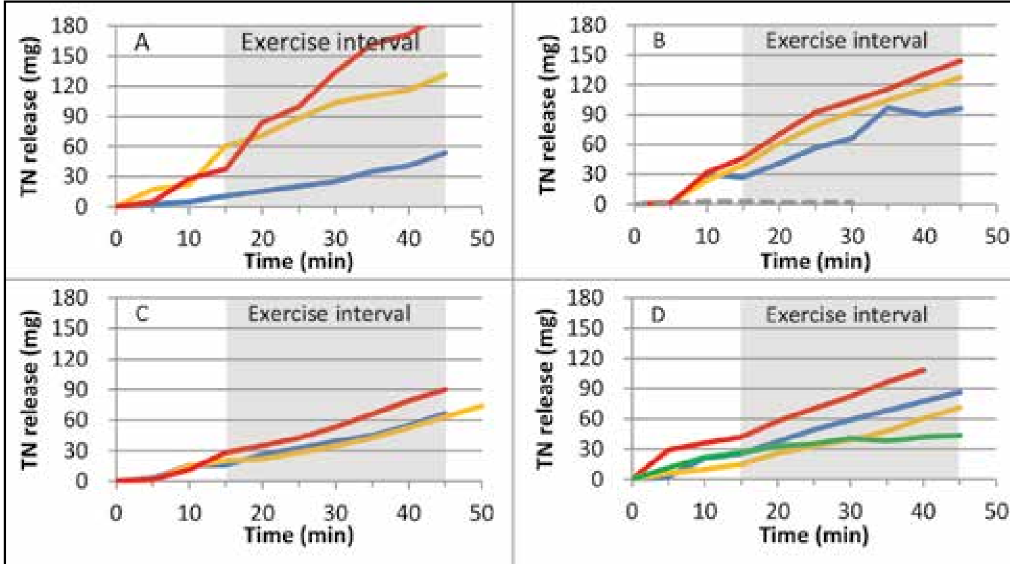


Figure S2.2 *Individual (subject A-D) TN release (mg) during laboratory time-series experiments at different temperatures (—25°C, — 30°C, — 35°C, — 35°C rest, — 35°C suit only).*

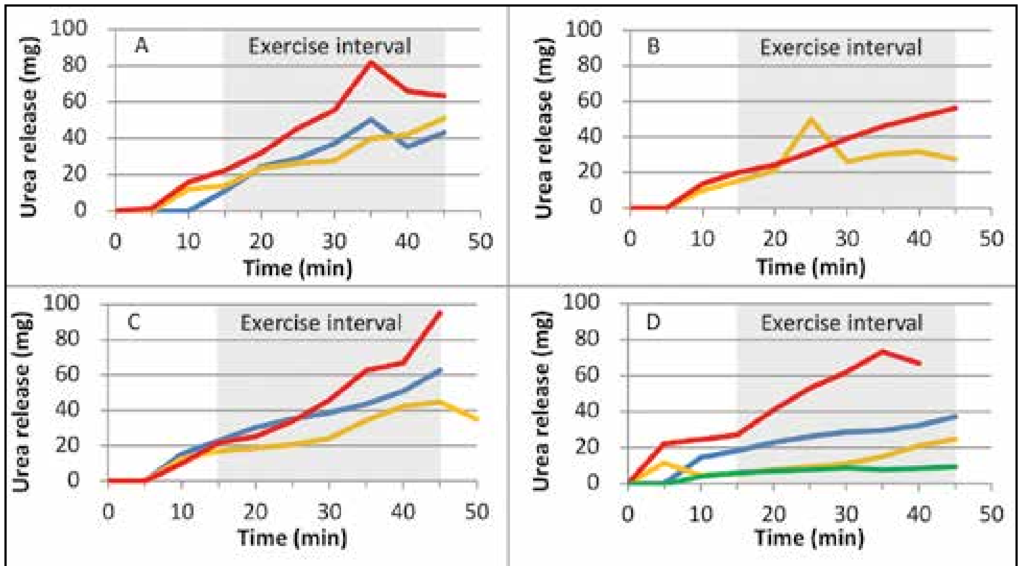


Figure S2.3 *Individual (subject A-D) urea release (mg) during laboratory time-series experiments at different temperatures (—25°C, — 30°C, — 35°C, — 35°C rest, — 35°C suit only).*

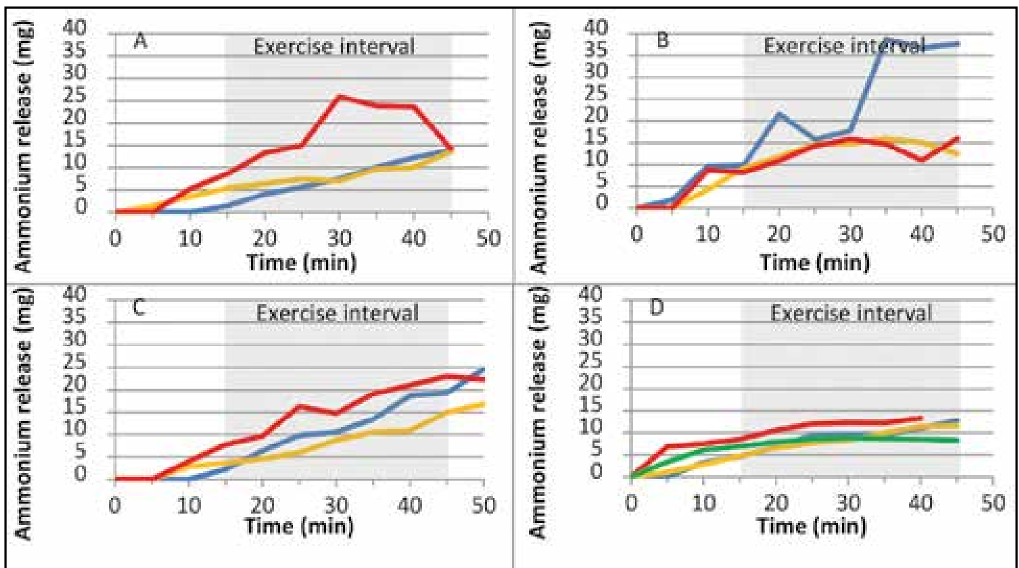


Figure S2.4 *Individual (subject A-D) ammonium release (mg) during laboratory time-series experiments at different temperatures (—25°C, — 30°C, — 35°C, — 35°C rest, — 35°C suit only).*

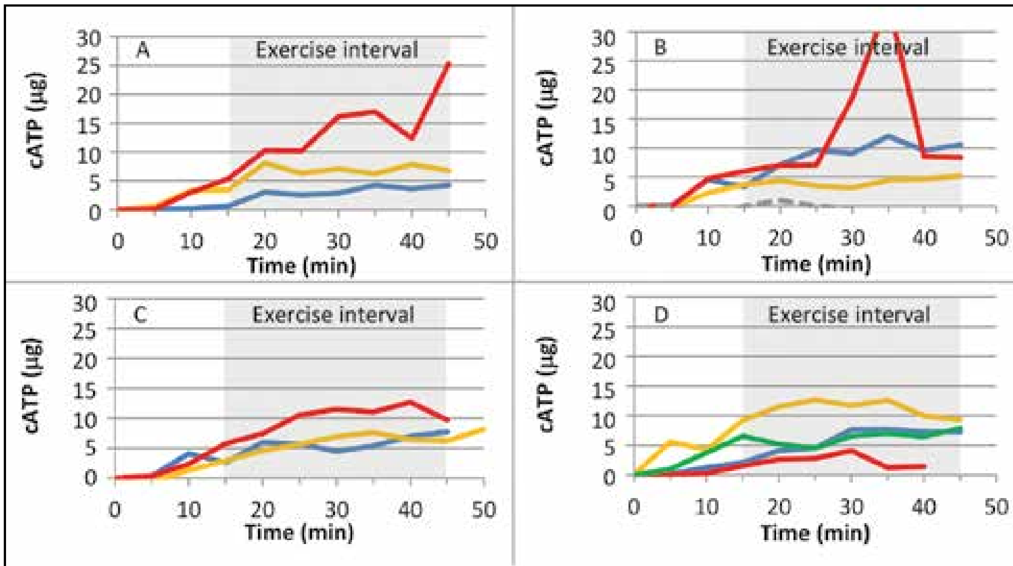


Figure S2.5 *Individual (subject A-D) cATP release (µg) during laboratory time-series experiments at different temperatures (—25°C, — 30°C, — 35°C, — 35°C rest, — 35°C suit only).*

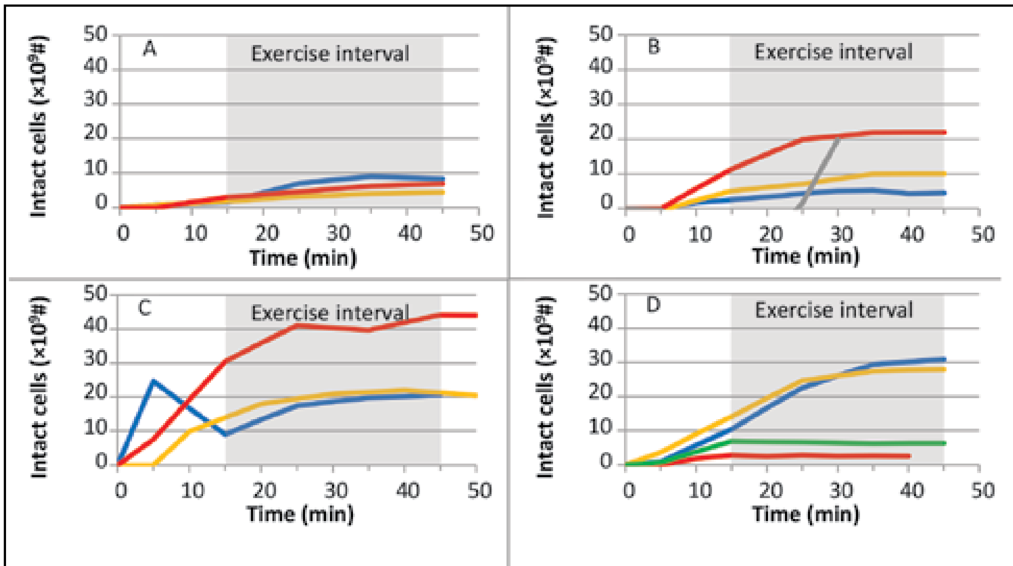


Figure S2.6 *Individual (subject A-D) release of intact cells (×10 ⁶#) during laboratory time-series experiments at different temperatures (—25°C, — 30°C, — 35°C, — 35°C rest, — 35°C suit only).*

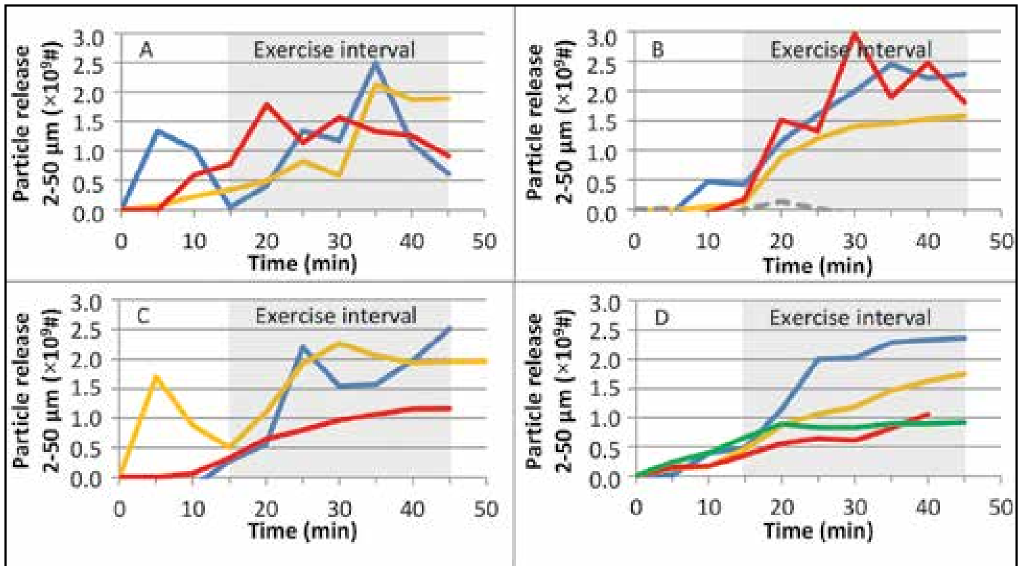
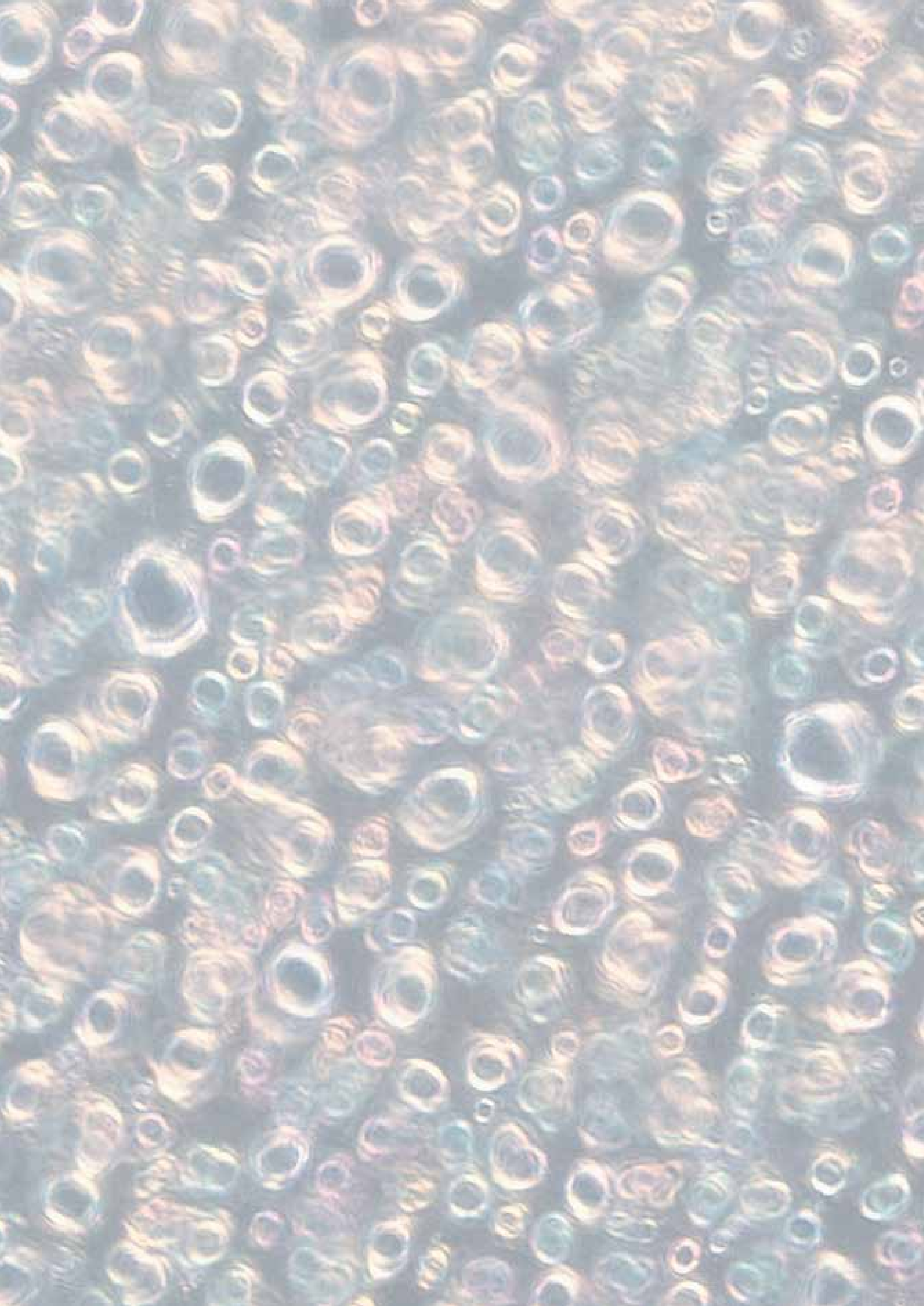


Figure S2.7 *Individual (subject A-D) particle release (×10 ^{9}# particles 2-50 µm) during laboratory time-series experiments at different temperatures(—25°C, — 30°C, — 35°C, — 35°C rest, — 35°C suit only).*



3 Characterization of the bacterial community in shower water before and after chlorination

Chlorine resistant bacteria entering swimming pools

Abstract

Bathers release bacteria in swimming pool water, but little is known about the fate of these bacteria and potential risks they might cause. Therefore shower water was characterized and subjected to chlorination to identify the more chlorine resistant bacteria that might survive in a chlorinated swimming pool.

The total community present in the concentrated shower water before and after chlorination (1 mg Cl_2 L^{-1} for 30 s) was characterized. More than 99% of the bacteria were Gram-negative. The bacterial families with a relative abundance of ≥ 10% of the non-chlorinated and chlorinated samples were *Flavobacteriaceae* (24-21%), *Xanthomonadaceae* (23-24%), *Moraxellaceae* (12-11%) and *Pseudomonadaceae* (10-22%). Because the relative abundance of *Pseudomonadaceae* increased after chlorination of the concentrated shower water, *Pseudomonadaceae* were suggested to be relatively more chlorine resistant than the other identified bacteria.

Characterizing the intact cell community present in the concentrated shower water reflects another way to determine chlorine resistance. The bacterial families with intact cell membranes present for ≥ 10% of the non-chlorinated and chlorinated samples were *Xanthomonadaceae* (21-17%) and *Moraxellaceae* (48-57%). *Moraxellaceae* were thus suggested to be more chlorine resistant than the other identified intact bacteria present.

Furthermore, the relative abundance of *Pseudomonadaceae* in the concentrated shower water increased with longer contact times at 1 mg Cl_2 L^{-1}. This suggests that *Pseudomonadaceae* were more chlorine resistant than the other identified bacteria present.

Within both families of *Pseudomonadaceae* and *Moraxellaceae* (opportunistic) pathogens were found, which are therefore likely to be released in swimming pools as well. Because these families were more chlorine resistant than others, there could be a potential health risk.

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3.1 Introduction

Chlorine-based products are used in most swimming pools as residual disinfectant because of its effectiveness and low costs (Shannon et al. 2008) as well as its mandatory use in many countries. In the Netherlands, the free available chlorine concentration in swimming pools is required to be between 0.5 and 1.5 mg Cl_2 L^{-1}, which is based on a 4-log removal of *Pseudomonas aeruginosa* at 1 mg Cl_2 L^{-1} within 30 s contact time (Anonymous 2011). To monitor the swimming pool water quality, different indicator organisms are used. Whereas *P. aeruginosa* is used as an indicator organism for disinfection efficiency, *Escherichia coli* is used as a faecal indicator (WHO 2006).

Indicator organisms have been used for many years because of their (assumed) similar response to water treatment processes as pathogens (WHO 2006). In addition, indicator organisms are usually present in higher concentrations than pathogens, and the analysis methods of indicator organisms are easier and cheaper to apply. However, it is unknown which microorganisms, including indicators, bathers introduce into swimming pools.

Faecally derived microorganisms may enter the pool water when residual faecal material on bathers' bodies is washed into the pool or when a person has an (accidental) faecal release (WHO 2006). Non-faecally derived microorganisms might enter the pool by being washed from skin or due to vomit, mucus or saliva (WHO 2006). Because these potentially infectious microorganisms enter the pool, a potential health risk exists when a pool is not well-operated. Different outbreaks or incidents of waterborne infections have been reported in the past (CDC 2000, Dziuban et al. 2006, Favero 1984). These incidents indicate that some microorganisms could be resistant to disinfects (Hingst et al. 1995), such as the (opportunistic) pathogens *Pseudomonas aeruginosa, Staphylococcus aureus* and *Klebsiella pneumoniae* (Papadopoulou et al. 2008), of which some bacterial families are related to skin infections (Al-Tatari et al. 2007, Chang et al. 2008, Jurado et al. 2002, Mashouf et al. 2008b, Wade et al. 1991). Although methicillin-resistant *Staphylococcus aureus* (MRSA) of the *Staphylococcaceae* family is associated with bacterial skin infections, *S. aureus* has also been found inside human noses (Burns et al. 2004, Noble et al. 1967), Verburgh and De Neeling 2006). Moreover, the bacterial family *Streptococcaceae* which is also associated with bacterial skin infections was not found on human skin by the Human Microbiome Project (Human Microbiome Project 2012) and only occasionally by Burns et al. (2004), while the bacterial skin infection related *Streptococcus pyogenes* was found inside human mouths (Human Microbiome Project 2012) and in 10% of human throats (Burns et al. 2004).

When bathers act hygienically in swimming pools, it might be assumed that most of the microorganisms brought into a pool are skin related because of the large exposed surface area. In addition, bacteria from oral and nasal cavities are released during swimming. The composition of an anthropogenic bacterial wash-off community released by bathers was characterized, and the impact of chlorination on the community was investigated.

3.2 Materials and methods

3.2.1 Shower procedure

The initial anthropogenic pollutant release is introduced into the pool water during the first minutes of body contact with the water and consists of the residue of evaporated sweat, microorganisms and pollutants as well as any cosmetics on the swimmer's skin (Keuten et al. 2012). Therefore, to obtain an initial anthropogenic community, standardised shower experiments were performed in a laboratory setting. Because specific factors like age, location, and sex contribute to the variability of the microbial flora of the skin (Grice and Segre 2011), an average anthropogenic community was obtained by collecting all shower water of 10 West-European adults (5 male and 5 female) between 20-40 years old. Each showered for 60 s in a standardised shower cabin in a laboratory (Keuten et al. 2012), creating ~10 L of shower water per person.

Before using the standardised shower cabin, the shower hose, shower nozzle, rinsing hose and sampling scoop were thermally disinfected (5 min, 70 °C). Next, the shower cabin was rinsed twice, disinfected by spraying a 70% ethanol solution (ethanol 96%, VWR chemicals, mixed with demineralized water to a 70% ethanol solution) on the surfaces and leaving it to disinfect for 2 min. Lastly, the cabin was rinsed with drinking water in triplicate and drained until only a few drops of water came out.

The water used for showering was non-chlorinated tap water (Anonymous 2013). Hot tap water was mixed with cold tap water using a thermostatic valve, ensuring a constant water temperature of 38 ± 0.5 °C. Blank samples from showering without a test person resulted in an average cell concentration of 5×10^3 intact cells mL^{-1}, while the average concentration of shower water from the test persons was 3.6×10^5 intact cells mL^{-1}. Because the intact cells in the blank samples were less than 2% of the used shower water, it was assumed that all microorganisms found in the shower water originated from the bathers.

Shower participants were not ill and were asked not to take a shower 12 h prior to the start of the experiment and not to use any cosmetics. The participants wore normal swimwear and were barefoot. To avoid introduction of dust and dirt from laboratory floors, participants wore slippers before entering the shower cabin. During showering, the participants rubbed themselves with their hands and rinsed their mouth with water. After 1 min, all shower water from one person was collected in a bucket and transferred into one large vessel of ~100 L. Both the bucket and vessel were disinfected similar to the shower cabin, by rinsing and usage of a 70% ethanol solution.

3.2.2 Anthropogenic community

All shower water from the 10 participants was collected within 2 h and mixed in one vessel. Subsequently, all bacteria were collected by filtration of the mixed shower water of all participants through a 0.2 μm pore size membrane filter cartridge (MediaKap-5, Spectrum laboratories). In 5 days, 59.2 L of the mixed shower water was filtrated at ~17 °C, and sufficient material was collected. The other ~40 L mixed shower water was discarded. After filtration, the plastic outer layer of the filter cartridge was removed, and the cells were dissolved in 1 L of a mineral salt medium. The mineral salt medium contained: KH_2PO_4 2.7 mg L^{-1}; K_2HPO_4 4.0 mg L^{-1}; Na_2HPO_4 3.2 mg L^{-1}; $CaCl_2$ 38 mg L^{-1}; $CoCl_2$ 0.03 mg L^{-1}; H_3BO_3 0.1 mg L^{-1}; $mgSO_4$ 24 mg L^{-1}; $CaSO_4$ 0.06 mg; $MnSO_4$ 2.7 mg L^{-1}; $ZnSO_4$ 0.06 mg L^{-1}; $FeSO_4$ 1.6 mg L^{-1}, with addition of 2.5 mg L^{-1} glucose, 3 mg L^{-1} peptone and 3.4 mg L^{-1} acetate as a carbon source at a pH of 6.8. The concentrated anthropogenic microbial community from bathers of 1.0×10^{10} cells mL^{-1} was used for chlorination and characterisation of the bacterial community on the same day of preparation of the cell suspension in the mineral salt medium and stored at room temperature (~20 °C).

3.2.3 Chlorination

Different samples were taken in duplicate in order to determine (i) the bacterial composition of the total and intact cell community before and after chlorination at one free available chlorine dose and (ii) the impact of different free available chlorine doses on the composition of the bacterial community. During all experiments, 15 ml of anthropogenic stock community from bathers was collected in sterile 50 mL tubes (Greiner Bio-One, sterile tubes 227261). An overview of the different experiments is given in Table 3.1.

Table 3.1. *Overview of the chlorination experiments performed with the anthropogenic community, whereafter the bacterial community was characterized.*

Initial free available chlorine concentration	Chlorine incubation time	Total/intact community characterized
0 mg Cl_2 L^{-1}	0 s	Intact
1 mg Cl_2 L^{-1}	30 s	Intact
0 mg Cl_2 L^{-1}	0 s	Total
1 mg Cl_2 L^{-1}	30 s	Total
1 mg Cl_2 L^{-1}	5 min	Total
1 mg Cl_2 L^{-1}	20 min	Total

Chlorine stock solutions were prepared by diluting a 12.5% sodium hypochlorite solution with demineralised water. These chlorine stock solutions were prepared to a concentration 10 times higher than the desired free available chlorine concentration of 0 or 1 mg Cl_2 L^{-1} during the experiments. After the addition of 1.5 mL of the chlorine stock solution to 13.5 mL of the anthropogenic community from bathers (pH = 6.8), the free available chlorine concentrations probably reduced during the contact time because of its reaction with microorganisms and oxidation of organics. Therefore, the free available chlorine concentrations presented in this paper represent the initial dosing. Free and total chlorine concentrations were analysed with

a Merck Millipore kit (Chlorine Test (free and total chlorine) Spectroquant®) with the use of a spectrophotometer (Photometer NOVA 60 A Spectroquant®).

To achieve a desired contact time, the reaction was stopped after 30 s, 5 min or 20 min contact time by adding 1.5 mL of 10 mM sodium thiosulphate (Fluka, Chemica 72049, sodium thiosulfate anhydrous, dissolved in demineralized water). The solution of sodium thiosulphate was also added to the non-chlorinated samples in order to treat all samples in the same way. All samples were manually shaken after addition of chlorine and after addition of sodium thiosulphate in order to create a homogeneous solution.

From the non-chlorinated and chlorinated (1 mg Cl_2 L^{-1} for 30 s) 15 mL batches, samples (2 mL) were taken for determination of total and intact cell counts using flow cytometry using live/dead staining (Prest et al. 2013). Thereafter, of all 15 mL batches, samples (2 mL) were taken for deoxyribonucleic acid (DNA) extraction to determine the composition of the total anthropogenic bacterial community. In order to characterise the intact cell community, and therefore the potentially living cells before and after chlorination, samples of 0.5 mL (~ 10^8 cells mL^{-1} ~ 500 ng DNA) of the 15 mL batches were taken and treated with DNase and proteinase K to remove DNA from injured and dead cells as well as the free genomic DNA as described by Villarreal et al. (2013). All samples for DNA extraction were stored at -20 °C.

3.2.4 DNA extraction and sequence analysis

DNA extraction was performed using the UltraClean Microbial DNA Isolation Kit of MO BIO laboratories, which is suitable for DNA isolation of both Gram-negative and Gram-positive bacteria (Guo and Zhang 2013).

Subsequently, the extracted DNA samples were characterised at the Regional Laboratory for Public Health, Haarlem, the Netherlands. For each sample, the 16S ribosomal ribonucleic acid (rRNA) was quantified by quantitative polymerase chain reaction (qPCR) according to Yang et al. (2002). Thereafter, 16S rRNA deep sequencing was done as described by Biesbroek et al. (2012) using the V5-V7 primers. NGS-data were automatically processed using the 'Full Processing Amplicon' pipeline available through the Run Wizard on the GS Junior Attendant PC (Roche). FASTA-formatted sequences were extracted from the .sff data file and processed using modules implemented in the Mothur v. 1.33.0 software platform (Schloss et al. 2009). Primer sequences were trimmed, and sequences with a length smaller than 200 bp were removed from the analysis. Potentially chimeric sequences were detected and removed with the Uchime command (Edgar et al. 2011).

The remaining aligned sequences were classified using a naïve Bayesian classifier with the SILVA SEED database release 119 as template and clustered into operational taxonomic units (OTUs) defined by 97% similarity. To reduce the effects of uneven sampling, all samples were rarefied to 1000 sequences per sample. For all samples, rarefaction curves were plotted (Figure 3.1) and the inverse Simpson's diversity index and Good's coverage were calculated (Table 3.3). The inverse Simpsons's diversity index was between 2.4 and 4.1 and the Good's coverage was for all samples ≥ 97.5%.

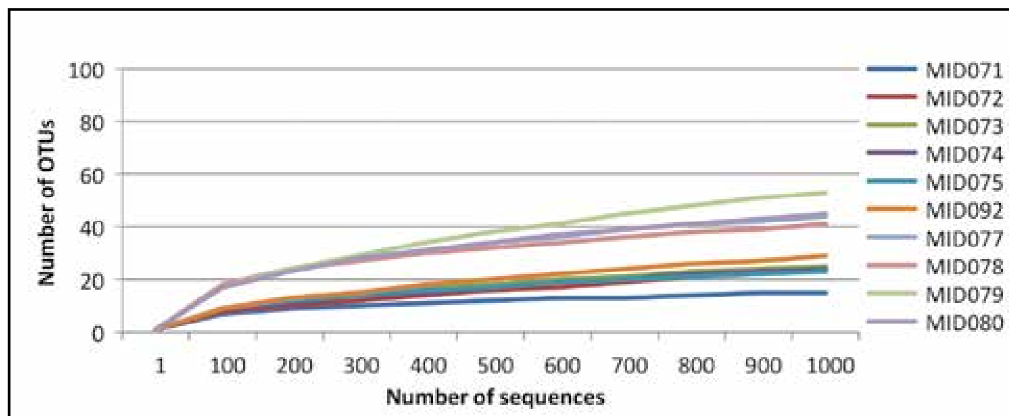


Figure 3.1 *Rarefraction analysis of the samples determining the richness in the different samples. Sample MID# from left to right are from highest to lowest number of OTUs after 1000 sequences and explained in Table 3.2.*

All OTUs were sequenced with SILVA SEED database release 119. Sequencing results were grouped on family level and whether the familial cells were Gram-positive or Gram-negative followed from literature.

Table 3.2 *Sample identification.*

Sample	Chlorine concentration	Chlorine incubation time	Total/intact community characterized
MID071	1 mg Cl_2 L^{-1}	30 s	Total
MID074	0 mg Cl_2 L^{-1}	0 s	Total
MID075	1 mg Cl_2 L^{-1}	5 min	Total
MID092	1 mg Cl_2 L^{-1}	20 min	Total
MID077	0 mg Cl_2 L^{-1}	0 s	Intact
MID078	1 mg Cl_2 L^{-1}	30 s	Total
MID079	1 mg Cl_2 L^{-1}	30 s	Intact
MID080	0 mg Cl_2 L^{-1}	0 s	Total

Table 3.3 *Sequencing results; the number of sequences includes reads longer than 200 that were aligned using the SILVA alignment release 119 as reference whereafter they were normalized at 1000. Number of OTUs. The inverse Simpson index indicated the probability that 2 randomly selected sequences will belong to different OTUs. The Good's coverage indicates the percentage of OTUs that are found more than once. Sample MID# explained in Table 3.1.*

MID	# sequences	# OTUs	Inverse Simpson index	Good's coverage
MID071	3360	15	2.7	99.5%
MID074	1769	24	3.4	98.8%
MID075	2751	23	3.5	99.1%
MID092	3277	29	2.4	98.3%
MID077	1946	44	4.1	98.4%
MID078	3047	41	3.9	98.4%
MID079	1789	53	3.0	97.5%
MID080	3058	45	4.0	98.1%

3.2.5 Cell counts

Flow cytometry was used in combination with live/dead staining to measure the concentration of intact cells and total cells, according to Prest et al. (2013). The samples were measured in duplicate and preheated for 5 min at 35 °C then stained with either SYBR® Green I (100x) and 10 mM EDTA to determine total cell counts or SYBR® Green I (100x) and Propidium Iodide (0.5 mg mL^{-1}) and 10 mM EDTA to determine intact cell counts. After 10 min of staining time at 35 °C, the samples were analysed using a BD Accuri C6® flow cytometer (BD Accuri cytometers, Belgium). During analysis, medium flow rate was used with a volume limit of 500 µL. Electronic gating was performed during the determination of total cells, after which the results were obtained as events per µL. Inside the gate, the events represent the number of intact cells per µL. If necessary, samples were diluted with Evian water (Evian, France) filtered over a 0.22 µm pore size filter (Millex-GP, Millipore) to prepare intact cell concentrations < 250 cells μL^{-1}.

3.3 Results

3.3.1 Composition of the total and intact cell community of non-chlorinated shower water

The composition of the bacterial community present in the mixed shower water of the 10 bathers is reported in Table 3.4. Characterisation of the anthropogenic bacterial community was conducted at family level. The abundance of a family was calculated by dividing the number of found sequences of that family by the total number of sequences per sample. Seven families had an abundance of more than 5% of the community: *Flavobacteriaceae* (24%), *Xanthomonadaceae* (23%), *Moraxellaceae* (12%), *Pseudomonadaceae* (10%), *Enterobacteriaceae* (9%), *Comamonadaceae* (6%) and *Burkholderiales_incertae_sedis* (5%). After removal of the extracellular DNA, the intact cell composition of the bacterial anthropogenic community was characterised. Within the intact cell community, five families had an abundance of more than 5%: *Moraxellaceae* (48%), *Xanthomonadaceae* (21%), *Burkholderiales_incertae_sedis* (8%), *Caulobacteraceae* (7%) and *Sphingomonadaceae* (6%).

3.3.2 Composition of the anthropogenic community after chlorination

The total and intact cell community was determined after chlorination (30 s with 1 mg Cl_2 L^{-1}) of the anthropogenic cell community (Table 3.4). Four families had an abundance of more than 5% of the chlorinated total community: *Flavobacteriaceae* (21%), *Xanthomonadaceae* (24%), *Moraxellaceae* (11%) and *Pseudomonadaceae* (22%). Within the chlorinated intact cell composition, the four families with an abundance of more than 5% of the community were: *Moraxellaceae* (57%), *Xanthomonadaceae* (17%), *Sphingomonadaceae* (7%), and *Burkholderiales_incertae_sedis* (6%).

Overall, similar shifts from the total to intact cell community were obtained for the non-chlorinated and chlorinated anthropogenic communities (Table 3.4). Taking into account the families with a relative abundance higher than 5%, *Comamonadaceae* was present (6%) only in the total non-chlorinated community and not detected above the background level in the intact cell community (≤1%) nor in the chlorinated ones (≤1%). Also in both non-chlorinated and chlorinated intact cell communities, *Sphingomonadaceae* was present in a higher relative abundance (6-7%) than in the total community (2%).

Comparing the total communities of the non-chlorinated and chlorinated samples showed that the dominant bacteria were similar (Table 3.4). The only exception is the relative abundance of *Pseudomonadaceae*, which is 12% higher in the chlorinated total community than in the non-chlorinated total community. The dominant families of the intact cell communities of the non-chlorinated and chlorinated samples were also similar (Table 3.4). However, the relative abundance of *Moraxellaceae* was 9% higher in the chlorinated intact community than in the non-chlorinated intact community. The relative abundance of the other families were within 5% difference.

Table 3.4 *Pyrosequencing results; the bacteria present in the anthropogenic community from bathers, before and after chlorination (30 s with an initial free available chlorine concentration of 1 mg* Cl_2 L^{-1}*), and before and after removal of the extracellular DNA resulting in the intact cell community. Numbers represent the relative abundance of the community in that sample.*

Bacterial family name	**Total community**	**Intact cell community**	**Total chlorinated community**	**Chlorinated intact cell community**
Flavobacteriaceae	24%	1%	21%	< 1%
Xanthomonadaceae	23%	21%	24%	17%
Moraxellaceae	12%	48%	11%	57%
Pseudomonadaceae	10%	< 1%	22%	< 1%
Enterobacteriaceae	9%	< 1%	5%	nd
Comamonadaceae	6%	< 1%	1%	< 1%
Burkholderiales_incertae_sedis	5%	8%	4%	6%
Burkholderiaceae	3%	2%	3%	1%
Caulobacteraceae	2%	7%	3%	4%
Sphingomonadaceae	2%	6%	2%	7%
Oxalobacteraceae	1%	3%	2%	2%
Rhodocyclaceae	1%	1%	< 1%	< 1%
Cytophagaceae	< 1%	1%	1%	3%
Methylophilaceae	< 1%	1%	< 1%	1%
Alcaligenaceae	< 1%	nd	< 1%	nd
Propionibacteriaceae	< 1%	nd	nd	< 1%
Methylobacteriaceae	< 1%	< 1%	nd	nd
Chitinophagaceae	nd	nd	< 1%	nd
Erythrobacteraceae	nd	nd	< 1%	nd
Rhodospirillaceae	nd	nd	nd	< 1%
Sphingobacteriaceae	nd	nd	< 1%	< 1%
Staphylococcaceae	nd	nd	nd	< 1%
Unclassified	< 1%	1%	< 1%	1%

< 1 = is considered to be background sequences and not exclusively referring to the sample
nd = not detected

3.3.3 Cell counts combined with deep sequencing results

Evaluation of the anthropogenic community with flow cytometry live/dead staining revealed a total cell concentration of 4.2×10^7 total cells mL^{-1} and an intact cell concentration of 9.6×10^6 intact cells mL^{-1} for the non-chlorinated sample. This means that only 23% of the cells in filtered shower water were intact, where on average 32% of the cells in the original shower water were intact (Table 3.5). This decrease may be explained by concentrating the water by filtration, whereas the shower water used for comparison was not concentrated.

Table 3.5 *Total and intact cells measurements of shower water.*

Total cells (#/mL)	Intact cells (#/mL)	Percentage intact cells
8.8×10^5	2.6×10^5	30%
1.2×10^6	3.1×10^5	27%
3.8×10^5	1.0×10^5	27%
6.6×10^5	1.1×10^5	17%
3.3×10^5	1.2×10^5	36%
4.5×10^5	1.6×10^5	36%
8.4×10^5	2.0×10^5	24%
8.5×10^5	3.1×10^5	37%
1.1×10^6	3.8×10^5	36%
1.9×10^6	9.0×10^5	47%
2.2×10^6	6.3×10^5	28%
3.2×10^6	1.2×10^6	39%
Average = 1.2×10^6	Average = 3.9×10^5	Average = 32%

In the chlorinated samples of the anthropogenic community, the cell concentrations were 4.63×10^7 total cells mL^{-1} and 4.75×10^6 intact cells mL^{-1}, respectively. Therefore, chlorination with 1 mg Cl_2 L^{-1} for 30 s decreased the percentage of intact cells of the anthropogenic community from 23% to 10%.

Combining cell counts with the relative deep sequencing results is a method for quantification (Prest et al. 2014). The higher the ratio between the intact cell concentration after and before chlorination, the more chlorine resistant the bacterial family is under these conditions. Of the identified families (Table 3.4), the most chlorine resistant were *Moraxellaceae* and *Sphingomonadaceae,* with a chlorine resistance of 59-60%.

3.3.4 Impact of varying initial free available chlorine doses

The effect of changing the contact time (0 s, 30 s, 5 min and 20 min) on the presence of bacterial families in the AMCB was investigated, while the initial free available chlorine concentration was kept at 1 mg Cl_2 L^{-1}, complying with the guideline for Dutch swimming pools (Anonymous 2011). After characterizing the total community, an impact of chlorine contact time on the community presence was observed (Figure 3.2). The relative abundance of both *Moraxellaceae* and *Xanthomonadaceae* decreased from 23% and 12% respectively to < 1% with increasing chlorine contact time. The relative abundance of *Enterobacteriaceae* (9-5%) and *Flavobacteriaceae* (16-29%) remained constant within a change of ±5%, except for the relative abundance of *Flavobacteriaceae*, which decreased from 29% after 5 min to 16% after 20 min. In time, the relative abundance of *Comamonadaceae* increased from 6% to 13%, but the highest increase observed was of *Pseudomonadaceae* from 10% to 62%. The large increase of the relative abundance of *Pseudomonadaceae* with increasing chlorine contact time suggests that *Pseudomonadaceae* is more chlorine resistant than the other identified bacteria.

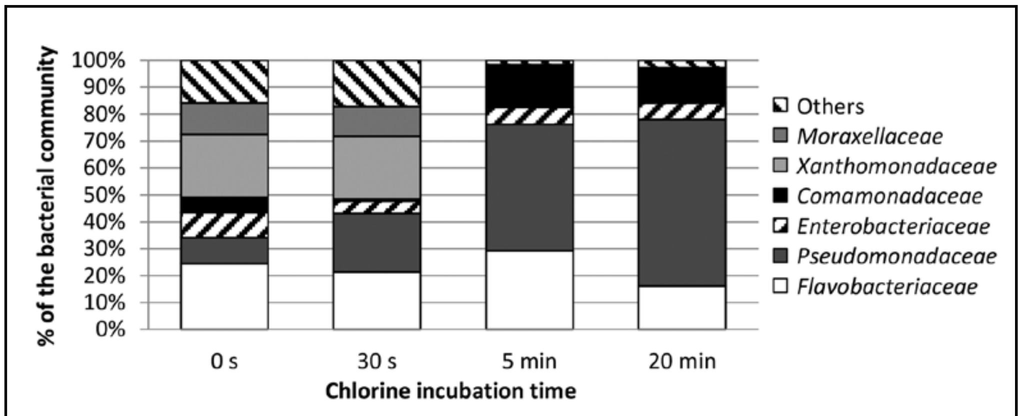


Figure 3.2 *The difference in anthropogenic bacterial communities from shower water after disinfection with an initial free available chlorine concentration of 1 mg* Cl_2 L^{-1} *at different contact times.*

3.4 Discussion

3.4.1 The bacterial anthropogenic community

An anthropogenic community was created from the wash-off water of 10 humans and concentrated over five days into 1 L with 1.0×10^{10} cells mL^{-1}. Because of the long filtration time and the time in the mineral medium, the relative abundance of the bacterial families could have changed between showering and sampling. Although samples for DNA extraction were taken in duplicate, suggestions for other research determining the wash-off community of humans might incorporate (i) shorter concentration time and (ii) duplication of showering to create different batches of shower water. However, based on these characterization results, the observed anthropogenic community consisted mainly of the bacterial families *Flavobacteriaceae*, *Xanthomonadaceae*, *Moraxellaceae*, *Pseudomonadaceae*, *Comamonadaceae* and *Enterobacteriaceae*.

Considering all 274 different found OTUs, the anthropogenic community contained only 23 different bacterial families, while 19% of the OTUs were unclassified families. Furthermore, 32 different genera were found, while 51% of the OTUs were unclassified genera. Of these 32 different genera, 30 genera were found to be Gram-negative bacteria. The only Gram-positive bacteria found were *Staphylococcaceae* and *Propionibacteriaceae*, with an abundance of < 1% each. Both are known to be present on human skin and commonly found on sebaceous areas like the side of the nostril, the back and the upper chest (Grice and Segre 2011). Also most of the other skin related bacteria, found after swabbing the skin, are known to be Gram-positive (Human Microbiome Project 2012). Gram-negative bacteria are not often found on human skin (Marples 1965) and assumed to be contaminants from the gastrointestinal tract (Chiller et al. 2001, Roth and James 1988). Because mostly Gram-negative bacteria were found in this anthropogenic community, this suggests that Gram-negative bacteria are more easily rinsed from the body than Gram-positive bacteria observed after swabbing. This was also observed by Lowbury (1969), who wrote that Gram-negative bacilli tend to appear in small numbers on human skin and mainly as "transient" organisms which are superficial and easily washed off. This suggests that in swimming pools, Gram-negative bacteria could be dominant over Gram-positive bacteria.

Whether the supposedly more chlorine resistant Gram-positive bacteria (Le Chevallier et al. 1980) are released in a later stage of swimming, is unknown.

3.4.2 The chlorine resistant community

Chlorination oxidizes cell membranes (Venkobachar et al. 1977), so a distinction between cellular membrane integrity indicates how many cells are oxidized and therefore less chlorine resistant (Joux and Lebaron 2000b, Ramseier et al. 2011). Based on quantitative cell counts, the intact cell concentration was reduced by 51% after chlorination with 1 mg Cl_2 L^{-1} for 30 s, indicating a 0.31-log removal. The Dutch disinfection regulation in swimming pools is based on a 4-log removal of *P. aeruginosa* at 1 mg Cl_2 L^{-1} in 30 s (Anonymous 2011) determined by plate counts from the research of Fitzgerald and Der Vartanian (1969). The difference between this guideline and the obtained log-reduction of the anthropogenic community might be partly explained by (i) the differences between intact cell counts and viability because a cell could be intact but not culturable and (ii) the cell distribution. Fitzgerald and Der Vartanian (1969) used a *P. aeruginosa* laboratory culture containing free planktonic cells whereas cells in the anthropogenic community could be aggregated and/or contain some debris acting as a protective layer. When this protective layer consists of organic material, the free available chlorine could oxidize the organic material and therefore reduce the effective dose.

In addition, the effect of chlorination on the relative abundance of the bacterial families present in the AMCB could be related to the interaction between chlorine and DNA. All DNA in the sample after chlorination was sequenced to determine the effect on the relative abundance of the bacterial families by characterizing the total community composition. Chlorine reacts randomly with biological molecules like enzymes and DNA (Campbell and Lyman 1961, Whiteman et al. 1997). DNA denaturation has been described in literature to occur from a chlorine dose of 1,500 mg Cl_2 L^{-1} (Prütz 1996, Suquet et al. 2010, Van Aken and Lin 2011). Although the chlorine doses utilized in this research were lower, a difference in community composition was observed, indicating that DNA was denatured and therefore impossible to amplify and sequence. It is likely that the damaged DNA was extracellular DNA because extracellular DNA is directly available while intracellular DNA is protected by the cell membrane. Characterisation of the total community in a chlorinated sample could therefore indicate which bacteria might be more chlorine resistant. However, removal of the extracellular DNA probably implies this observation directly, as in this case only the intracellular protected DNA was sequenced. Because of the increase of relative abundance of *Moraxellaceae* in the intact cell community and *Pseudomonadaceae* in the total cell community, both are considered to be more chlorine resistant than the other bacteria present in those samples. This is in contradiction with the findings of Wolfe et al. (1985), who showed that *Flavobacteriaceae* were more chlorine resistant in drinking water reservoirs.

3.4.3 Bacterial origin and (opportunistic) pathogens

Most of the bacterial families found in the shower water are related to aquatic environments or have been detected as part of the human skin community. To determine the human health risks of swimming pools, assuming this wash-off community is released during swimming, the species present should be identified as not all species in a bacterial family are human pathogens. Within the family *Xanthomonadaceae*, the genus *Stenotrophomonas* was found.

The species *S. maltophilia* is an opportunistic pathogen that is most commonly associated with respiratory infections in humans (Brooke 2012). Of the 274 *Xanthomonadaceae* OTUs found, 20 different OTUs were detected. 12 of these 20 OTUs were defined as *Stenotrophomonas*, of which 11 OTUs belonged to the species *S. malthophilia*. *Stenotrophomonas* is found in water, soil, petroleum (Chang and Zylstra 2010), moist places such as shower heads (Crossman et al. 2008) and sporadically in Dutch drinking water (Van der Wielen and Van der Kooij 2011). However, *Stenotrophomonas* could also originate from the participants, because Dekio et al. (2005) detected *Stenotrophomonas* on the foreheads of people.

Other bacteria which have been found in shower areas, e.g. in a biofilm on shower curtains, could be related to wash-off bacteria as well. Those families include *Cytophaga*, *Flavobacteria*, *Bacteroides*, *Sphingomonas* spp. and *Methylobacterium* spp. (Kelley et al. 2004), of which the family *Sphingomonadaceae* was detected as the most abundant family among the biofilm organisms. Within the identified *Sphingomonaceae* family, the genus *Sphingomonas* was the most common with 13 OTUs out of the 24 OTUs detected in the various samples. This genus includes the opportunistic pathogen *Sphingomonas paucimobilis*, found with 4 OTUs in the shower water, which is known to infect immune-compromised patients. *S. paucimobilis* has also been found in fluids in humidifiers and in tap water in hospitals (Hsueh et al. 1998).

The genus *Cloacibacterium*, of the large family *Flavobacteriaceae*, was identified in 9 of the 31 *Flavobacteriaceae* OTUs. This microorganism lives in aquatic environments, e.g. in municipal sewage (Allen et al. 2006). However, *Cloacibacterium* was not detected in human stool samples (Allen et al. 2006, Koskey 2013) and could therefore originate from shower water, as found in our experiments and confirmed by Oh et al. (2013). *Flavobacterium*, found in 10 of the 31 OTUs, has been detected in swimming pools (Favero and Drake 1966). Within this genus, three species are known to be fish pathogens (Touchon et al. 2011). *Flavobacteriaceae* may create a symbiotic relationship with the pathogen *Legionella pneumophila* because of the extracellular products secreted by the *Flavobacterium breve* strain (Wadowsky and Yee 1983).

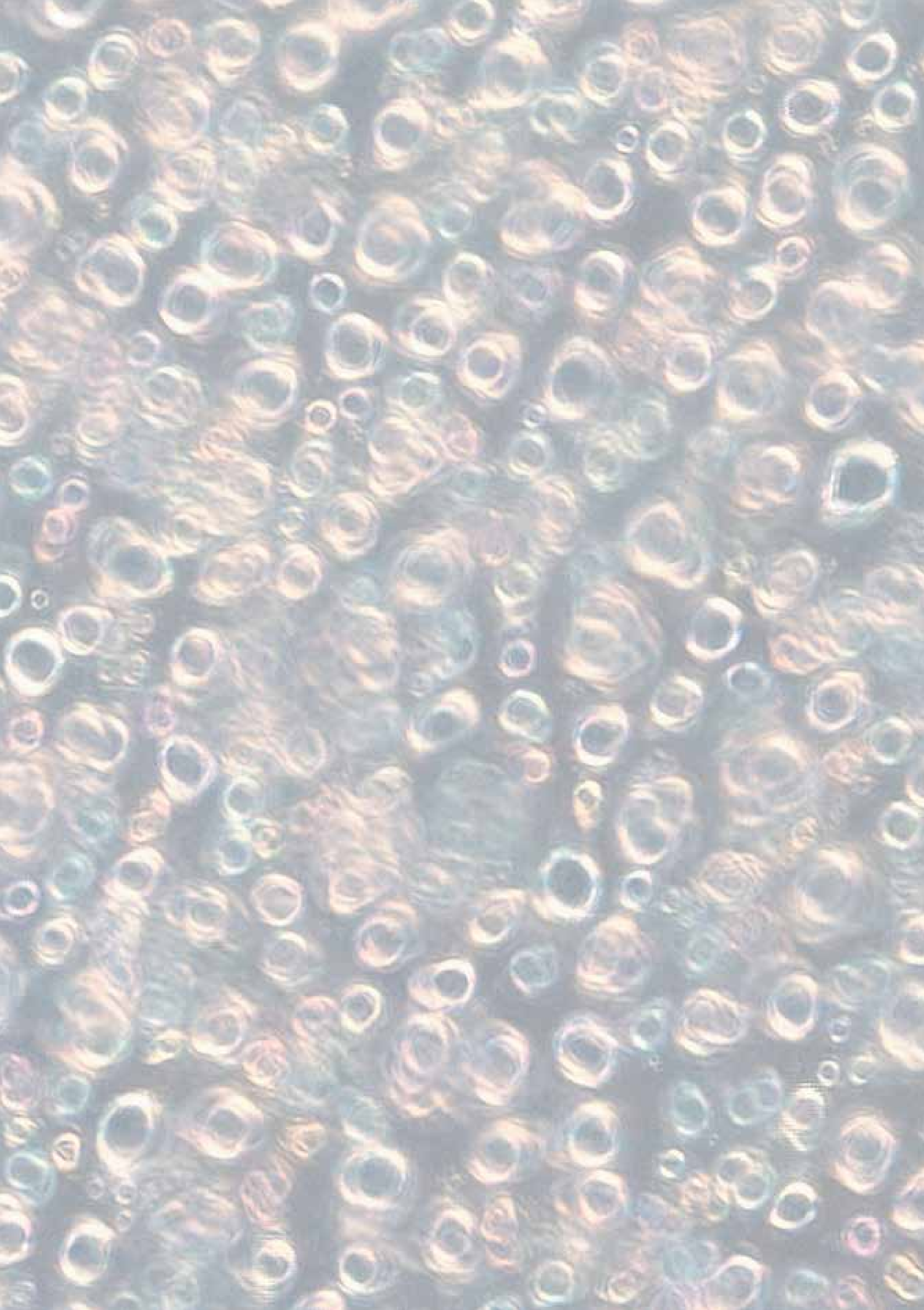
Like *Pseudomonadaceae, Moraxellaeae* is a family that belongs to the order *Pseudomonales*. The genus *Acinetobacter*, belonging to the detected family *Moraxellaeae,* was found in 11 of the 18 OTUs of *Moraxellaeae*. This genus is often found in water, soil, living organisms and on human skin (Berlau et al. 1999, Fournier and Richet 2006, Seifert et al. 1997). Regarding the family *Moraxellaceae* and its detected genus, there are more than 30 species of *Acinetobacter* known (Seifert and Dijkshoorn 2008). Only *A. baumannii* strains have an implication for infection control. 10 of the 11 OTUs of the *Acinetobacter* genera belonged to *A. baumannii*, supporting literature that shows this genus is rarely found on healthy human skin (Berlau et al. 1999, Seifert et al. 1997). Furthermore, the persistence of *A. baumannii* in a hospital environment might be due to its resistance to major antimicrobial drugs, desiccation and disinfectants (Seifert and Dijkshoorn 2008).

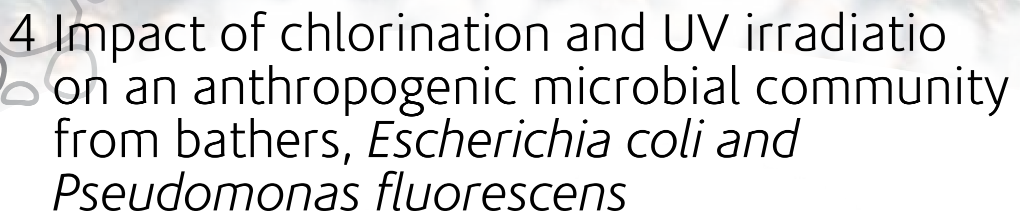
The genus *Pseudomonas,* of the family *Pseudomonadaceae,* was found in all 11 OTUs detected as *Pseudomonadaceae*. *Pseudomonas* is present in insects, humans, soil, plants and water (Nikel et al. 2014). *P. aeruginosa* was detected in 1 of the 11 OTUs and is one of the best-known opportunistic pathogens related to swimming pools as it causes infections and could be resistant to antibiotics (Papadopoulou et al. 2008). *P. aeruginosa* is commonly found in the human mouth, nose and throat (Eriksson et al. 2002). However, the environment surrounding the swimming pool might also be a source of contamination (Jacobson 1985) as temperature and humidity forms an ideal environment for the growth of *Pseudomonas* (Price and Ahearn 1988).

Also, faecally derived *Enterobacteriaceae* were detected in 25 OTUs in which mainly *Escherichia_Shigella* was found. Both *E. coli* and *Shigella* are pathogens (Kaper et al. 2004) whose strains are closely related because of the many genetic and phenotypic similarities (Pupo et al. 2000). Both *E. coli* (O157) and *Shigella* spp. are considered to be responsible for faecally derived microbial hazards in swimming pool water (WHO 2006). However, 6 of the 25 detected *E. coli* species were of strain K-12, which is non- pathogenic (Blum et al. 1994, McDaniel et al. 1995, Muhldorfer and Hacker 1994). Non-faecal bacterial hazards in (pool) water selected by the World Health Organization are caused by *Mycobacterium* spp. and *Staphylococcus aureus* (WHO 2006) The family *Mycobacteriacea* was not found in this anthropogenic community and only 1 OTU was detected of the family *Staphyloccaceae*. This OTU was characterized as *S. aureus*, which is detected on the anterior nasal mucosa and skin as well as in the faeces of a substantial portion of healthy individuals (WHO 2006). Furthermore, Robinton and Mood (1966) found that *S. aureus* was released by bathers under all swimming conditions as also in this shower water.

3.5 Conclusions

This research showed that the bacteria present in human wash-off shower water mainly consists of Gram-negative bacteria. The bacterial families present with a relative abundance of ≥ 10% were *Flavobacteriaceae*, *Xanthomonadaceae*, *Moraxellaceae* and *Pseudomonadaceae*. The most chlorine resistant families were *Moraxellaceae* of the intact cell community and *Pseudomonadaceae* of the total cell community. Within both families, (opportunistic) pathogens were found, which are therefore likely to be released in swimming pools as well. Because these families were more chlorine resistant than others, there could be a potential health risk.





4 Impact of chlorination and UV irradiatio on an anthropogenic microbial community from bathers, *Escherichia coli and Pseudomonas fluorescens*

Disinfection response of anthropogenic community and indicators

Abstract

When bathers enter a swimming pool, they will release microorganisms: an anthropogenic microbial community from bathers (AMCB). To ensure microbial safety, swimming pool water is disinfected, whereafter the remaining presence of the AMCB is monitored by the presence of indicators. To determine whether the indicators represent similar disinfection efficiencies, this research compared the response of the AMCB to the response of two indicator microorganisms used for water quality in swimming pools: *Escherichia coli* and *Pseudomonas fluorescens*.
Samples of an AMCB and indicator organisms were treated with a single shock dose of hypochlorite (0-15 mg Cl_2 min L^{-1}) or UV light (0-70 J m^{-2}). Their response to disinfection was determined by heterotrophic plate count (HPC) and flow cytometry cell count based on live/dead staining (FCM) over time.
After chlorination, AMCB were still culturable on applied media, while indicator organisms were below the detection limit. Based on intact cell counts measured by FCM, the log reductions of the AMCB were similar to *P. fluorescens* after chlorination, while *E. coli* showed higher log reductions. This suggests that *P. fluorescens* would be a suitable indicator organism for an AMCB in chlorinated systems.
After UV irradiation, the intact cell concentration remained constant, probably because of DNA damage instead of membrane damage. HPC results revealed similar log reductions for the AMCB and *E. coli*. Both showed a log reduction of 0.5 and higher from doses of 47 J m^{-2} and higher, while *P. fluorescens* showed higher log reductions from lower UV doses. This suggests that *E. coli* would be a suitable indicator organism for an AMCB in UV irradiated systems.

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4.1 Introduction

Different types of water, such as drinking water and swimming pool water, are disinfected to ensure microbial safety. The most frequently used disinfectants are chlorine based products, because of their effectiveness and low costs (Shannon et al. 2008) as well as their mandatory use in many countries. In the Netherlands, drinking water is produced without chlorination (Anonymous 2009), but the use of chlorine is still mandatory in swimming pools (Anonymous 2011).

The free available chlorine concentration in Dutch swimming pools must be between 0.5 and 1.5 mg $Cl_2\,L^{-1}$, which follows a regulation based on a 4-log removal of *Pseudomonas aeruginosa* within 30 seconds (Anonymous 2011). In order to ensure microbial safety, the swimming pool water is regularly analysed for the presence of different indicator organisms. Besides *P. aeruginosa*, *Escherichia coli* is often used as a faecal indicator (WHO 2006). Although these indicator organisms have been used for many years, a study comparing their response to disinfection to that of an anthropogenic microbial community released by bathers (AMCB), to the best of our knowledge, has not been reported.

The number of microorganisms released by bathers has been investigated before (chapter 2, Keuten et al. 2012). Furthermore, an AMCB derived from shower water has been characterised (chapter 3). The study assumes the same bacteria are released during 1 min showering as during a jump into a swimming pool, therefore representing the initial microbial pollution of a swimming pool (Keuten et al. 2012). Characterisation of the AMCB revealed that both families of the indicator organisms, *P. aeruginosa* and *E. coli,* were present in this community (chapter 3). After chlorinating the AMCB, *Pseudomonadaceae* became the dominant bacterial family of the total population, suggesting that *Pseudomonadaceae* was more chlorine resistant than the other identified bacteria present (chapter 3).

During disinfection by chlorination, chlorine reacts with the cell membrane (Venkobachar et al. 1977). Thereafter, different intercellular molecules are oxidised, resulting in complete disruption of bacterial ATP production as a consequence of inhibition of the inner membrane systems (Barrette et al. 1989). Although chlorination is widely applied as disinfection method, one considerable disadvantage is the chlorine resistance of some microorganisms and waterborne pathogens such as *Cryptosporidium* and *Giardia* (Hijnen et al. 2006).

UV treatment, on the other hand, is a widely used alternative disinfection method. Disinfection with UV light at 254 nm is based on the formation of pyrimidine dimers, which distort the deoxyribonucleic acid (DNA) helix and blocks cell replication (Lado and Yousef 2002). Furthermore, cross-linking of aromatic amino acids occur at their carbon–carbon double bonds, resulting in denaturation of proteins, which contributes to membrane depolarization and abnormal ionic flow (Lado and Yousef 2002). In addition to DNA damage, UV irradiation also independently damages other cell components, such as the cell membrane and cytoplasm (Schwarz 1998).

Disinfection efficiency is often measured by heterotrophic plate count (HPC) (Pernitsky et al. 1995). Disadvantages of any culture-based method is the long period of time before results are known. Aside from practical considerations, only a small fraction of the microbial population is culturable (Hoefel et al. 2005a). Besides, bacterial cells can reach a viable but non-culturable (VBNC) state under certain conditions, e.g. after chlorination

(Oliver 2005) and UV irradiation (Zhang et al. 2015). Therefore, even if no colony forming units (CFU) were detected with HPC, living microorganisms (VBNC) could still be present and pose a potential health risk upon human contact.

With new techniques such as flow cytometry (FCM), cell counts can be performed faster and without constraints from microorganisms in the VBNC state (Hoefel et al. 2005a). One way to determine viable microbial cells with FCM is based on cell membrane integrity by means of live/dead staining; when the cytoplasmic cell membrane is not intact, propidium iodide (PI) can enter the cell and stain its DNA (Novo et al. 2000). All intact cells are assumed to be viable, as suggested in previous studies (Phe et al. 2005, Wang et al. 2010).

The aim of this research was to determine whether indicator organisms have a similar response to chlorination or UV irradiation as an AMCB obtained from shower water collected from the wash-off of 10 different persons. The used indicator organisms were *E. coli* and *P. fluorescens*, assuming *P. fluorescens* has a similar response to disinfection as *P. aeruginosa*. The response in terms of inactivation and repair after disinfection was determined by culturable cell (HPC) measurements and intact cells (FCM live/dead staining) measurements at regular time intervals up to 29 h after disinfection.

4.2 Materials and methods

4.2.1 Shower procedure

The initial anthropogenic pollutant release is introduced into the pool water during the first minutes of body contact with the water and consists of the residue of evaporated sweat, microorganisms and pollutants as well as any cosmetics on the swimmer's skin (Keuten et al. 2012). Therefore, to obtain an initial anthropogenic community, standardised shower experiments were performed in a laboratory setting. Because specific factors like age, location, and sex contribute to the variability of the microbial flora of the skin (Grice and Segre 2011), an average anthropogenic community was obtained by collecting all shower water of 10 West-European adults (5 male and 5 female) between 20-40 years old. Each showered for 60 s in a standardised shower cabin in a laboratory (18), creating ~10 L of shower water per person.

Before using the standardised shower cabin, the shower hose, shower nozzle, rinsing hose and sampling scoop were thermally disinfected (5 min, 70 °C). Next, the shower cabin was rinsed twice then disinfected by spraying a 70% ethanol solution (Ethanol 96%, VWR chemicals, mixed with demineralized water to a 70% ethanol solution) on the surfaces and leaving it to disinfect for 2 min. Lastly, the cabin was rinsed in triplicate with tap water and drained until only a few drops of water came out.

The water used for showering was non-chlorinated tap water (19). Hot tap water was mixed with cold tap water using a thermostatic valve, ensuring a constant water temperature of 38 ± 0.5 °C. Blank samples from showering without a test person resulted in an average cell concentration of 5×10^3 intact cells mL^{-1} while the average concentration of shower water from the test persons was 3.6×10^5 intact cells mL^{-1}. Because the intact cells in the blank samples were less than 2% of the used shower water, it was assumed that all microorganisms found in the shower water originated from the bathers.

Shower participants were not ill and were asked not to take a shower 12 h prior to the start of the experiment and not to use any cosmetics. The participants wore normal swimwear and were barefoot. To avoid introduction of dust and dirt from laboratory floors, participants wore slippers before entering the shower cabin. During showering, the participants rubbed themselves with their hands and rinsed their mouth with water. After 1 min, all shower water from one person was collected in a bucket and transferred into one large vessel of ~100 L. Both the bucket and vessel were disinfected in a similar manner as the shower cabin, by rinsing and usage of a 70% ethanol solution.

4.2.2 Communities

All shower water from the 10 participants was collected within 2 h and mixed in one vessel. Subsequently, all bacteria were collected by filtration of the mixed shower water of all shower participants over a 0.2 µm pore size membrane filter cartridge (MediaKap-5, Spectrum laboratories). After 1.8 L shower water was filtrated, the microorganisms were collected by backwashing the filter with 1 L 0.01 M phosphate buffer saline (PBS) with a pH of 6.8. The purpose of backwashing with PBS was to remove any carbon and nitrogen resources from the shower water and to suspend the microorganisms in a medium with a known composition. First measurements were conducted within one day after showering the persons. The experiments were performed with a total cell concentration of 2.2×10^6 cells mL^{-1} and an intact cell concentration of 1.1×10^6 cells mL^{-1}, both measured with FCM, and 2.2×10^5 colony forming units (CFU) mL^{-1} measured with HPC.

A *P. fluorescens* strain P17 culture was prepared in a mineral salt medium containing the following salts per litre of demineralized water: KH_2PO_4 2.7 mg; K_2HPO_4 4.0 mg; Na_2HPO_4 3.2 mg; $CaCl_2$ 38 mg; $CoCl_2$ 0.03 mg; H_3BO_3 0.1 mg; $mgSO_4$ 24 mg; $CaSO_4$ 0.06 mg; $MnSO_4$ 2.7 mg; $ZnSO_4$ 0.06 mg; $FeSO_4$ 1.6 mg, with addition of 3.42 mg L^{-1} acetate as a sole carbon source with a pH of 6.8. The culture was incubated at 25 °C until a stationary cell concentration was reached. The experiments were performed with a total cell concentration of 5.7×10^5 cells mL^{-1} and an intact cell concentration of 4.3×10^5 cells mL^{-1}, both measured with FCM, and 3.6×10^5 CFU mL^{-1} measured with HPC.

The pure culture of *E. coli* strain WR1 was prepared in the same mineral salt medium as used for *P. fluorescens* except for the substitution of 2.5 mg L^{-1} glucose as a sole carbon source. The culture was incubated at 25 °C and reached a stationary phase after approximately two weeks. The experiments were performed with a total cell concentration of 2.2×10^6 cells mL^{-1} and an intact cell concentration of 1.1×10^6 cells mL^{-1}, both measured with FCM, and 2.6×10^5 CFU mL^{-1} measured with HPC.

4.2.3 Flow cytometry

FCM was used in combination with live/dead staining to measure the concentration of intact cells and total cells according to Prest et al. (2013). The samples were preheated for 5 min at 35 °C and stained with 5 µL SYBR® Green I (100x) (Sigma Aldrich art.nr. S9430) and 5 µL 10 mM EDTA (Merck, art.nr.51008418) to determine total cell counts, or with 5 µL SYBR® Green I (100x) and Propidium Iodide (0.5 mg mL^{-1}) (Sigma Aldrich art.nr. P4864) and 5 µL 10 mM EDTA to determine intact cell counts. After 10 min of staining at 35 °C, the samples

were measured using a BD Accuri C6® flow cytometer autosampler (BD Accuri cytometers, Belgium), applying medium flow mode with a volume limit of 500 µL. Electronic gating was performed during the determination of total cells, after which the results were obtained as events per µL. Inside the gate, the events represent the number of intact cells per µL. If necessary, samples were diluted with Evian water (Evian, France) filtered over a 0.1 µm pore size filter (Millex-GP, Millipore) to prepare intact cell concentrations < 250 cells μL^{-1}.

4.2.4 Heterotrophic plate count

Culturable cells were determined by HPC with the use of Lab Lemco Agar (LLA) plates (Oxoid CM0017, Oxoid LTD, Hampshire, United Kingdom). Two to three different dilutions of all samples were prepared and depending on the dilution needed, 0.05-0.1 ml of a dilution was plated in duplicate. Plates were incubated for 2-3 days at 25 ± 2 ˚C. Hammes et al. (2008) described that HPC has a practical standard error of > 30%. Following these statistics, between 30-300 CFU should be detected per plate to consider them statistically valid. However, due to inactivation, plates with few to no colonies are to be expected for non-diluted samples. When all plates of a sample, plated in different decimal dilutions, contained CFU outside the range of 30-300 CFU per plate, the colony count closest to 30-300 CFU was used.

4.2.5 Sample preparation

From the manually homogenized solution of AMCB, *E. coli* culture or *P. fluorescens* culture, a sample of 45 or 50 mL, for chlorination and UV irradiation experiments respectively, was added to an open PVC jar of Ø75 mm (Hellebrekers Technieken, Nunspeet, the Netherlands). Before use, the PVC jars were disinfected with 70% ethanol for 2 min and rinsed with demineralized water.

Next, disinfection was performed by the addition of 5 mL hypochlorite or UV irradiation to the community suspension in the PVC jar. After disinfection, the PVC jars were placed at room temperature (~20 ˚C) on a table exposed to daylight and artificial light, but shielded from direct sunlight. The response, in terms of inactivation or repair, after disinfection was determined in time by taking samples from these jars to measure the culturable and intact cell concentration. The time between disinfection and sampling is referred to as response time.

4.2.6 Chlorination

Chlorine stock solutions of 5, 10, 15 and 30 mg Cl_2 L^{-1} free available chlorine were prepared by diluting a 12.5% sodium hypochlorite solution with demineralised water. 5 mL of the chlorine stock solution was added to a PVC jar containing 45 mL of a manually stirred community suspension to achieve a final volume of 50 mL. The chlorine reaction was stopped after 0.5 or 6 min of contact time by adding 5 mL of a 10 mM sodium thiosulphate solution, while the jar was manually stirred.

The free available chlorine concentration was determined after each chlorine contact time of 0, 0.5 and 6 min and measured with a Merck Millipore kit (1.00597 Chlorine Test (free and total chlorine) Spectroquant ®) with the use of a spectrophotometer (Photometer NOVA

60 A Spectroquant®). The average free available chlorine dose was calculated by multiplying the average free available chlorine concentration by the contact time, which was linearly interpolated between 0-0.5 and 0.5-6 min (Table 4.1).

Table 4.1 *The applied free available chlorine dose present for disinfection inside the experimental jars.*

Initial free available chlorine dose ($mg\ Cl_2\ min\ L^{-1}$)	**Free available chlorine dose AMCB* ($mg\ Cl_2\ min\ L^{-1}$)**	**Free available chlorine dose *E. coli* ($mg\ Cl_2\ min\ L^{-1}$)**	**Free available chlorine dose *P. fluorescens* ($mg\ Cl_2\ min\ L^{-1}$)**
0.0	0.0	0.0	0.0
0.3	0.1	0.2	0.2
0.5	0.2	0.5	0.3
0.8	0.4	0.7	0.4
1.5	1.1	1.3	0.9
3.0	0.5	1.6	1.4
6.0	2.6	4.9	2.2
9.0	2.1	6.5	4.4
18.0	9.1	14.2	6.9

* AMCB = anthropogenic microbial community of bathers

4.2.7 UV dose

The UV irradiation experiments were performed with 50 mL of the community suspension in a PVC jar. For UV irradiation of the communities, a bench-scale collimated beam apparatus (Fabr. Van Remmen UV Techniek, Wijhe, the Netherlands) was used to spread a homogeneous light on the surface of the jars (Fabr. Hellebrekers Technieken, Nunspeet, the Netherlands). A 12 W low-pressure lamp was placed in the apparatus.
The average irradiance ($mW\ cm^{-2}$) was measured with a jar-sensor (SiCONORM-I-LP). To calculate the applied UV dose, the irradiation time was multiplied with the fluence rate (Fr), EQ 4-1, 2 and 3.

$$Fr = E0 \times Pf \times (1 - R) \times Df \times Wf \qquad \text{(EQ 4-1)}$$

Where
Fr = Fluence rate ($mW\ cm^{-2}$)
$E0$ = Irradiance on the centre of the medium surface ($E0$ = 0.128 $mW\ cm^{-2}$)
Pf = Petri factor, average relative irradiance on the jar (Pf = 0.997)
R = Reflectance at air water interface at 254 nm (R = 0.02500)
Df = Divergence factor
Wf = Water factor, average relative fluence in medium

$$Df = \frac{L}{L+d} \qquad \text{(EQ 4-2)}$$

Where
L = Distance from the lamp to the suspension surface (L = 16.4 cm)
d = depth of the suspension (d = 2.0 cm)

$$Wf = \frac{1-10^{-a\times d}}{a\times d\times 2.3}$$ (EQ 4-3)

Where
a = UV absorption coefficient (a = 0.05 cm^{-1})

The UV lamp was preheated and turned on for 15 min, then shut down for 15 min, whereafter the lamp was turned on again for 15 min before use in the experiments to ensure a constant and reproducible lamp output. The collimated beam was equipped with an automated shutter to ensure controlled irradiation times. The applied UV doses on the communities were: 0, 6, 9, 12, 17, 23, 47, 70 J m^{-2} with a standard error of the mean of ≤1%.

4.3 Results

In this study, the response of an AMCB was compared to the response of indicator organisms *E. coli* and *P. fluorescens* after exposure to chlorination and UV irradiation. The efficiency of inactivation was evaluated by HPC (CFU mL^{-1}) and FCM live/dead staining (intact cells mL^{-1}) over time.

4.3.1 Response to chlorination

4.3.1.1 Anthropogenic microbial community of bathers

Determination of the response of the AMCB to chlorination by HPC showed inactivation of the microorganisms when hypochlorite was added. Non-disinfected samples contained on average $2.2\times10^6 \pm 1.1\times10^6$ CFU mL^{-1} over time, while the chlorinated samples contained on average $2.1\times10^5 \pm 5.6\times10^5$ CFU mL^{-1} over time. Higher free available chlorine doses caused higher inactivation, resulting in lower CFU mL^{-1}. Log reductions were calculated using the cell concentration in the non-disinfected sample of the same time series (N0) as a reference, correcting the data for possible growth/decay. The maximum log reduction possible was 4.0 ± 0.2-log on average over time. The maximum log reduction was reached from chlorine doses of ≥ 1.1 mg Cl_2 min L^{-1} within the first hours of response time (Figure 4.1). After 29 h of response time, a higher concentration of CFU mL^{-1} was found, resulting in a lower log reduction per free available chlorine dose compared to the log reductions of the other response times. The concentration increase of CFU mL^{-1} between the first measurement at 1.1 h and 29 h response time was 0.5-log for the non-disinfected sample and on average 2.3 ± 0.7-log for the chlorinated samples.

Measurements of the intact cell concentration with FCM (Figure 4.2A) showed lower log reductions than their corresponding samples measured with HPC. While the maximum log reduction determined with FCM was 6.6 ± 0.2-log on average over time, only a 2.3-log reduction was observed at the highest chlorine dose of 9.1 mg Cl_2 min L^{-1} after 27 h response time. FCM also indicated a reduction of intact cell concentration with increasing free available chlorine concentrations and a cell concentration increase after one day of response time. The concentration increase of intact cells mL^{-1} between the first measurement at 0.6 h and 27 h response time was 0.3-log for the non-disinfected sample and on average 0.0 ± 0.4-log for the chlorinated samples.

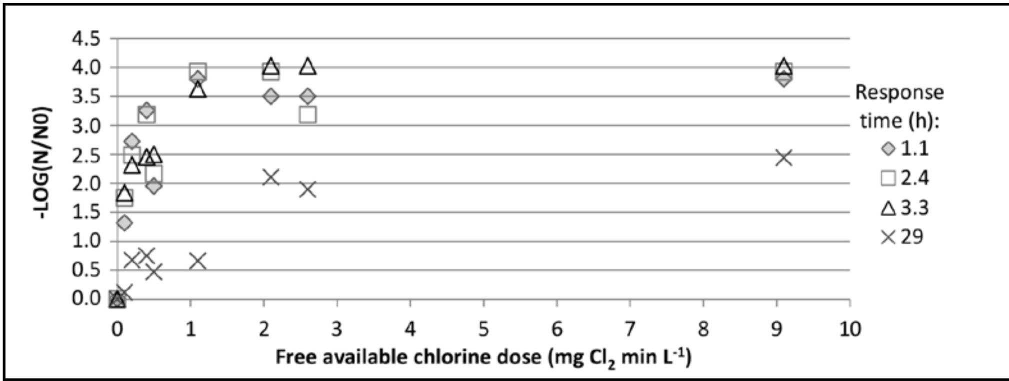


Figure 4.1 *Log reduction of the anthropogenic microbial community of bathers (AMCB) over time after disinfection with different free available chlorine doses measured with heterotrophic plate count (HPC).*

4.3.1.2 E. coli

While the non-disinfected *E. coli* samples had on average $2.2\times10^5 \pm 5.6\times10^4$ CFU mL^{-1}, all chlorinated *E. coli* HPC samples resulted in ≤10 CFU mL^{-1} (detection limit), which corresponds to the maximal log reduction of ≥ 4.3 ± 0.1-log. This means that the chlorinated *E. coli* cells were damaged in such way that they were unable to repair themselves and remain culturable within 27 h.

The intact cell concentration measured with FCM decreased with increasing free available chlorine dose (Figure 4.2B). The data points of the series between 4 and 11 h of response time partly overlapped. This indicates that the response time did not influence the concentration of measured intact cells, meaning that both repair and decay between 4 and 11 h was similar, if not absent.

The series with a response time of 2.2 h and 27 h showed lower log removals than the series 4-11 h (Figure 4.2B). A lower log reduction could be caused by a higher cell concentration (N) or a lower cell concentration in the non-disinfected sample (N0). After a response time of 2.2 h, the cell concentrations (N) were higher than the cell concentrations after 4 h response time. After 27 h, the cell concentration of the non-disinfected sample (N0) was lower than the cell concentrations between 4-11 h response time and the cell concentrations (N) higher.

4.3.1.3 P. fluorescens

The non-disinfected *P. fluorescens* samples contained on average $3.4\times10^5 \pm 1.2\times10^5$ CFU mL^{-1} over time, where all chlorinated *P. fluorescens* HPC samples resulted in ≤ 20 CFU mL^{-1} (detection limit), corresponding to the maximal log reduction of 4.2 ± 0.1-log.

The intact cell concentration was measured with FCM and decreased with increasing free available chlorine dose (Figure 4.2C). The first measurement after 1.1 h response time showed lower log reductions than the other measurements between 2.1 and 27 h response time. The lower log reductions after 1.1 h response time resulted from higher intact cell concentration (N) of this time series in comparison with the other time series. The intact cell concentrations decreased until a response time of 6.5 h, after which the intact cell concentration increased

again. The maximum log reduction found was 2.5-log at 6.9 mg Cl_2 min L^{-1} after 2.1 h of response time and on average 1.4 ± 0.6-log, while the maximum log reduction could be 4.3 ± 0.1-log on average over time.

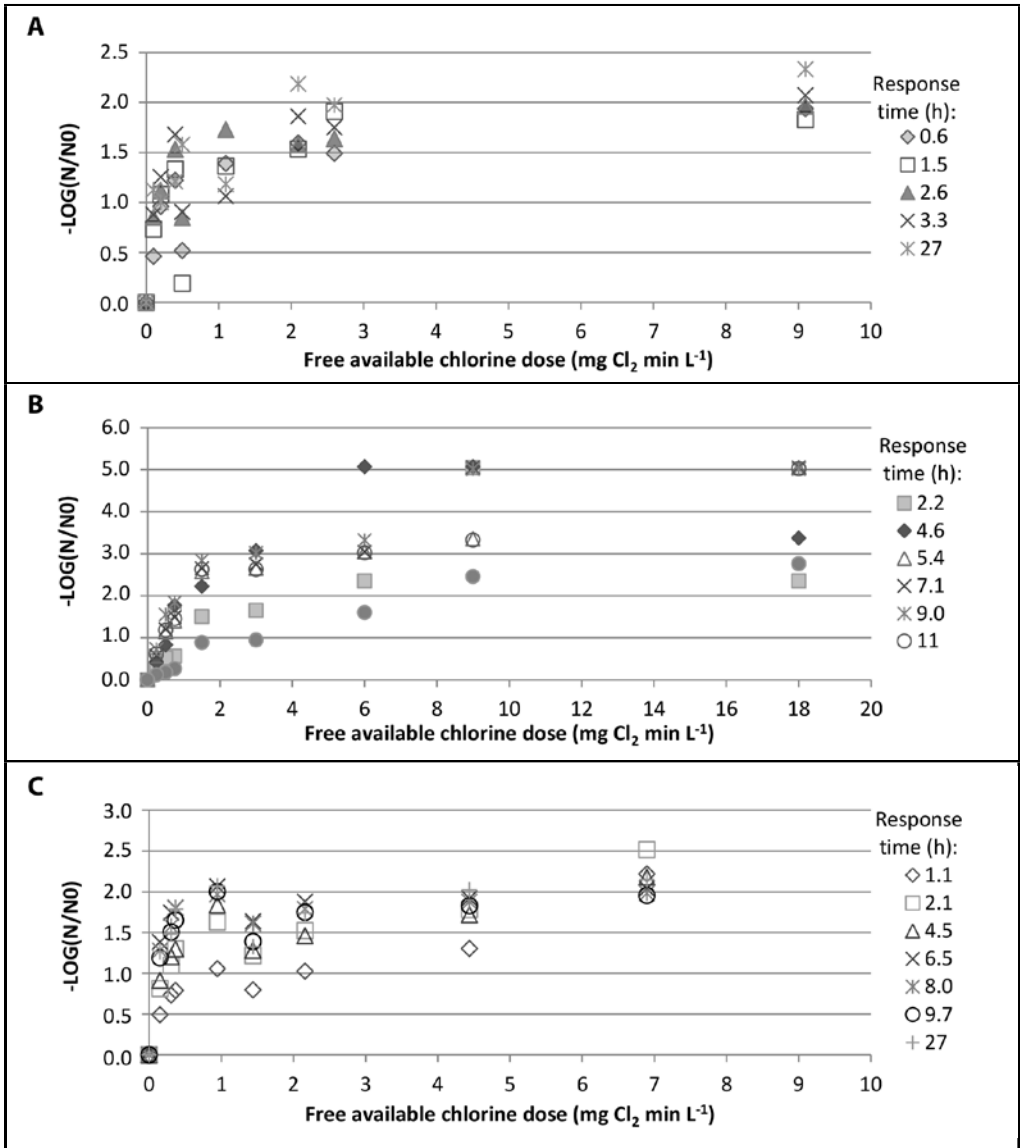


Figure 4.2 *Log reduction of the AMCB (A), E. coli (B) and P. fluorescens (C) over time after disinfection with different free available chlorine doses measured with flow cytometry (FCM) using live/dead staining.*

4.3.2 Response after UV irradiation

The effect of UV irradiation on the AMCB and the indicator organisms was determined in combination with light repair. Light repair, or photoreactivation, is an enzymatic reaction where light energy is used to split the dimers (Quek and Hu 2008). Light repair was chosen over dark repair because of the shorter repair times reported under these conditions (Hijnen et al. 2006), indicating a worst case scenario.

4.3.2.1 AMCB

The HPC results of the AMCB after UV irradiation showed a higher log reduction with increasing UV dose (Figure 4.3A). The maximum log reduction possible was 3.0 ± 0.1-log, which was not reached. UV doses of 47 and 70 J m^{-2} resulted in average log reductions of 0.5 ± 0.2-log and 1.0 ± 0.1-log respectively over time. Lower UV (0-23 J m^{-2}) doses revealed only low inactivation effects of 0.1 ± 0.2-log on average over time.
The FCM measurements indicated that the concentration of intact cells mL^{-1} remained more or less constant, independent of the UV dose; the average log reduction was 0.0 ± 0.1-log.

4.3.2.2 E. coli

The HPC results of UV irradiated *E. coli* were influenced by the magnitude of the UV dose (Figure 4.3B). The log reductions at low UV doses (0-23 J cm^{-2}) were on average 0.1 ± 0.1-log over time. The log reductions at a UV doses of 47 and 70 J m^{-2} were on average 0.5 ± 0.2-log and 1.1 ± 0.4-log respectively, while the maximum log reduction possible was 4.4 ± 0.1-log on average over time.

The FCM results indicated a constant intact cell concentration with an average log removal over all response times of -0.01 ± 0.03-log, suggesting that these results were not influenced by UV irradiation.

4.3.3.3 P. fluorescens

Determination of the log reductions of *P. fluorescens* after UV irradiation with HPC resulted in a lower concentration of CFU mL^{-1} at higher UV doses (Figure 4.3C). The log reductions at UV doses higher than 12 J cm^{-2} were higher than 0.8-log. The highest log removal possible of 4.3 ± 0.2-log was found after a UV dose of both 47 and 70 J m^{-2} within 5.8 h of response time. Also for *P. fluorescens*, the intact cell concentrations remained constant showing a log removal of 0.0 ± 0.1-log, suggesting that these results were also not influence by UV irradiation.

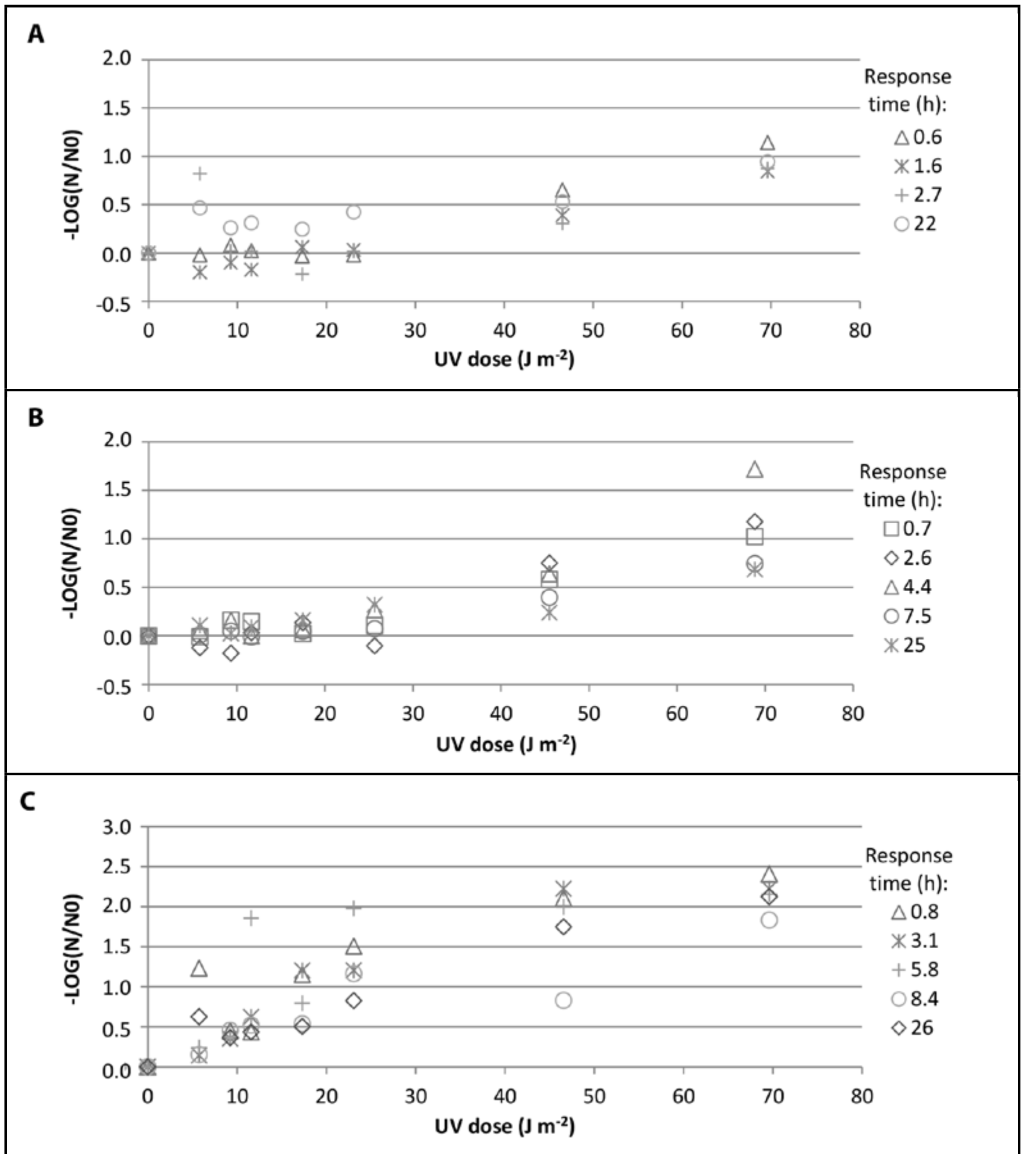


Figure 4.3 *Log reduction of AMCB (A), E. coli (B) and P. fluorescens (C) determined over time after disinfection with different UV doses measured with HPC.*

4.4 Discussion

The objective of this study was to determine the disinfection efficiency of an AMCB and compare it to the efficiency of the recreational water quality indicator organisms *E. coli* and *P. fluorescens*. After exposure to chlorine, the AMCB could still be cultured on applied medium while indicator organisms were below detection level. Still, intact cells were measured for the AMCB and both indicators. The samples treated with UV irradiation showed constant intact cell concentrations despite the UV dose, whereas measured HPC values were related to the UV dose.

4.4.1 Response to chlorination based on FCM

When evaluating the log reduction of the AMCB after chlorination, lower log reductions were measured using FCM than with HPC. The log reductions obtained with HPC were 1.0-log higher for free available chlorine concentrations of 0.2-0.5 mg Cl_2 min L^{-1} and 1.4-1.7-log higher for free available chlorine concentrations of 1.1-9.1 mg Cl_2 min L^{-1} than for FCM. The underestimation of the bacterial counts by HPC has been observed before (Hammes et al. 2008, Hammes and Egli 2010).

In order to compare the response to chlorination of the AMCB and the indicator organisms, FCM live/dead staining results were used. FCM live/dead staining was chosen over HPC because FCM could determine bacterial cells despite of their state (starved, stressed, VBNC – (Oliver 2005, Rezaeinejad and Ivanov 2013) and has a higher sensitivity and detection limit (Hammes and Egli 2005, Vital et al. 2012). Furthermore, FCM live/dead staining could be considered to be a suitable method to determine inactivation with hypochlorite (Joux and Lebaron 2000a) because it measures the cell membrane damage caused by oxidants like hypochlorite (Ramseier et al. 2011).

Calculated log reductions of the different response time series of the AMCB determined by FCM showed similar trends (Figure 4.2A). However, this was not the case for all time series of the indicator organisms, such as the series of 1.1 h response time of *P. fluorescens* (Figure 4.2C) and of 2.2 h and 27 h response time of *E. coli* (Figure 4.2B). An explanation for lower log reductions in the first hours of response time could be that the outer membrane of the cells was not yet disrupted. It is generally accepted that chlorination oxidizes the cell membrane (Ramseier et al. 2011, Venkobachar et al. 1977) and that membrane integrity plays an important role in bacterial resistance (Virto et al. 2005). However, Virto et al. (2005) found that extensive membrane damage is not the key factor of inactivation of bacteria by chlorine, as enzyme inactivation might occur earlier and play a larger role than membrane damage. When there is lack of extensive membrane damage, the PI molecule could probably not enter the cells, causing the cell to be measured as an intact cell instead of a cell without intact cell membrane.

The ongoing inactivation after the first hours of response time could indicate that chlorine was not completely removed. It is known that sodium thiosulphate, used for dechlorination, removes free chlorine in water samples (Schmittinger et al. 2006). However, literature about removing chlorine inside cells was not found, although it is known that thiosulfate could enter cells (Dreyfuss 1964). Since chlorine could have still been present inside cells, the damage of the intracellular components could continue such that the cell could be indicated as damaged after some time when the membrane is also damaged.

The *E. coli* series of 27 h of response time showed a lower log reduction over free available chlorine concentrations compared to the log reductions of lower response times (Figure 4.2B). This was caused by the decrease of the intact cell concentration of the non-chlorinated sample and an increase of the intact cell concentration of the chlorinated samples, suggesting growth or repair. Because of low carbon and nitrogen concentrations in the medium, growth resulting from added components is not likely. However, (in)organic nutrients could have been released by the inactivated cells (Fabrizio et al. 2004), from which the living cells could benefit. This would suggest that at an increasing free available chlorine dose, more *E. coli* cells were disrupted, resulting in more food for the survivors and therefore showing the highest growth potential. As illustrated in Figure 4.2, the log reduction from an average free available chlorine dose of 1.3 mg Cl_2 min L^{-1} after 27 h was 1-log lower than the minimum log reductions determined before, suggesting some growth or repair of damaged cells. Growth was less at an average free available chlorine dose of 14.2 mg Cl_2 min L^{-1} probably because of a higher log reduction efficiency at these doses. The decrease of intact cell concentration in the blank after 27 h could be explained by the lack of disrupted cells, leading to limitation of nutrients and, consequently, cell decay over time.

4.4.2 Different response of the 3 communities to chlorination

At all free available chlorine doses (0-15 mg Cl_2 min L^{-1}), the AMCB could still be cultured on applied media while indicator organisms were below detection level. The culturability of the AMCB after chlorination could be caused by clustering of microorganisms or formation of a protective layer of skin debris. The cultures of the indicator organisms, however, consisted of free cells and were probably more vulnerable. Besides, the AMCB consists of different microorganisms, some of which might have been more chlorine resistant than the tested indicators. As described in chapter 3, the dominant family in the AMCB was *Flavobacteriaceae*. The response of *Flavobacteriaceae* to chlorination and UV irradiation was not investigated here, but literature suggests that *Flavobacteriaceae* is less sensitive to chlorine (Hwang et al. 2012, Wolfe et al. 1985). Furthermore, the AMCB was exposed to different stress levels of hot, cold, dry, moistured e.g. on the human skin, which might have resulted in a more resistant community (Ridgway and Olson 1982).

Comparing the average log reductions of the time series per community per free available chlorine dose (Figure 4.4), the log reductions of *P. fluorescens* were lower than the log reductions of *E. coli* (Borgmann-Strahsen 2003). It was also observed that *E. coli* showed higher log reductions over time than *P. aeruginosa* under swimming pool conditions. The log reductions of the AMCB were similar to the log reductions of *P. fluorescens* and lower than the log reduction of *E. coli*. Therefore, the indicator organism *P. fluorescens* is more suitable to predict the response of an AMCB to chlorination than *E. coli*.

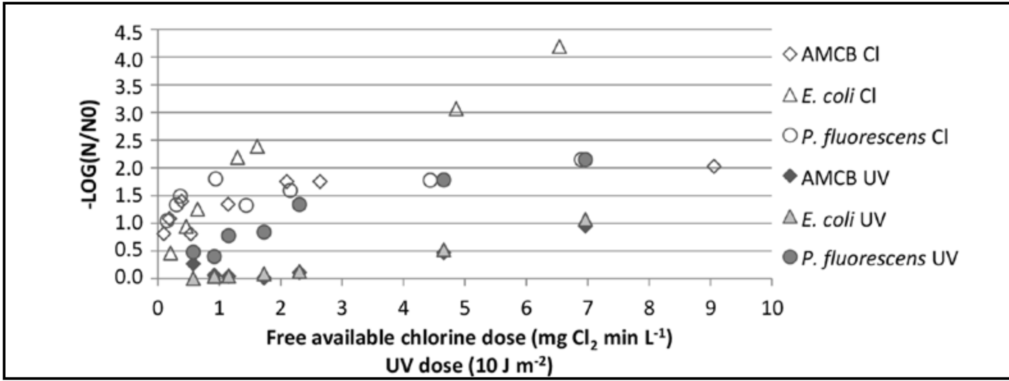


Figure 4.4 *Average log reductions of all response times per disinfection dose of the AMCB, E. coli and P. fluorescens. Log reductions of the UV experiments were measured with HPC as CFU mL^{-1} while the log reductions of the chlorination experiments were measured with FCM live/dead staining as intact cells mL^{-1}.*

4.4.3 Viability and culturability after chlorination

The AMCB is a mixture of different microorganisms, and probably not all microorganisms could be cultured on the selected agar medium. Wade (2002) described that the culturability of microorganisms of body sites is assumed to be similar to the culturability of the oral microflora, which is about 50%. The average concentration of intact cells of the non-disinfected samples measured with FCM within 4 h was 3.2×10^6 intact cells mL^{-1}, where the average of the CFU mL^{-1} found with HPC was 1.7×10^6 CFU mL^{-1}. Dividing the concentration of CFU by the concentration of intact cells indicates that 52% of the AMCB was culturable, which is closely related to the assumption of Wade (2002). After chlorination, the culturability percentage decreased to an average of 2% for the chlorinated samples measured within a response time of 4 h.

The same sample showed different log reductions when measured with HPC or FCM after chlorination. Intact cells were detected in all non-disinfected and chlorinated samples (Figure 4.2). However, the culturability of the indicator organisms was below the detection limit, suggesting that the intact cells of the indicator communities were VBNC. VBNC microorganisms might still pose a threat for human health as ingestion creates different conditions that might trigger repair, a phenomenon called resuscitation (Milbauer and Grossowicz 1959). Determination of microbial levels of indicator organisms in chlorinated swimming pools with HPC might not reflect the actual microbial levels, resulting in an overestimation of the disinfection efficiency and thus an underestimation of the health risks.

4.4.4 VBNC state after UV irradiation

The results of the experiments with UV irradiation show that despite the increasing UV dose, the FCM results remained constant while the HPC results decreased. Even after one day of response time, no relation between FCM and HPC was found for any of the communities. Because the cells were irradiated with UV, the DNA could have been damaged resulting in an inactivated cell with an intact cell membrane. However, Zhang et al. (2015) observed that UV induces the VBNC state in *E. coli* and *P. aeruginosa*. Therefore, it might be assumed that most of the cells with an intact cell membrane that were not culturable were also in the VBNC state, so metabolic routes might still be working. Without any change of environment, microorganisms might survive. The results of this study found that the membrane of the tested microorganisms remained intact even 26 h after UV irradiation (Figure 4.3). However, other researchers observed some membrane damage after UV irradiation. Ito and Ito (1983) described that the damage caused by UV irradiation would only allow small molecules to pass. In addition, Sosnin et al. (2004) distinguished living from dead cells after UV irradiation based on membrane leakage. This suggests that the cell membrane damage caused by UV irradiation is generally smaller than 1.5 nm, because Bowman et al. (2010) determined that PI could enter the cell through such a hole. Berney et al. (2006) has previously described that FCM live/dead staining is not a proper tool to determine UV inactivated cells, and this research confirms this once more.

4.4.5 Response to UV irradiation based on HPC

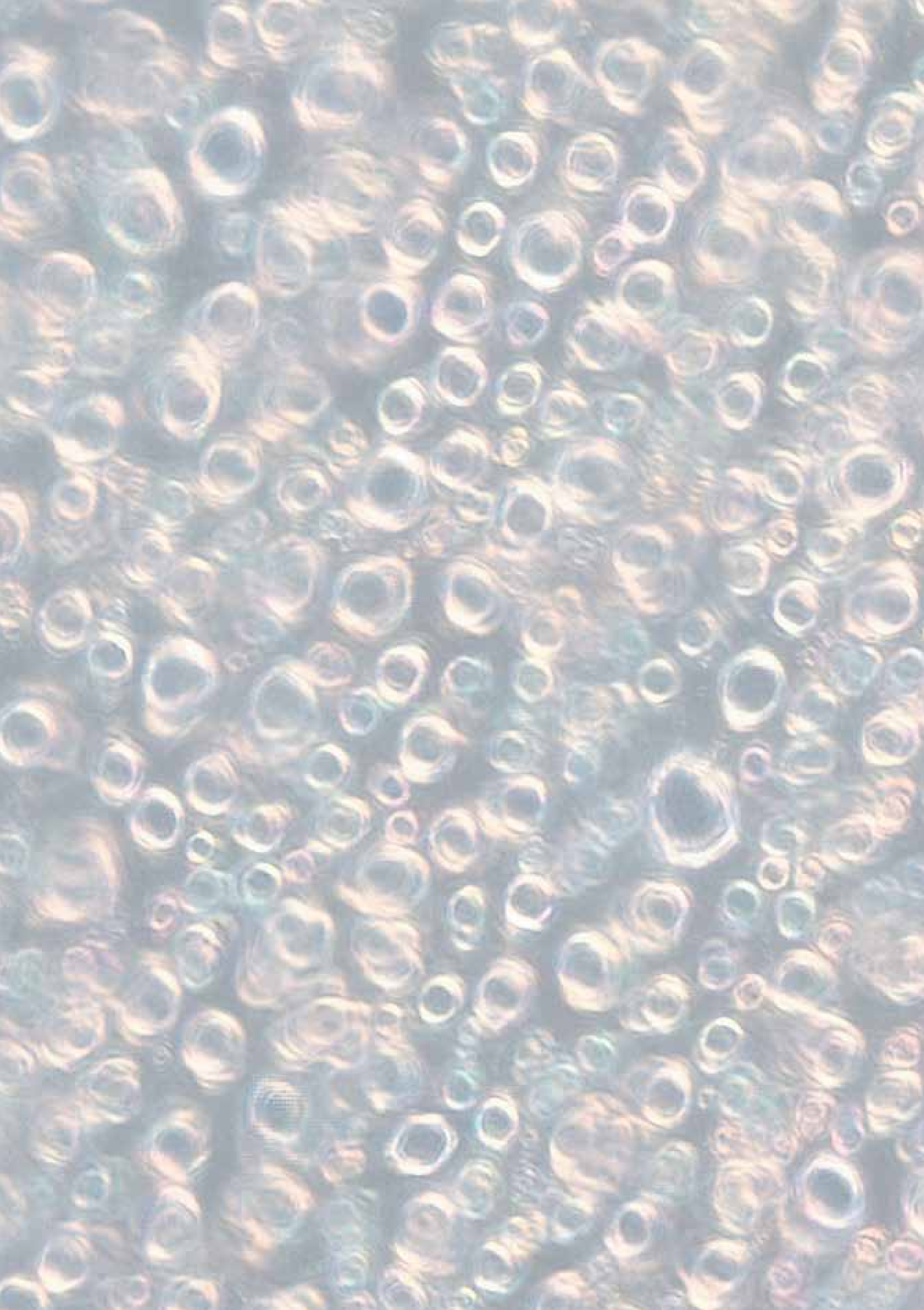
HPC results were used to compare the log reductions of the AMCB and the indicator organisms after UV irradiation (Figure 4- 4). The log reductions of the AMCB were similar to the ones of *E. coli*, whereas the log reductions of *P. fluorescens* were higher, indicating that *P. fluorescens* is more sensitive to UV irradiation than the AMCB and *E. coli*. Previous studies have observed that the log reductions of *E. coli* were lower than of *Pseudomonas* spp. (Zhang et al. 2015).

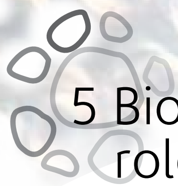
Average log reductions of *E. coli* over time were 0.5 ± 0.2-log and 1.1 ± 0.4-log for a UV dose of 47 and 70 J m^{-2} respectively, while slightly higher log reductions were obtained by Hijnen et al. (2006), Zimmer-Thomas et al. (2007) and Van Aken and Lin (2011) who found log reductions of 1 and 2 for UV doses of 50 and 90 J m^{-2}, respectively. An explanation for the slightly lower log reductions could be that during UV irradiation the samples were not stirred and cells might have partially been settled or flocculated. Another explanation for the lower reductions could be due to light repair, indicating that log reductions were probably slightly higher and better in range with literature.

4.5 Conclusions

This research determined the log reduction of an anthropogenic microbial community of bathers (AMCB) and the indicator organisms *E. coli* and *P. fluorescens* after chlorination and UV irradiation with heterotrophic plate count (HPC) and flow cytometry, live/dead staining (FCM). Although both methods could be used after chlorination, FCM analysis is more useful than HPC because the technique also measures non-culturable organisms. Based on FCM results, chlorination of an AMCB showed similar log reductions as the recreational water quality indicator *P. fluorescens*, while both showed lower log reductions than *E. coli*. Therefore, *P. fluorescens* would be a more suitable indicator to predict the log reduction of an AMCB in chlorinated water systems, e.g. chlorinated swimming pools, than *E. coli*.

Using FCM after UV irradiation was not considered to be useful, as intact cells were measured in spite of the UV dose, probably due to DNA damage instead of membrane damage. Based on HPC results after UV irradiation, the AMCB showed similar log reductions as *E. coli*, while both showed lower log reductions than *P. fluorescens*. Therefore, in UV disinfected water systems, *E. coli* would be a more suitable indicator to predict the log reduction of an AMCB than *P. fluorescens*.





5 Biofouling in swimming pools: role of material properties and nutrients

Abstract

Biofouling occurs in aqueous environments but is not desired in swimming pools. This paper investigated biofilm development on 11 swimming pool materials (PVC on stainless steel, PVC, stainless steel, rough foil, smooth foil, concrete, rough tiles, smooth tiles, pultrusion polyester, polyester and polypropylene) in a laboratory-scale set-up. Tap water was used as inoculum and nutrients were dosed (simulating highly occupied swimming pool conditions) to determine the influence of nutrient release by bathers. The biofilm quantity determined by cellular adenosine-tri-phosphate (cATP) measurements was an order of magnitude higher when high bathing load conditions were applied than application of plain tap water conditions.

The biofilm development on the different materials under high bathing load conditions was quantified over time (4-28 days). After 14 of the 28 days, the highest biofilm quantity was observed; therefore, the biofilm quantity on the different materials was compared on day 14. The lowest average biofilm quantity was found on polypropylene (3.3×10^4 pg ATP cm^{-2}), while the highest average quantity was measured on stainless steel (2.0×10^5 pg ATP cm^{-2}) and concrete (2.2×10^5 pg ATP cm^{-2}). Roughness and hydrophobicity (based on cohesion energy) of the materials were expected to play a role in the biofilm development. However, it was found that the extent of biofilm formation was not related to the surface roughness. The biofilm quantity after 4 days increased with increasing hydrophobicity of the materials, but after 14 days, this effect was no longer observed. This suggest that in time the role of material characteristics on the extent of biofilm formation diminish.

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5.1 Introduction

In water, microorganisms can be found as planktonic cells, aggregates or attached to a surface. When microorganisms attach to a surface, in time, the attachment becomes irreversible (not removable by gentle rinsing) because microorganisms excrete extracellular polymeric substances (EPS) (Donlan 2002, Dufrêne et al. 1996) and form a biofilm. EPS may account for 50% to 90% of the total organic carbon of biofilm (Flemming et al. 2000) and can be considered the primary matrix material of the biofilm. While swimming, bathers release microorganisms (chapter 2), which could form a biofilm. Also, pathogens have been found in swimming pools (Papadopoulou et al. 2008), which might be shielded in those biofilms. This introduces a microbiological health risk and therefore are biofilms unwanted in swimming pools.

Different methods have been used to prevent biofouling, such as disinfection of water containing planktonic microorganisms and use of materials with biofouling limiting characteristics. While most swimming pools use residual disinfectants, like chlorine-based products, biofilms still have been found on the surface of the pool itself (Davis et al. 2009). This suggests that the applied method is not sufficient to inactivate all suspended microorganisms and biofilms. Insufficient disinfection and possibilities for biofilm growth have also been observed in food (Bagge-Ravn et al. 2003, Weese and Rousseau 2006), medicine (Deva et al. 1998, Martin et al. 2008) and domestic environments (Cooper et al. 2008). Biofouling is even more likely to occur in a swimming pool without residual disinfectants, like a pool with UV-based treatment. Whereas a traditional swimming pool treatment consists of a sand filter and chlorination, the UV-based treatment consists of a biological sand filter followed by ultrafiltration and UV irradiation. Although multiple barriers are implemented to prevent survival of microorganisms within the UV-based treatment, microorganisms are not directly inactivated as soon as they enter the pool water. Therefore, prevention of biofouling is important.

The degree of bacterial adhesion on materials depends on various material characteristics such as surface charge (Kerr et al. 1998), surface roughness (Kerr et al. 1999) and hydrophobicity. It has been observed that biofouling increased as the surface roughness increased (Characklis et al. 1990) because shear forces at the direct surface of the material diminish (microorganisms can be shielded) and the absolute surface area is higher when surfaces are rougher (Donlan 2002). Moreover, biofouling occurred more rapidly on hydrophobic and nonpolar surfaces than on hydrophilic materials (Bendinger et al. 1993, Fletcher and Loeb 1979, Pringle and Fletcher 1983).

In order to determine which materials should be used in swimming pools to prevent biofouling as much as possible, biofilm formation on 11 different swimming pool materials was investigated. Experimental settings were based on a swimming pool with a UV-based treatment and a variable load of nutrients (to determine the influence of bathing load): a high concentration of biodegradable compounds was used, simulating a high bathing load, as well as plain tap water with lower nutrient content.

5.2 Materials and methods

5.2.1 Experimental setup and operation

Biofouling experiments were performed in a climate controlled set-up (Hellebrekers Technieken, Nunspeet, the Netherlands). The air temperature was maintained between 30-33 ˚C and the incoming medium (water) temperature was controlled at 30 ± 0.5 ˚C.

Biofilm growth was determined on 11 different swimming pool materials, of which plates with similar dimensions (Ø 80-85 mm) were used. Each plate was placed in the inner ring of a jar (Figure 5.1) in order to create a laminar flow over the whole plate to create equal distribution of nutrients. The jars themselves were made of PVC pipes. For each swimming pool material, four jars were connected to each other and operated in series. The four jars were sampled simultaneously and the accumulated biomass was quantified using cellular adenosine-tri-phosphate (cATP) measurements as a measure for active biomass (Velten et al. 2007).

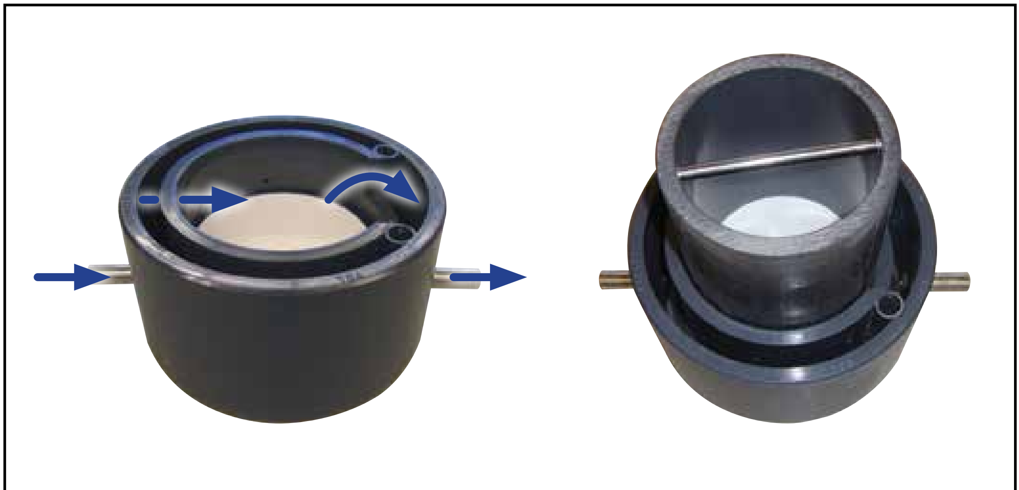


Figure 5.1 *The arrows indicate the flow through the jars (left). From the outer ring, the medium flows through holes in the inner ring over the plates. Next, the medium goes via an overflow to the next jar. Determination of the biofilm on the plate only was conducted by inserting a PVC pipe in the inner part of the jar to exclude remaining surfaces (right).*

5.2.2 Biomass sampling

In order to determine relevant sampling moments in time, the biofilm quantity was determined on days 4, 7, 11, 14, 18, 23 or 28, for three different materials. The maximum biofilm quantity for all three materials was found on day 14 (Figure 5.4). It was therefore chosen to sample the other 8 materials on days 4, 7, 11 and 14 only.

In order to avoid interference of the PVC jar on the determination of the biofilm growth on the plates of the different materials, the remaining surfaces of the jar were covered by a clean PVC pipe before sampling (Figure 5.1). Thereafter, the medium was removed from the jar and replaced by 100 mL of demineralised water. To remove the biofilm from the plate upon

measuring, the demineralised water in the jar was sonified with a high energy sonifier (HES, Branson Sonifier digital Cell Disruptor 250 W) for 2 min with an amplitude of 20%. Thereafter, the water was replaced and sonified another two times. According to Van der Kooij et al. (2006), application of this treatment three times per plate achieves > 90% removal of biofilm.

5.2.3 Selection of swimming pool materials

All materials used in this research are frequently used in swimming pools: different tiles and materials are used in movable floors, foils are used to cover the tiles and concrete is mainly used in water buffer tanks. The used swimming pool materials and their suppliers are: concrete (Hellebrekers Technieken, Nunspeet, the Netherlands); polyester, pultrusion polyester, stainless steel 316, PVC on stainless steel 316, polypropylene and PVC (all from Variopool, Oudkarspel, the Netherlands); Rough and smooth tiles (Agrob Buchtal, Germany); Rough and smooth foil (DLW Delifol, Germany).

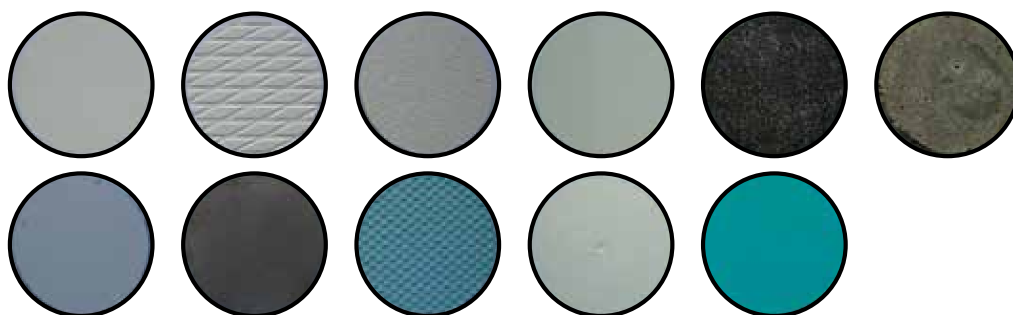


Figure 5.2 *Photograph of the used materials. Upper row, from left to right: polypropylene, polyester, pultrusion polyester, smooth tiles, rough tiles, concrete. Under, from left to right: PVC on stainless steel 316, stainless steel 316, PVC, smooth (white) foil, rough (blue) foil.*

5.2.4 Medium composition

To determine the influence of bathing load, a variable load of nutrients was used, ranging from plain tap water with low nutrient content to plain tap water with addition of extra nutrients, simulating a high bathing load. The flow rate was 500 mL h^{-1}.

The high bathing load medium was based on available data on the compounds released by bathers during a swim. The medium contained 404 mg L^{-1} urea (VWR, the Netherlands), 31.4 mg L^{-1} creatine (Sportfood, the Netherlands), 16.8 mg L^{-1} sodium citrate (VWR, the Netherlands) and 9 mg L^{-1} potassium phosphate (VWR, the Netherlands). The non-purgable organic carbon (NPOC) and total nitrogen (TN) loading rates were set higher than high occupancy levels in swimming pools, which are about 30 mg NPOC h^{-1} and 60 mg TN h^{-1} respectively (chapter 2), to create optimal growing nutrient conditions. The average NPOC and TN concentrations during all experiments in all 4 jars (>600 samples) were 94.8 mg C L^{-1}, with a standard error of the mean of 3%, and 199.1 mg N L^{-1}, with a standard error of the mean of 1%, respectively. The C/N ratio (0.48) was closely related to the C/N ratio present in swimming pools (0.58) according to Judd and Black (2000).

5.2.5 Biomass quantification

The biofilm quantity was determined with cATP, which was measured with the QGA™ kit of Aqua-tools (Poissy, France). The samples were filtered over a 0.7 μm filter to collect the microorganisms and remove the dissolved ATP. Thereafter, cells were treated with a trisodium phosphate solution to destroy the cell membrane and release the cATP. After dilution (Ultralute – ATP dilution buffer) of the extracted cATP, a luciferine/luciferase complex was added to induce the emission of light. The generated light signal (Relative Light Units, RLU) was measured within 30 s with a Luminometer (Junior LB 9509, Aqua-tools). The concentration of cATP was calculated from the RLU values using a conversion factor determined from calibration measurements performed after six analyses (EQ 5-1). The standard solution contained 1 ng ATP mL^{-1}. With the measured cATP concentration, the cATP concentration per surface could be calculated (EQ 5-2).

$$\mathrm{cATP} = \frac{\mathrm{RLU_{sample}}}{\mathrm{RLU_{standard\ solution}}} \times \frac{10^4}{\mathrm{V}} \qquad \text{(EQ 5-1)}$$

Where
cATP = cellular ATP (pg mL-1)
RLU = Relative Light Units
V = volume (mL)

$$\mathrm{cATP}\ (\mathrm{pg\ cm^{-2}}) = \frac{\mathrm{cATP} \times 1000}{\frac{\mathrm{S}}{\mathrm{V}}\mathrm{ratio}} \times \frac{1}{100} \qquad \text{(EQ 5-2)}$$

Where
S/V ratio = the surface/volume ratio of the plates, being $\frac{1.77\ \mathrm{dm^2}}{0.35\ \mathrm{dm^3}} = 5\ \mathrm{dm^{-1}}$

5.2.6 NPOC and TN

NPOC and TN were measured to determine whether the nutrient concentrations were constant in all jars and for all experiments. NPOC was determined according to NEN-EN-1484 (1997) using a Shimadzu TOC-Vcph analyser. After acidifying with 2 M hydrochloric acid and purging with high purity air, the water samples were injected into the combustion chamber at 680 °C to oxidise all carbon into CO_2, which was subsequently detected by using infrared spectrometry. TN was determined according to NEN-EN-12260 (2003) using a Shimadzu TNM-1 analyser connected to the Shimadzu TOC-Vcph analyser. The water samples were injected into the combustion chamber at 720 °C, where nitrogen compounds were converted into nitric oxide and subsequently exposed to ozone to induce emission of light, which was detected by a chemiluminescent detector.

5.2.7 Roughness

The root mean square (RMS) roughness (Rq) and the arithmetic average roughness (Ra) of all materials were determined with a DektakXT (Bruker) and the program Vision64. With a needle, the troughs and crests of the materials were determined over a 1 cm length. The Ra and Rq roughnesses of each material were determined using at least at three different spots on the material plate. Using Ra and Rq, the roughness area ratio (r = Ra/Rq) was calculated.

5.2.8 Determination of cohesion energy as a measure of hydrophobicity

The hydrophobicity of the swimming pool materials was determined by calculation of the cohesion energy. The parameter ΔG_{MLM} represents the free energy of cohesion, which is associated with the relative energetic favourability of water molecules maintaining contact with the solid material rather than with each other (Subramani and Hoek 2008, van Oss 2006). When is positive, the material could be considered non-cohesive when immersed in water, and thus hydrophilic; when is negative, the material is cohesive in water, or hydrophobic (van Oss 2007, 2008). The free energy of cohesion can be determined from the Lifshitz-van der Waals and the acid-base components of the surface tension of the material and the water (EQ 5-3).

$$\Delta G_{MLM}^{LW} = 2\left(\sqrt{\gamma_M^{LW}} - \sqrt{\gamma_L^{LW}}\right)^2 - 4\left(\sqrt{\gamma_M^{+}\gamma_M^{-}} + \sqrt{\gamma_L^{+}\gamma_L^{-}} - \sqrt{\gamma_L^{+}\gamma_M^{-}} - \sqrt{\gamma_M^{+}\gamma_L^{-}}\right) \quad \text{(EQ 5-3)}$$

Where

γ_i^{LW} = the apolar (Lifshitz-van der Waals) component of the surface tension of species i

γ_i^{+} = the apolar electron-accepting component of the surface tension of species i

γ_i^{-} = the apolar electron-donating component of the surface tension of species i

The surface tension components were calculated via the Young-Dupré equation that links the contact angle of a drop of liquid (L) deposited on a flat solid surface (M) with the surface tension of the liquid (), and the surface tension components of the solid (M) and the liquid (L) according to van Oss (2007). However, the contact angles should be corrected for the interfacial tensions as a result of the increased surface area due to roughness. Therefore, the roughness area ratio (r) as described by Wenzel (1949) was implemented in the Young-Dupré equation, which resulted in the extended Young-Dupré equation (EQ 5-4).

$$\left(1 + \frac{\cos\theta}{r}\right)\gamma_L^{TOT} = 2\sqrt{\gamma_M^{LW}\gamma_L^{LW}} + \sqrt{\gamma_M^{+}\gamma_L^{-}} + \sqrt{\gamma_M^{-}\gamma_L^{+}} \quad \text{(EQ 5-4)}$$

Where

θ = the measured contact angle

r = the roughness area ratio

γ_i^{TOT} = the surface tension

The contact angles were determined using a goniometer (Krüss DSA10, Krüss GmbH, Germany) equipped with contact angle calculation software (Drop Shape Analysis, Krüss GmbH, Germany). At least 10 measurements per material were performed on different locations on the plate with three liquids: ultrapure water, glycerol and diiodomethane. Ultrapure water was obtained from a Millipore water purification system, while glycerol and diiodomethane were supplied by Sigma Aldrich (St Louis, MO). The surface tension properties (mJ m^{-2}) of the probe liquids at 20 °C used are shown in Table 5.1. The contact angle measurements and surface tension properties of all three probe liquids were used in a set of three extended Young-Dupré equations and solved in order to calculate the free energy of cohesion.

Table 5.1 *Surface tension properties (mJ/m2) of probe liquids at 20 °C.*

Probe liquid	γ^{LW} **(mJ m^{-2})**	γ^{+} **(mJ m^{-2})**	γ^{-} **(mJ m^{-2})**	γ^{AB} **(mJ m^{-2})**	γ^{TOT} **(mJ m^{-2})**
Water	21.8	25.5	25.5	51.0	72.8
Glycerol	34.0	3.9	57.4	30.0	64.0
Diiodomethane	50.8	0.0	0.0	0.0	50.8

5.3 Results and discussion

5.3.1 Influence of nutrient presence on formed biofilm quantity

Bathers introduce different pollutants in swimming pools, ranging from suspended and colloidal compounds to microorganisms and soluble substances (Powick 1989). A clean pool will contain very low concentrations of nutrients and organic contaminants. However, after intensive bathing, these concentrations could increase substantially (chapter 2). To determine the influence of human activity in the pool, biofilms were grown on tap water with and without addition of compounds released by bathers during a swim. The biofilm quantity was 1-log higher when high bathing load conditions were applied than application of plain tap water conditions (Figure 5.3). In order to be able to distinguish differences between biofilm growth on the different materials, biofilm growth was maximized in subsequent experiments by adding bathers compounds in order to prevent growth limitation due to nutrient deficits.

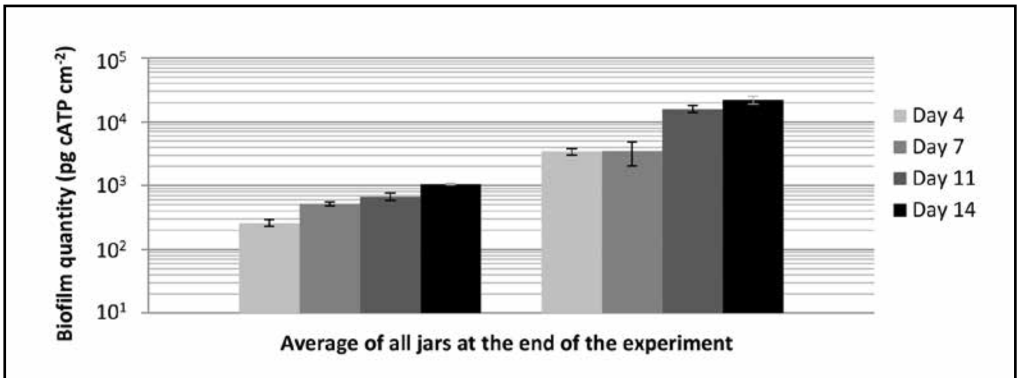


Figure 5.3 *Comparison of the biofilm quantity on concrete plates with a medium consisting only of Dutch tap water (left) and one with extra added nutrients simulating a high bathing load (right). On day 0, the biofilm quantity was 12 pg cATP cm^{-2}.*

5.3.2 Maximum biofilm quantity formed in 14 days

The biofilm formation was studied during a period of 28 days on three swimming pool materials, namely smooth foil, pultrusion polyester and concrete (Figure 5.4). Results indicate that a similar biofilm development was found on concrete and pultrusion polyester: the biofilm quantity increased exponentially during 14 days, whereafter the biofilm quantity decreased again. The exponential growth during the first two weeks was in line with the findings of Kerr et al. (1999). The majority of biofilm formation on smooth foil happened during the first 7 days, whereafter the quantity was stabilised during the remainder of the experiment. The maximum biofilm formation of all three materials was found at day 14.

5.3.3 Biofilm development on different materials

Biofilm development on the 11 swimming pool materials was determined over 14 days when highest biofilm quantities were found to underline the differences, using the same set up and growth conditions, including bathers compounds addition, for all materials. Biofilms were exponentially growing between day 4 and day 14 on most of the materials: rough foil, concrete, pultrusion polyester, PVC, smooth tiles, stainless steel and PVC on stainless steel (Figure 5.5).

After 11 days, the highest biofilm quantity was observed on concrete and stainless steel, while the lowest biofilm quantity was found on polypropylene, polyester and rough tiles. The biofilm quantity on polyester and rough tiles increased until the end of the experiment (14 days), while the biofilm quantity on polypropylene stayed low during the whole experiment. These results indicate that polypropylene may be a suitable material to use in swimming pools in order to suppress biofouling, while the use of concrete and stainless steel should be avoided or would require more cleaning.

These results were obtained after three sonification treatments, which should have removed >90% of the biofilm (Gasik et al. 2012, Maeng et al. 2011, Magic-Knezev and van der Kooij 2004, Van der Kooij et al. 2006). However, the removal of attached biomass in this research was studied on hard materials only and was found to be dependent on the material used; while after three of the five times HES on average 85% of the biofilm was removed from the concrete plates, the biofilm removal from rough tiles was on average 92% of the biofilm in three of five times HES. This suggests that the biofilm removal was more efficient for the smoother materials than on rough materials, suggesting that the biofilm quantity on concrete was probably even higher and thus the difference in measured biofilm growth between concrete and polypropylene even larger.

The higher biofilm quantity on stainless steel than on the polymeric materials is in line with Hallab et al. (2001) who reported that more cells were found on metallic materials than on polymeric materials, although these experiments were not carried out in a submerged mode. Furthermore, literature reports different results, e.g. Zacheus et al. (2000) and Pedersen (1990) found similar biofilm quantities on stainless steel and PVC for drinking water and chemically purified lake water respectively, while Rogers et al. (1994) found more biofouling on PVC than on stainless steel in drinking water. And while Hallam et al. (2001) found a higher biofilm quantity on PVC than on cement in potable tap water, the biofilm growth on concrete was found to be higher than on PVC from 11 days onwards under the high bathing load conditions. An explanation of the different observations of biofilm

growth over time on the different materials compared to literature could be due to the use of different experimental set-ups. Furthermore, in this research, extra nutrients were added to tap water to simulate a high bathing load, whereas in literature less or no extra nutrients were added to simulate growth limiting conditions. Besides, material properties can differ from the one supplier to the other, depending on the production process.

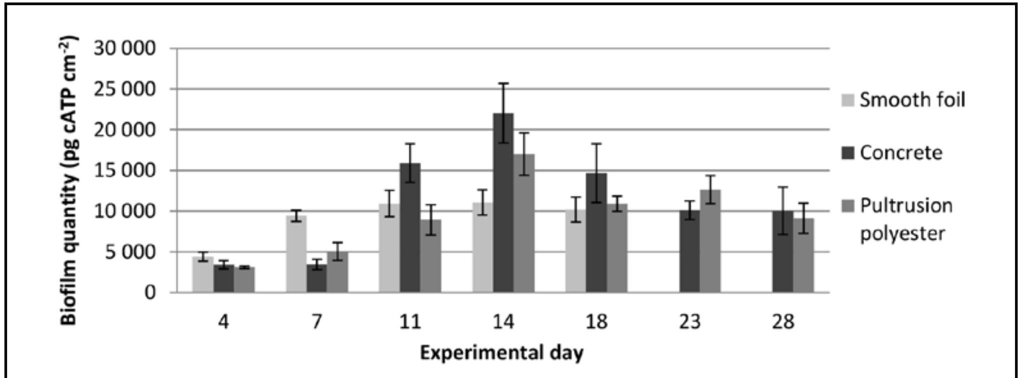


Figure 5.4 *The biofilm quantity over time after growth on different swimming pool materials, reaching maximum growth on day 14. The error bars represent the standard error of the mean.*

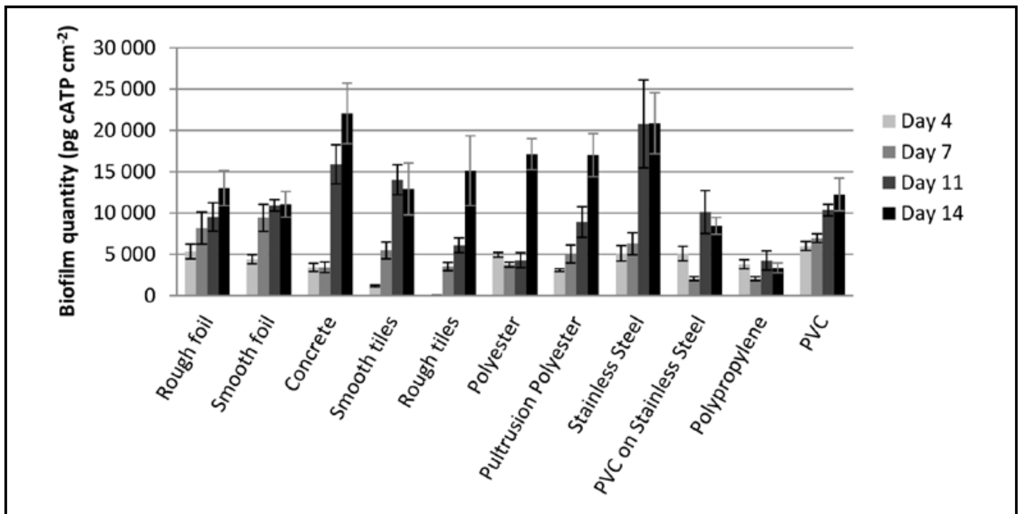


Figure 5.5 *The biofilm quantity over time after growth on different swimming pool materials. The error bars represent the standard error of the mean.*

5.3.4 Effect of material roughness on biofilm growth

The influence of roughness on biofilm formation was determined for the different swimming pool materials. Higher biofilm quantities were expected on the rougher materials (Kerr et al. 1999, Tran et al. 2012) such as concrete, which felt rough upon touching. After determination of the material roughness, concrete was found to be the roughest material tested (19.0 μm), followed by pultrusion polyester (13.7 μm) and polypropylene (9.2 μm), while the other average roughnesses (Ra) were lower than 6.2 μm (Table 5.2).

The roughness of both pultrusion polyester and polypropylene were unexpectedly high and can be explained by the patterns engraved in these plates (Figure 5.2).

Table 5.2. *The roughness of the swimming pool materials in which Ra is the arithmetic average roughness of the material, Rq is the root mean square roughness of the material and r the roughness area ratio.*

Materials	**Ra (μm)**	**Rq (μm)**	$r = Ra/Rq$
Concrete	18.95	22.88	0.83
Pultrusion polyester	13.68	16.04	0.85
Polypropylene	9.20	10.00	0.92
Rough foil	6.08	8.39	0.72
Rough tiles	4.03	4.93	0.82
PVC	1.98	2.34	0.85
Polyester	1.56	1.73	0.90
Smooth foil	1.07	1.40	0.76
PVC op stainless steel	0.49	0.57	0.87
Smooth tiles	0.28	0.34	0.83
Stainless steel	0.13	0.20	0.65

The biofilm quantity determined over time was plotted against the average roughness of the different materials (Figure 5.6). The highest biofilm quantity was found on the roughest surface (concrete), but there is no clear relation found between roughness and biofilm growth.

Literature suggest that initial biofilm formation is easier on rough surfaces than on smooth surfaces (Lecleroq-Perlat and Lalande 1994, Pedersen 1990, Teughels et al. 2006, Verran and Maryan 1997). Taking into account that the adhesion of cells to a surface occurs in a few hours (Flemming 1994, Fletcher 1996), the effect of the roughness becomes less over time (days) because cells overgrow the surface to form a biofilm with all similar properties and onto which new cells can attach. This suggests that over time, the roughness of the material becomes a less important factor for biofilm growth. Therefore, a relation between the biofilm growth at day 4, 7, 11 and 14 and the roughness of the material was difficult to observe.

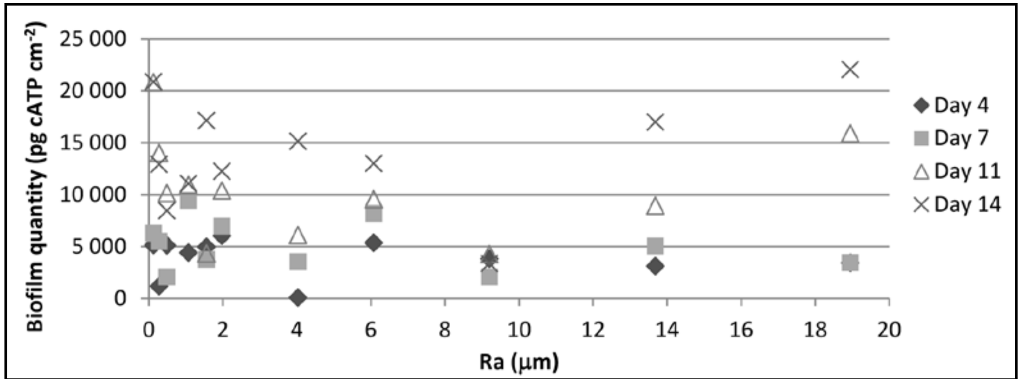


Figure 5.6 *The average roughness (Ra) plotted versus the biofilm quantity determined over time.*

5.3.5 Effect of material cohesion energy on biofilm growth

Besides roughness, the cohesion (surface) energy could also have an influence on the attachment of on average negatively charged microorganisms (Pedersen 1990), as the cohesion energy can be used to determine whether a material is hydrophobic (negative cohesion energy) or hydrophilic (positive cohesion energy). To calculate the cohesion energy, contact angle measurements were performed with ultrapure water, glycerol and diiodomethane (Table 5.3), which yielded a set of three extended Young-Dupré equations to be solved. Hydrophobic materials were, in order of most hydrophobic to least hydrophobic: PVC on stainless steel, rough foil, PVC and stainless steel. The other materials were all hydrophilic, of which the smooth tiles and concrete were the most hydrophilic.

Comparing these results to literature, Guillemot et al. (2006) found higher contact angles for water on polypropylene (θw = 103.0°) and stainless steel 316 (θw = 84.1°) than found in this research (θw = 69.6° and θw = 74.2° respectively). Also, Šíra et al. (2005) found a higher contact angle with water on polypropylene (θw = 97°), as well as Schönherr et al. (1998) on isotactic polypropylene (θw = 99.8°). The contact angle of water on PVC determined in this research (θw = 74.7°) was lower than found in literature (Tamai et al. (1977) θw = 84.0°; Jańczuk et al. (1999) θw = 89.18°). Furthermore, the contact angle of water with copolyester found in literature was between 80-85° (Charpentier et al. 2006), while in this research the contact angle found was lower (θw = 62.8°). The lower water contact angles found in this research may have been a result from the time the materials had been submerged in water (medium). The longer materials stay in water, the more hydrophilic the material surface becomes (Botton et al. 2012).

Table 5.3 *The material contact angle measurements, ± the standard error of the mean, and calculated cohesion energy. Negative values for the cohesion energy indicate hydrophobic materials, while positive values indicate hydrophilic materials.*

Materials	**Contact angle measurements (degrees)**			**Coheshion energy (mJ m^{-2})**
	Diiodomethaan	**Glycerol**	**Water**	
PVC on stainless Steel	46.2 ± 0.1	90.9 ± 0.1	85.2 ± 0.2	-23.2
Rough foil	43.8 ± 0.3	77.8 ± 0.2	74.1 ± 0.2	-19.1
PVC	38.5 ± 0.3	85.1 ± 0.2	74.7 ± 0.1	-9.2
Stainless steel	48.9 ± 0.2	85.1 ± 0.1	74.2 ± 0.3	-3.2
Polypropylene	47.0 ± 0.3	82.8 ± 0.2	69.6 ± 0.1	3.9
Rough tiles	55.4 ± 0.2	85.1 ± 0.2	68.1 ± 0.3	17.3
Smooth foil	53.3 ± 0.2	72.7 ± 0.1	62.5 ± 0.3	17.6
Polyester	49.2 ± 0.3	83.8 ± 0.1	62.8 ± 0.4	21.0
Pultrusion polyester	43.3 ± 0.2	75.8 ± 0.3	53.5 ± 0.2	29.8
Concrete	24.4 ± 0.5	61.3 ± 0.5	35.3 ± 0.2	38.9
Smooth tiles	49.1 ± 0.1	73.7 ± 0.2	51.0 ± 0.4	40.0

The biofilm quantity determined over time was plotted against the calculated cohesion energies (Figure 5.7). After 4 days, the biofilm quantity seems to be slightly decreasing with increasing cohesion energy, meaning that the more hydrophilic materials showed lower biofilm quantities. This suggests that in the beginning, cells attach more easily onto hydrophobic materials, which is in line with literature (Gilbert et al. 1991, Liu et al. 2004, Peng et al. 2001, van Loosdrecht et al. 1987).

From day 7 onward, the relation between the biofilm quantity and the cohesion energy became less clear. However, after 14 days, the biofilm quantity seemed to be increasing with the cohesion energy, indicating that a higher biofilm quantity was observed on the more hydrophilic materials. Increased biofouling over time on hydrophilic materials has been previously observed (Everaert et al. 1997, Ploux et al. 2007, Quirynen et al. 1989, Quirynen et al. 1990).

As an exception, after 14 days, a high biofilm quantity was also observed on the hydrophobic stainless steel. Because the biofilm growth on both hydrophilic (concrete) and hydrophobic (stainless steel) materials was similar at day 14, the cohesion energy might not have been the sole determining factor for growth at this point. This effect may be explained by the adhesion of cells to a surface occurring in a few hours instead of days (Flemming 1994, Fletcher 1996).

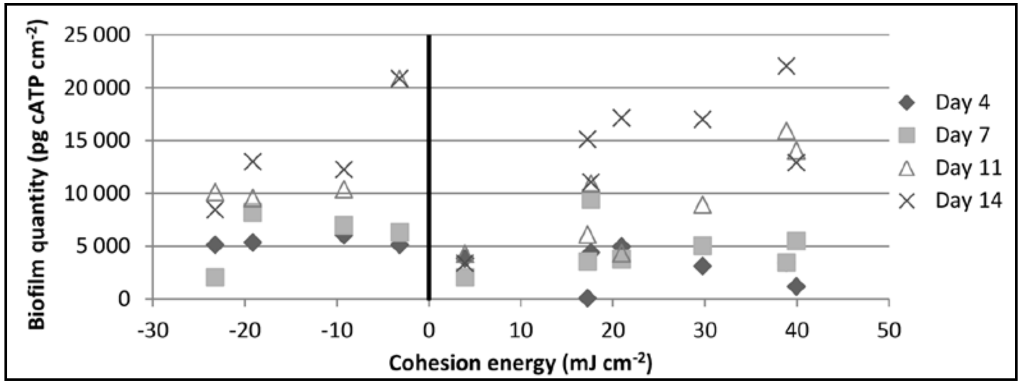


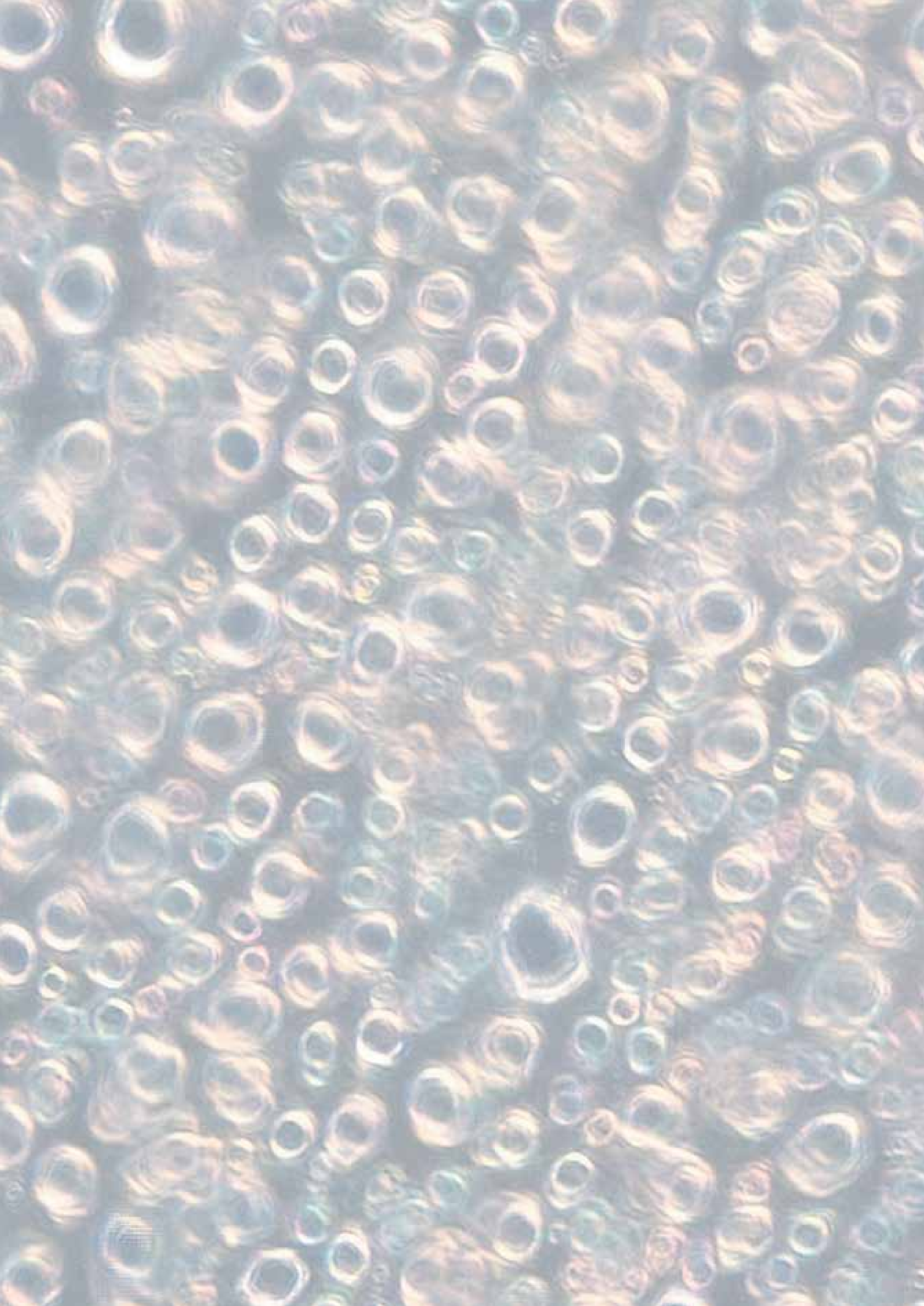
Figure 5.7 *The cohesion energy plotted versus the biofilm quantity determined in time. Negative values for the cohesion energy indicate hydrophobic materials, while positive values indicate hydrophilic materials.*

5.3.6 Biofouling more influenced by surface energy than by roughness

Whether roughness or cohesion energy has a larger influence on the extent of biofouling is difficult to determine as the surface roughness is used for calculation of the cohesion energy and therefore influences the results. However, research has been conducted on the effect of surface roughness and surface energy on super hydrophobic materials (Busscher et al. 1984, Miwa et al. 2000, Yoshimitsu et al. 2002). This was tested by sliding of a water droplet over surfaces with different roughnesses (Bikerman 1950), which resulted in an increase of the measured hydrophobicity with increasing surface roughness in the low roughness region ($r < 1.7$), while the hydrophobicity decreased drastically when the roughness became large ($r \geq 1.7$) (Miwa et al. 2000). Furthermore, the adhesion sheer strength was not statistically different at different surface roughness of titanium (Ti-6-Al-4V) (Hallab et al. 2001). This indicated that roughness had a lower effect on high energy (i.e. metallic) substrates than the surface energy, which is in line with the findings by Bundy et al. (1992) and Bundy et al. (1993). However, an increase of the roughness on low surface energy materials (polymers) resulted in greater tissue adhesion (Bundy et al. 1991, Hallab et al. 2001), which is more in line with the general assumption that initial biofilm formation (adhesion) is easier on rough surfaces than on smooth (Characklis et al. 1990).

5.4 Conclusion

Based on cATP measurements, the highest biofilm quantity was found on concrete and stainless steel, while, by approximately a factor of 6, the lowest quantity of biofilm was found on polypropylene. Therefore, this research suggests that polypropylene would be a good material to use in order to prevent large quantities of biofilm in swimming pools. The material characteristics roughness and hydrophobicity were determined for all materials. Roughness did not show any correlation with the measured biomass, while the cohesion energy showed that in in general the beginning more biofilm was likely to be attached to hydrophobic materials, but in time, more biofilm was grown on the more hydrophilic materials. From these results, it was concluded that both surface roughness and hydrophobicity might influence initial attachment, but that the influence on a growing biofilm will decrease over time. In addition, the biofilm quantity was larger by approximately a factor of 11 when extra nutrients were added (simulating a high bathing load) in comparison to plain tap water. Therefore, bathing load is expected to have a stronger influence than material properties on biofilm growth on materials in swimming pools.



6 Biofilm disinfection by UV irradiation and removal by brushing

Abstract

Biofilms were developed on UV transmitting foil and concrete plates under laboratory conditions, to simulate biofilms growing in swimming pool water without residual disinfectants. The biofilm was subjected to (i) UV irradiation at varying doses and frequencies, (ii) brushing and (iii) brushing followed by UV irradiation. The impact of UV irradiation (disinfection) and brushing (removal) on biofilm activity was evaluated using adenosine triphosphate (ATP, a measure for active biomass) analyses of the biofilm before and after the treatment. The applied UV doses were 0, 25, 50, 100, 250 and 1500 J m^{-2}.
First, the penetration of UV light through a biofilm was determined. UV irradiation was found to pass through biofilm thicknesses up to 104 µm and, therefore, the subsequent studies were done with biofilms thinner than 100 µm. UV irradiation doses ≥ 50 J m^{-2} lowered the cellular ATP concentration of biofilms developed on concrete plates, while 250 J m^{-2} stabilised the biofilm growth and 1500 J m^{-2} reduced the active biofilm amount. The biofilm reduction of the different treatments determined with total and cellular ATP was for (i) only brushing 9-7%, (ii) only 50 J m^{-2} UV irradiation 36-48% and (iii) brushing followed by 50 J m^{-2} UV irradiation 45-57% respectively, indicating that the combination of subsequent brushing and UV irradiation showed to be the most effective strategy for biofilm control.

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6.1 Introduction

Microorganisms can be present in water as planktonic cells, flocks or attached to a surface as biofilms. The microorganisms in a biofilm are embedded in an extracellular matrix composed of water, extracellular polymeric substances (EPS) and proteins (Costerton et al. 1995). In swimming pools, biofilms are unwanted because they can shelter pathogens and give a slimy feeling to surfaces, introducing slipping risks. Therefore, swimming pools are chlorinated with 0.5-1.5 mg Cl_2 L^{-1} free available chlorine in the Netherlands, to disinfect microorganisms inside the pool, according to legislation (Anonymous 2011). However, biofilms have still been found in pools with residual disinfectants (Davis et al. 2009, Schets et al. 2014).

In order to prevent or control biofouling, research has been conducted on disinfection of water containing planktonic cells, e.g. by chlorination (chapter 4; Van Aken and Lin 2011). Chlorination of microbial cells is based on the random oxidation of proteins resulting in damaging the cell membrane, the deoxyribonucleic acid (DNA) helix and disruption of the adenosine triphosphate (ATP) production (Barrette et al. 1989, Venkobachar et al. 1977). Chlorination could also be used to disinfect biofilms, although lower log reductions have been observed compared to planktonic cells (Flemming 2002, Nett et al. 2008, Smith and Hunter 2008, Wong et al. 2010). Diffusion of chlorine into biofilms is the rate limiting factor for disinfection (Chen and Stewart 1996), being related to physicochemical interactions between the disinfectant and the EPS (Bridier et al. 2011). Therefore, microorganisms in the deeper regions of the biofilm might be exposed to a lower concentration of disinfectant, resulting in adaptive response to sub-lethal concentrations of the disinfectant (Bridier et al. 2011), which could lead to disinfectant resistance. Other disadvantages of chlorination of swimming pools are (i) the formation of by-products, such as trihalomethanes, which are a potential human health risk (Font-Ribera et al. 2010, Glauner et al. 2005, LaKind et al. 2010) and (ii), the chlorine resistance of some microorganisms and waterborne pathogens, such as *Cryptosporidium* and *Giardia* (Hijnen et al. 2006).

As an alternative disinfection method, UV treatment could be applied. Disinfection with UV light at 254 nm is based on the formation of pyrimidine dimers, which distort the DNA helix and blocks cell replication (Lado and Yousef 2002). Furthermore, cross-linking of aromatic amino acids occur at their carbon–carbon double bonds, resulting in denaturation of proteins, which contributes to membrane depolarization and abnormal ionic flow (Lado and Yousef 2002). Besides DNA damage, UV irradiation also independently damages other cell components, such as the cell membrane and cytoplasm (Schwarz 1998).

Disinfection with UV irradiation might be considered as an alternative to chemical disinfection of biofilms on submerged surfaces, although little literature on this exists. Therefore, in this research the effect of biofilm disinfection by UV irradiation was investigated. Experiments were done to determine UV passage through a biofilm in relation to biofilm thickness, as well as different biofilm treatments with various UV doses and frequencies. In addition, to remove biofilms, the mechanical stability of the EPS matrix should be broken in the cleaning process (Bridier et al. 2011, Flemming 2002, Maukonen et al. 2003), which could be done by creating shear stress, by for example brushing. Therefore, the effect of brushing on the presence of active biofilm was determined, as well as the combination of subsequent brushing and UV irradiation.

6.2 Materials and methods

6.2.1 Experimental setup

Biofouling experiments were performed in a climate controlled set-up (Hellebrekers Technieken, Nunspeet, the Netherlands). The air temperature was maintained between 30-33 ˚C and the incoming medium (water) temperature was controlled at 30 ± 0.5 ˚C.

To determine the effect of the different UV and brushing treatments, a highly active biofilm was grown on concrete plates (Ø 85 mm), while the UV transmission through a biofilm was determined on plates with UV transmitting foil after a biofilm was grown (Ø 85 mm). A plate was placed in the inner ring of a jar (Figure 6.1). In order to create a laminar flow over the entire plate to create equal distribution of divide nutrients. The jars themselves were made of PVC pipes. Four jars were connected to each other and operated in series. The four jars were sampled simultaneously and the accumulated biomass was quantified using ATP measurements as a measure for active biomass (Velten et al. 2007).

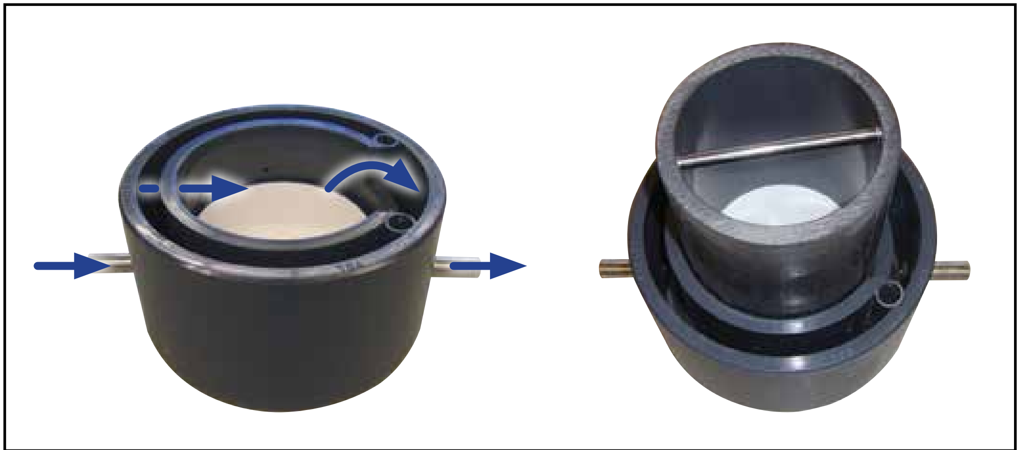


Figure 6.1 *The arrows indicate the flow through the jars (left). From the outer ring, the medium flows through holes in the inner ring over the plates. Next, the medium goes via an overflow to the next jar. Determination of the biofilm on the plate only was conducted by inserting a PVC pipe in the inner part of the jar to exclude remaining surfaces (right).*

6.2.2 Medium composition

The medium flow rate was 500 mL h^{-1} and the composition was based on available data on the compounds released by bathers during a swim. The medium contained 404 mg L^{-1} urea (VWR, the Netherlands), 31.4 mg L^{-1} creatine (Sportfood, the Netherlands), 16.8 mg L^{-1} sodium citrate (VWR, the Netherlands) and 9 mg L^{-1} potassium phosphate (VWR, the Netherlands). The non-purgeable organic carbon (NPOC) and total nitrogen (TN) loading rates were set higher than high occupancy levels in swimming pools, being 30 mg NPOC h^{-1} and 60 mg TN h^{-1}, respectively (chapter 2), to create non-limiting nutrient conditions. The average NPOC and TN concentration during the experiments was 100.2 mg C L^{-1}, with a standard error of the mean of 1%, and 192.2 mg N L^{-1}, with a standard error of the mean of 1%, respectively. The C/N ratio (0.52) was closely related to the one present in swimming pools (0.58) according to Judd and Black (2000).

6.2.3 Biofilm treatment

Previous research has shown that under these experimental conditions the highest biofilm activity was observed after 14 days (Figure 5.4). Therefore, in this research a highly active biofilm was developed in 14 days, whereafter the biofilm was treated either with UV light, by brushing or a combination of the two (Table 6.1). The biofilm was treated (i) every three days on day 15 and day 18 or (ii) every day on day 15 until day 20. Sampling was done on day 21.

Table 6.1. *Treatments performed on a biofilm grown on concrete plates.*

Biofilm treated with	Disinfection applied on day	Dose applied
UV irradiation	15, 16, 17, 18, 19 and 20	0, 25, 50, 100, 250 and 1500 J m^{-2}
UV irradiation	15 and 18	50 J m^{-2}
Brushing	15 and 18	3 wrist movements of 180°
Subsequent brushing and UV irradiation	15 and 18	3 wrist movements of 180° and 50 J m^{-2}

6.2.3.1 UV irradiation

Disinfection of the biofilm with UV irradiation was done with a bench-scale collimated beam apparatus (Fabr. Van Remmen UV Techniek, Wijhe, the Netherlands), which was used to spread a homogeneous light on the surface of the plates inside the jars (Fabr. Hellebrekers Technieken, Nunspeet, the Netherlands) (Figure 6.2).

In the apparatus a 12 W low pressure UV lamp was placed. The UV lamp was preheated by switching it on for 15 min, then switching it off for 15 min, whereafter the lamp was switched on again for 15 min before usage, to ensure a constant and reproducible lamp output. The collimated beam was equipped with an automated shutter to ensure exact irradiation times.

The average irradiance (W m^{-2}) was measured with a jar-sensor (SiCONORM-I-LP) before and after application on the plates. To calculate the applied UV dose, the irradiation time was multiplied by the fluence rate (Fr) (EQ 6-1, 2 and 3).

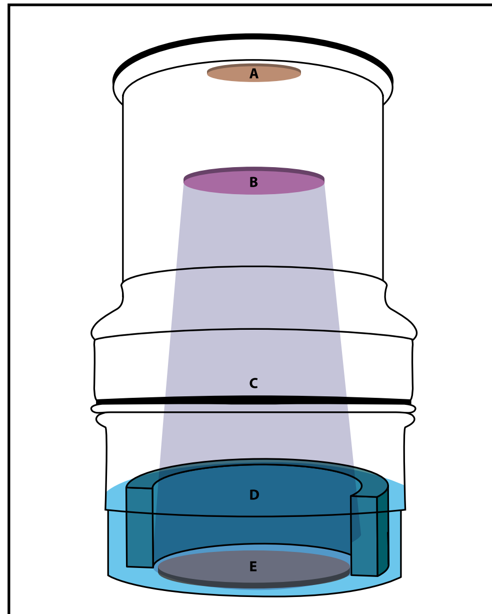


Figure 6.2 *Schematic drawing of the UV device placed on a jar, with a relative light sensor (A) for checking the output of the UV lamp (≥ 99%), a LP 10 W UV lamp (B), a camera diaphragm (C) which was used to ensure exact irradiation times, water body (D) on top of the biofilm, which was grown on a concrete plate (E).*

$$Fr = E0 \times Pf \times (1 - R) \times Df \times Wf \quad \text{(EQ 6-1)}$$

Where
Fr = Fluence rate (mW cm^{-2})
E0 = Irradiance at the centre of the plates (E0 = 0.128 mW cm^{-2})
Pf = Average relative irradiance on the jar (petri factor = 0.997)
R = Reflectance at air water interface at 254 nm (R = 0.02500)
Df = Divergence factor
Wf = Average relative fluence in medium (water factor)

$$Df = \frac{L}{L+d} \quad \text{(EQ 6-2)}$$

Where
L = Distance from the lamp to the medium surface (L = 16.4 cm)
d = depth of the medium (d = 2.0 cm)

$$Wf = \frac{1-10^{-a \times d}}{a \times d \times 2.3} \quad \text{(EQ 6-3)}$$

Where
a = UV absorption coefficient (cm^{-1})

Because the biofilm was grown at the bottom of a water body, the absorbance of the water body itself was measured separately for each jar and radiation times were calculated per jar, taking the absorbance into account. The UV doses, applied on the biofilm on the concrete plates, were 0, 25, 50, 100, 250 and 1500 J m^{-2}.

In order to quantify UV light passage through biofilms, biofilms were grown on UV transmitting fluorinated ethylene propylene foil (Holscot Fluoroplastics Limited, Grantham, England). UV emission was measured below this foil for 20 s. Longer irradiation times were not used, because this could disturb the biofilm.

6.2.3.2 Brushing

As shown in Table 6.1, two experiments were performed in which brushing was used as a treatment to control biofilm growth. A brush with the same size as the plates (Ø 85 mm) was used to clean the whole concrete plate at once (Figure 6.3) by manual wrist movements of three times 180° without pressing the brush onto the plate. The brush was cleaned with demineralised water before usage in order to remove the biofilm remnants.

Figure 6.3 *Brush (Ø 85 mm) used to remove the biofilm from the concrete plates.*

6.2.4 Microscopy

The thickness of the biofilm was determined with a microscope (Keyence digital microscope VHX-5000, universal zoom lens Keyence VH-Z100 UR) before and after treatment. A 3D image was made (Keyence digital microscope VHX-5000) to calculate the thickness of the biofilm (in μm). The thickness was determined on different spots on a plate with a minimum of 27 and a maximum of 61 determinations per plate. Because a plate was placed in each jar and a series of four jars was studied, the number of biofilm thickness determinations was between 127 and 194 per experimental day, while the biofilm thickness was determined on five experimental days.

6.2.5 Biomass sampling

In order to avoid interference of biofouling on the PVC jar during determination of the biofilm growth on the concrete plates, the remaining surfaces of the jar was covered by a clean PVC pipe before sampling (Figure 6.1). Thereafter, the medium was removed from the jar and 100 mL of demineralised water was added to the jar containing the concrete plate. To remove the biofilm from the plate upon measuring, the demineralised water in the jar was sonified with a high energy sonifier (HES, Branson Sonifier digital Cell Disruptor 250 W) with an amplitude of 20% for 2 min. Based on ATP measurements, after three of the five times HES, on average, 85% of the biofilm was removed from the concrete plates, with a standard error of the mean of 1% (Table 6.2). The ATP results presented in this research were based on three times HES, accomplished by mixing 25 mL of the sample from each HES treatment. The measured ATP concentrations were converted to ATP per surface area, assuming that the biofilm was equally distributed over the plate material.

Table 6.2 *The percentage of cATP removed from the concrete plates in three of five times High Energy Sonification (HES). The grey row was used as an example showing the percentage of cATP removed per HES treatment, Figure 6.4.*

Biofilm jar 1	Biofilm jar 2	Biofilm jar 3	Biofilm jar 4
97%	80%	nd	nd
87%	90%	88%	82%
96%	95%	83%	84%
90%	91%	53%	nd
37%	83%	84%	95%
80%	85%	84%	79%
83%	90%	80%	83%
85%	85%	92%	90%
nd	93%	87%	87%
87%	91%	86%	80%
90%	92%	74%	89%
87%	nd	89%	79%

nd = not determined

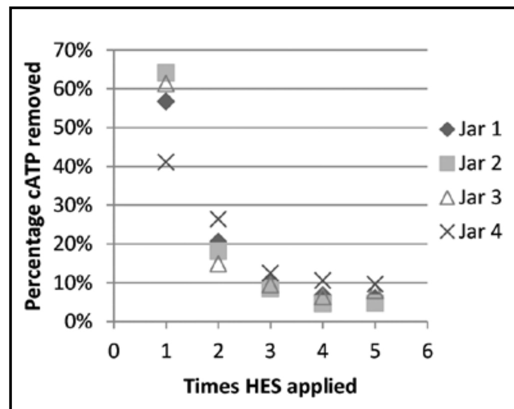


Figure 6.4 *Percentage of cATP removed every time HES was applied of the in total five times HES. This graph represents the data of the grey row in .*

6.2.6 Active biomass (ATP)

The accumulated biofilm activity on each concrete plate was determined by measuring the total ATP (tATP) and cellular ATP (cATP) of the mixed sample after sonification. All samples were measured in duplicates.

cATP was measured with the QGA™ kit of Aqua-tools (Poissy, France). To determine cATP, samples were filtered over a 0.7 µm filter to collect the microorganisms and remove the dATP. Thereafter, cells were treated with a trisodium phosphate solution to destroy the cell membrane and release the ATP. After dilution (Ultralute – ATP dilution buffer) of the extracted cATP, a luciferine/luciferase complex was added to induce the emission of light. The generated light signal (Relative Light Units, RLU) was measured with a luminometer (Junior LB 9509, Aqua-tools) within 30 s. The concentration of cATP was calculated from the RLU values using a conversion factor determined from calibration measurements performed after six analyses (EQ 6-4). The standard solution contained 1 ng ATP mL^{-1}.

$$\text{cATP} = \frac{\text{RLU}_{\text{sample}}}{\text{RLU}_{\text{standard solution}}} \times \frac{10^4}{\text{V}} \qquad \text{(EQ 6-4)}$$

Where
cATP = cellular ATP (pg mL^{-1})
RLU = Relative Light Units
V = volume (mL)

tATP was determined with the QG21WTM kit of Aqua-tools (Poissy, France). The principle is the same as the cATP measurement. However, dATP is not filtered out and the sample is directly added to a trisodium phospate solution. The concentration of tATP was calculated using EQ 6-5.

$$\text{tATP} = \frac{\text{RLU}_{\text{sample}}}{\text{RLU}_{\text{standard solution}}} \times 11 \qquad \text{(EQ 6-5)}$$

Where
tATP = total ATP (ng mL^{-1})

With the measured ATP concentration, the ATP concentration per surface area could be calculated (EQ 6-6).

$$\text{ATP (pg cm}^{-2}\text{)} = \frac{\text{ATP}\times 1000}{\frac{\text{S}}{\text{V}}\text{ratio}} \times \frac{1}{100} \qquad \text{(EQ 6-6)}$$

Where
S/V ratio = the surface/volume ratio of the plates, being $\frac{1.77\ \text{dm}^2}{0.35\ \text{dm}^3} = 5\ \text{dm}^{-1}$

6.2.7 NPOC and TN

NPOC and TN were measured to determine whether the nutrient concentrations were constant in all jars and for all experiments. NPOC was determined according to NEN-EN-1484 (1997) using a Shimadzu TOC-Vcph analyser. After acidifying with 2 M hydrochloric acid and purging with high purity air, the water samples were injected into the combustion chamber at 680 °C to oxidise all carbon into CO_2, which was subsequently detected by using infrared spectrometry.

TN was determined according to NEN-EN-12260 (2003) using a Shimadzu TNM-1 analyser connected to the Shimadzu TOC-Vcph analyser. The water samples were injected into the combustion chamber at 720 °C, where nitrogen compounds were converted into nitric oxide and subsequently exposed to ozone to induce emission of light, which was detected by a chemiluminescent detector.

6.3 Results and discussion

Biofilm disinfection (UV irradiation) and removal (brushing) were studied by determination of the presence of active biomass before and after the two different treatment methods. The relation between the penetration of UV light into the biofilm and the biofilm thickness was investigated by measuring the UV passage through a biofilm grown on UV transmitting foil. Furthermore, the activity of biofilms grown on concrete plates were determined after different treatments with (i) UV irradiation with various doses and frequencies, (ii) brushing and (iii) a combination of subsequent brushing and UV irradiation.

6.3.1 Impact of biofilm thickness on UV passage

In order to disinfect the complete biofilm, UV irradiation should penetrate through the entire biofilm to reach the microbes attached to the surface. The transmission of UV light was studied by irradiation of a biofilm cultivated on UV transmitting foil. 0.751 W m^{-2} of UV light was able to pass clean UV transmitting foil. The thickness of the developing biofilm was measured every day by microscopy on different spots on the UV transmitting foil (n >100 per day). Plotting the UV passage through the biofilm covered foil against the average biofilm thickness of all experimental days (Figure 6.5), a linear regression was found with a R^2 of 0.9892 indicating that the UV passage was linearly related to the thickness of the biofilm (EQ 6-7). Although a high correlation was found between the UV passage and biofilm thickness, it should be noted that probably also biofilm density has an impact.

$$\text{UV passage (W m}^{-2}) = -0.0071\ \text{Thickness}_{\text{Biofilm}}\ (\mu\text{m}) + 0.7389 \qquad \text{(EQ 6-7)}$$

Complete disinfection of a biofilm can only occur when the UV dose throughout the entire biofilm is sufficient, and thus when UV light could be measured below the biofilm and the UV transmitting foil. Light passing biofilms has been found in other studies too, focusing on phototrophic biofilms (Buhmann et al. 2012, Roeselers et al. 2007). Based on the linear relation between the biofilm thickness and the UV passage described in EQ 6-7, UV light could maximally penetrate a biofilm of 104 µm thick. This leads to a decrease of the UV irradiance (E0, EQ 6-1) with ~1% per 1 µm biofilm. Because of lower UV irradiance in the deeper regions of the biofilm, irradiation times should be increased to obtain a lethal dose in the deeper regions.

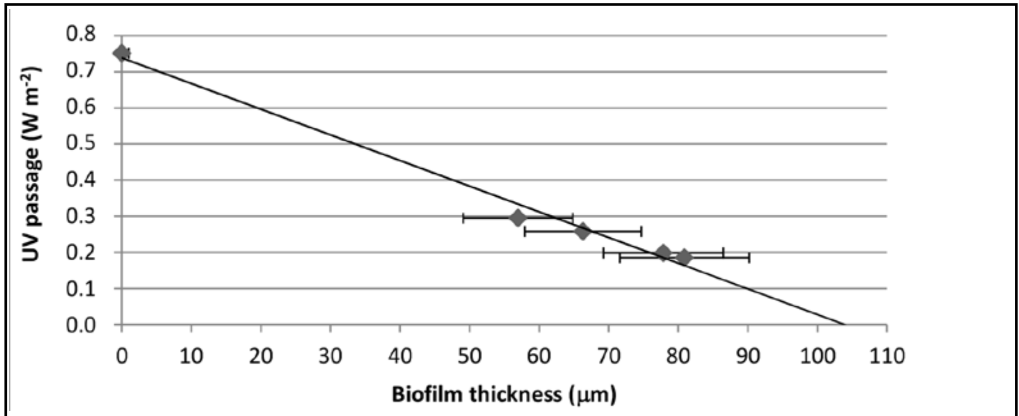


Figure 6.5 *The biofilm thickness and UV passage through the biofilm and UV transmitting foil determined per jar (average in time). The data is linear related (EQ 6-7) with a R^2 of 0.9892.*

6.3.2 Impact of UV irradiation on biofilm thickness

The effect of UV light on the biofilm thickness was determined as well. The average biofilm thickness developed during 14 days of incubation on concrete plates without any treatment was 55.1 ± 3.9 µm (standard error of the mean). After daily treatment of these biofilms with a UV dose of 1500 J m^{-2}, the average biofilm thickness was increased to 78.4 ± 5.9 µm (standard error of the mean) on day 20. This means that despite UV irradiation, the biofilm thickness increased.

Determination of the biofilm activity showed that after 14 days without treatment the tATP and cATP concentration were 16,219 ± 1,733 and 6,504 ± 992 pg ATP cm^{-2}, respectively, while at day 21 with daily UV treatment of 1500 J m^{-2} the tATP and cATP concentrations were 9,145 ± 1,176 and 5,426 ± 685 pg ATP cm^{-2}, respectively. The reduction of ATP per biofilm surface area, indicated that despite the increased biofilm thickness, part of the biofilm was inactivated during this period, suggesting that the death cells were not removed from the biofilm. Because the UV light penetration is linearly related to biofilm thickness, also the inactive cells potentially protect the microorganisms in the lower regions of the biofilm from disinfection (Chen et al. 2009, Wang et al. 2007), allowing these regions to remain active, grow and avoid detachment of the biofilm from the surface.

6.3.3 Effect of different UV doses on biofilm inactivation

In order to determine the UV dose needed to decrease bacterial activity in the biofilms, biofilms <100 µm thick were grown on concrete plates. Different UV doses between 0-1500 J m^{-2} were applied once every day from experimental day 15 to day 20, whereafter cATP and tATP measurements were used to determine the activity of the biofilm at day 21 (Figure 6.6).

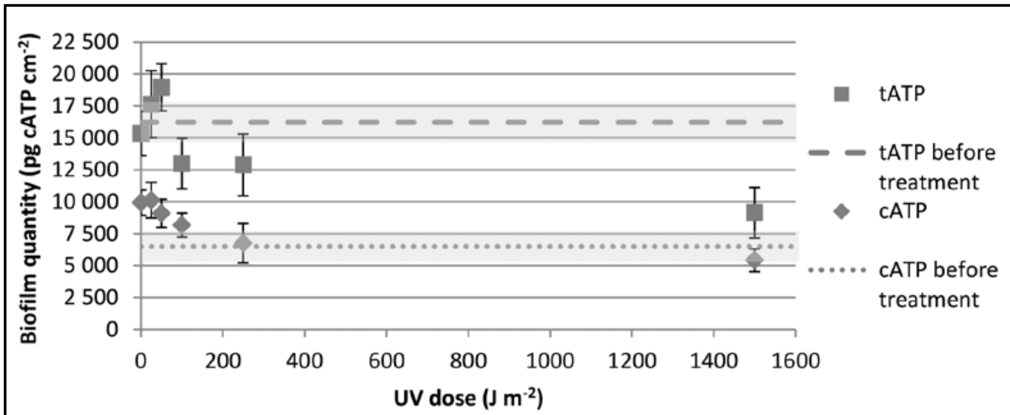


***Figure 6.6** The total ATP (tATP) and cellular ATP (cATP) content of the biofilm developed on concrete plates in 21 days after different UV treatments applied daily on day 15-20. The errorbars represent the standard error of the mean. The grey areas represent the standard error of mean of the ATP concentration before treatment on day 14 to determine whether UV irradiation could control biofilm growth.*

The average activity of the biofilm determined with cATP, showed a clear decrease with increasing UV dose starting at a UV dose of 50 J m^{-2} and higher (Figure 6.6). However, to determine whether UV irradiation stopped biofilm growth, the biofilm activity, after the different UV treatments at day 21, were compared to the biofilm activity before treatment at day 14. Based on cATP levels, daily application of 250 J m^{-2} showed similar biofilm activity at day 21 as on day 14, indicating stabilisation of the biofilm activity (Figure 6.6). A dose of 1500 J m^{-2} showed a lower biofilm activity, indicating some reduction of biofilm activity in comparison to day 14.

Based on tATP levels, the average biofilm activity at day 21 after daily application of 100 J m^{-2} showed already a reduction of biofilm activity compared to the biofilm activity at day 14, although the reduction was only significant after 1500 J m^{-2}, taking the standard error of the mean into account.

The biofilm activity reduction after a dose of 1500 J m^{-2} was 38% based on tATP levels and 17% based on cATP levels, when compared to the ATP levels after 14 days of cultivation without treatment.

These results suggest that a daily UV dose of ≥ 50 J m^{-2} lowered the cellular ATP concentration of biofilms developed on concrete plates in comparison to no UV treatment, while 250 J m^{-2} stabilised the biofilm growth and 1500 J m^{-2} reduced the active biofilm amount.

Usually, a dose of 400 J m^{-2} is used to disinfect drinking water, which is based on inactivation of enteric viruses (Shin et al. 2001) and leads to a 4-log reduction of planktonic cells based on plate counts (Hijnen et al. 2006). However, the reduction of attached biofilms under similar UV irradiation conditions was less than 1-log, based on ATP measurements (Figure 6- 6; Figure 6- 7). This confirms that biofilms are less susceptible to UV irradiation than free planktonic cells, which is in line with previous literature (Martinez and Casadevall 2007). Higher efficiencies were found when plate counts (CFU) were used to determine biofilm disinfection with UV radiation (Figure 6- 7, Bak et al. 2009, Jahid et al. 2014). Apparently, not all cells that are active are also culturable (Hammes et al. 2008, Hoefel et al. 2003), indicating that a relation between ATP and CFU does not exist (Hammes et al. 2010, Siebel et al. 2008, Venkateswaran et al. 2003). However, cells can be in a viable but non-culturable state (Oliver 2005). Because dead cells do not contain any ATP (Mason 1999), determination of the effectiveness of UV irradiation based on activity (ATP) is thus a representative method.

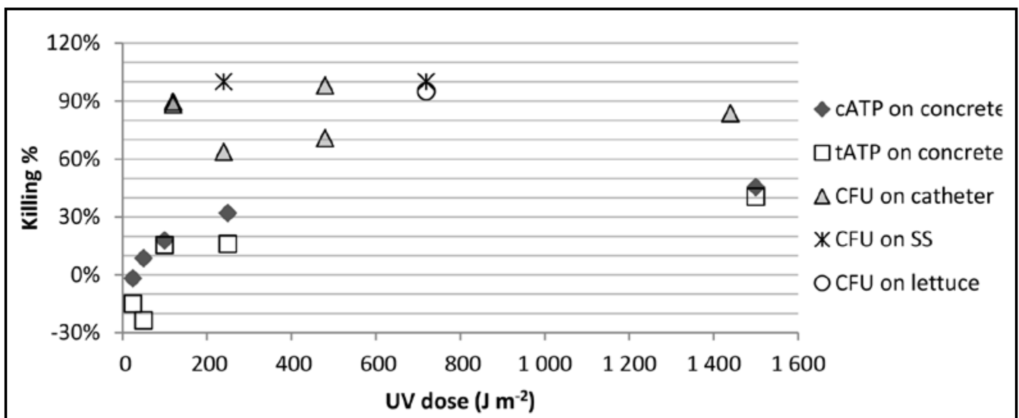


Figure 6.7 *Disinfection of biofilm determined with (i) cATP and tATP measurements on concrete plates, and (ii) with CFU measurements on catheters (Bak et al. 2009), stainless steel (SS) (Jahid et al. 2014) and lettuce (Jahid et al. 2014).*

6.3.4 Impact of frequency of UV irradiation on biofilm inactivation

The effect of the applied frequency of UV irradiation on biofilm development was determined by daily UV treatment and two UV treatments at an interval of three days (Table 6.3). Because the cATP levels decreased from a dose of 50 J m^{-2} and higher, compared to the reference at day 21 (Figure 6.6), 50 J m^{-2} was used to investigate the effect of UV application frequency. The average ATP surface concentration was about a factor two higher after daily UV irradiation than after two UV treatments (Table 6.3). A lower ATP level after application of a lower frequency of UV irradiation (Table 6.3) was not expected and cannot be explained from these results.

Table 6.3. *Average total and cellular ATP concentrations at day 21 after a UV dose of 50 J m^{-2} applied at different frequencies ± standard error of the mean.*

	tATP (pg ATP cm^{-2})	**cATP (pg ATP cm^{-2})**
Daily treatment (UV irradiation at day 15, 16, 17, 18, 19 and 20)	18,955 ± 1,976	9,093 ± 949
Treatment once in three days (UV irradiation at day 15 and 18)	9,885 ± 1,234	5,168 ± 846

6.3.5 Biofilm control by brushing and/or UV irradiation

Besides UV irradiation, also brushing was applied to control biofilm development. Treatments (brushing, 50 J m^{-2} UV and the combination of brushing followed by 50 J m^{-2} UV) were performed twice; on day 15 and 18 respectively. Results (Figure 6.8) showed that while the tATP concentration remained more or less constant between 14 and 21 days without treatment, the percentage of cATP increased in time from 40% to 65% respectively, suggesting a more active biofilm after 21 days. After brushing, the tATP and cATP contents of the biofilm were rather similar to the ATP contents after 21 days without treatment (Figure 6.8). Disinfection of the biofilms with UV light decreased the tATP concentration. For both treatments, UV irradiation and the combination of subsequent brushing and UV irradiation, the percentage of cATP decreased to 52% and 51%, respectively. The lower percentage of cATP after UV treatment indicates inactivation of the cells resulting in a less active biofilm, while brushing did not result in a lower bioactivity, unless cells would have been brushed away.

The ATP reduction enhanced by the combination treatment of subsequent brushing and UV irradiation compared to the biofilm activity after 21 d without treatment was 6,892 ± 905 and 5,619 ± 724 pg tATP cm^{-2} and cATP cm^{-2}, respectively (Figure 6.8), and indicated a reduction of 45% of tATP and 57% of cATP. This reduction was similar to the sum of the ATP reduction caused by brushing (9% of tATP and 7% of cATP) and UV irradiation (36% of tATP and 48% of cATP).

These results have shown that brushing was less efficient than UV treatment (Figure 6.8). A bigger effect of brushing was expected because brushing would remove the biofilm from the surface, such as dental plaque (Schifter et al. 1983, Stoltze and Bay 1994, Walsh and Glenwright 1984), while UV treatment with these tested doses only inactivates the organisms. Probably higher efficiencies of biofilm inactivation could be obtained with electric/sonic brushing (Khocht et al. 1992, Schifter et al. 1983, Stoltze and Bay 1994, Walsh and Glenwright 1984, Wilcoxon et al. 1991) and in combination with the application of a higher UV doses.

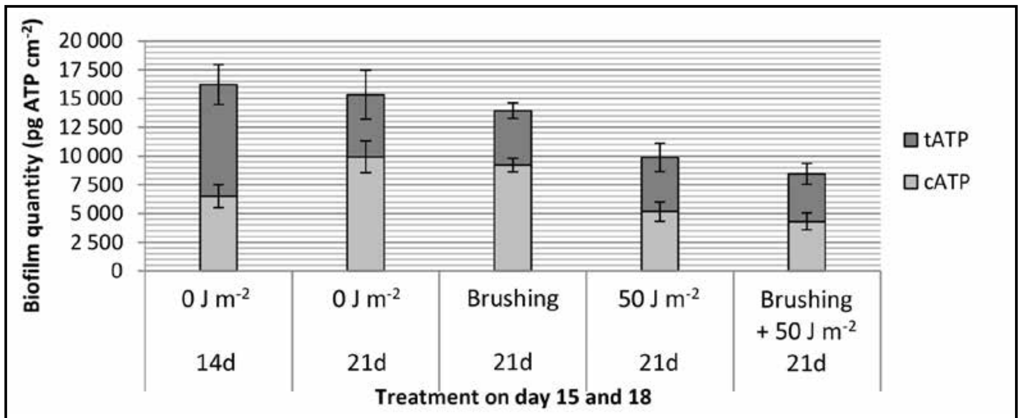
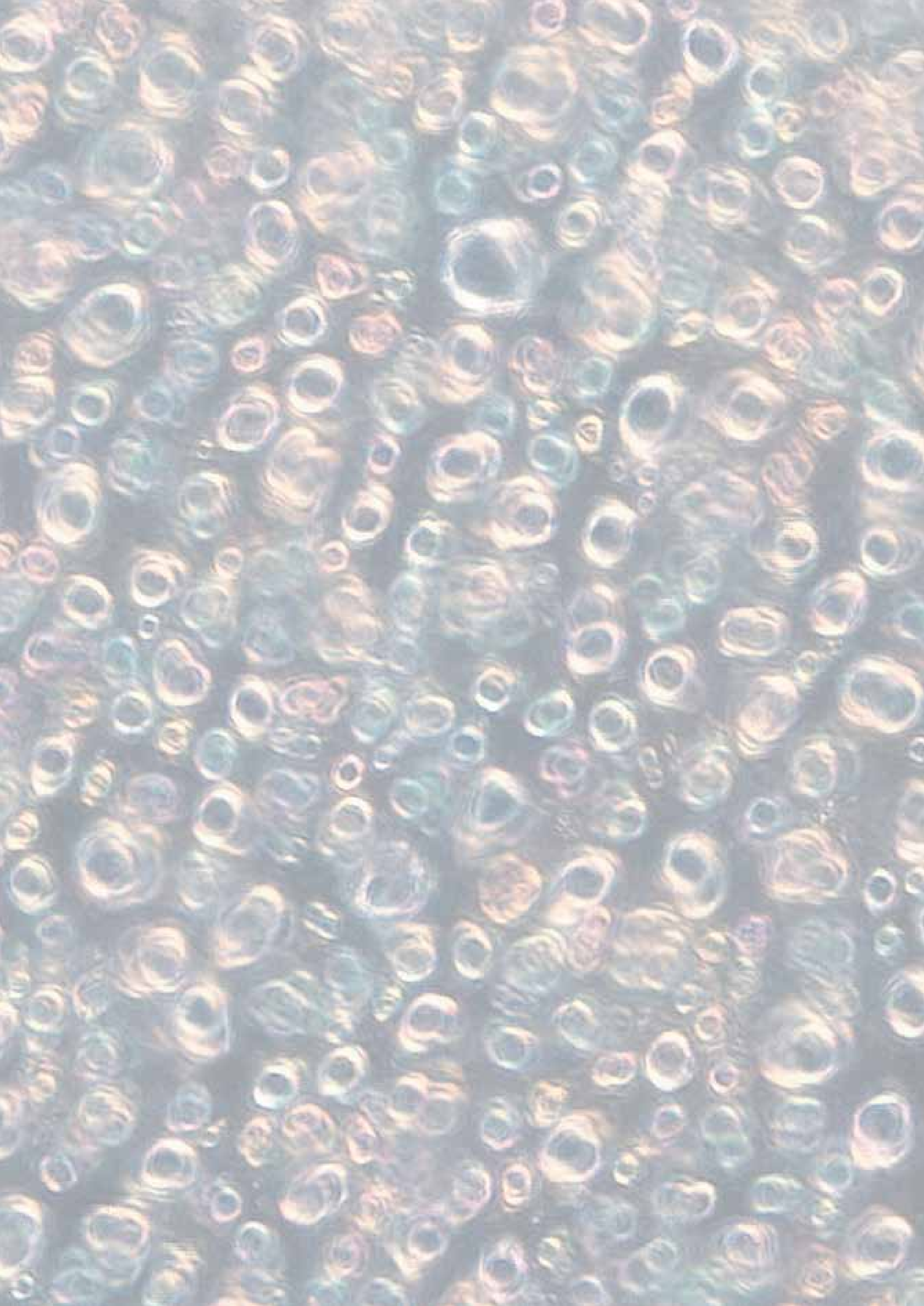
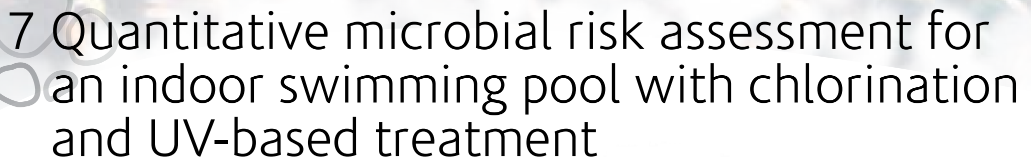


Figure 6.8 *The tATP and cATP content of the biofilm determined before treatment on day 14 and on day 21 after different treatments on day 15 and 18. The errorbars represent the standard error of the mean.*

6.4. Conclusion

The effect of UV irradiation and mechanical brushing on biofilms present in swimming pool water without residual disinfectant was studied. The log reduction is based on the reduction of the biofilm activity (ATP), which showed to be a suitable method to evaluate the efficiency of biofilm control strategies. These studies showed that (i) the full biofilm thicknesses of <100 µm applied in our studies were exposed to UV irradiation and that (ii) a UV dose of 50 J m^{-2} was found suitable to lower the biofilm growth, a dose of 250 J m^{-2} stabilized the biofilm development and a dose of 1500 J m^{-2} reduced the active biofilm amount. Finally, comparing the biofilm control strategies showed clear differences in cleaning efficiency. The biofilm reduction efficiency determined by tATP-cATP was for (i) mechanical brushing 9-7%, (ii) 50 J m^{-2} UV irradiation 36-48% and (iii) brushing followed by 50 J m^{-2} UV irradiation 45-57%.





7 Quantitative microbial risk assessment for an indoor swimming pool with chlorination and UV-based treatment

Abstract

Most swimming pools use residual disinfectants like chlorine for disinfection. The use of chlorine has several drawbacks: some waterborne-pathogens are chlorine resistant and disinfection by-products (DBPs) may be formed which are associated with various health risks. Therefore, an alternative treatment was developed which consists of biological sand filtration, coagulation, ultrafiltration and disinfection with UV-light at 400 J m^{-2}. In the presented research a quantitative microbial risk assessment was made of an indoor swimming pool with such a UV-based treatment, and of a chlorinated pool for comparison. The used reference pathogens were the bacterial enteric pathogens *Campylobacter jejuni*, *E. coli* O157:H7 and *Salmonella enterica*.

The average bacterial cell concentration during opening hours in a UV-based swimming pool were the highest for *C. jejuni* (3.1×10^{-3} cells L^{-1}) > *S. enterica* (9.5×10^{-4} cells L^{-1}) > *E. coli* (7.2×10^{-4} cells L^{-1}). These calculated pathogen concentrations were about 180 times higher than calculated pathogen concentrations in a chlorinated swimming pool in which the averaged concentration was 4.0×10^{-6} cells L^{-1} for pathogenic *E. coli* cells. The yearly risks of infection of a UV-based treated swimming pool were the highest for *C. jejuni* (1.7×10^{-3}) > *E. coli* (1.8×10^{-5}) > *S. enterica* (3.5×10^{-7}).

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7.1 Introduction

Because bathers release microorganisms during swimming (chapter 2), swimming pools are disinfected to ensure microbial safety. Most frequently used disinfectants are chlorine based products, because of their effectiveness and low costs (Shannon et al. 2008) and because of mandatory use in swimming pools in many countries. Also in the Netherlands, chlorination is mandatory and the free available chlorine concentration should be between 0.5 and 1.5 mg Cl_2 L^{-1} (Anonymous 1984). Although chlorination is widely applied as disinfection method, a considerable disadvantage is the chlorine resistance of some waterborne pathogens, such as *Cryptosporidium* and *Giardia* (Hijnen et al. 2006). Furthermore, bathers also release particles and soluble substances which could be oxidised by chlorine, resulting into a variety of disinfection by-products (DBPs) (Aggazzotti et al. 1995, Florentin et al. 2011, Richardson et al. 2010, Zwiener et al. 2007). DBPs in swimming pools are associated with various health risks, like impaired respiratory health and possibly asthma, while others may be carcinogenic (Font-Ribera et al. 2010, Glauner et al. 2005, LaKind et al. 2010). They also have potential genotoxic effects (Kogevinas et al. 2010a), or irritate the skin, eyes or the respiratory system (Eichelsdörfer et al. 1975b, Erdinger et al. 1998b). To prevent DBPs to be formed by residual disinfectants and to address chlorine resistance of some microorganisms, UV irradiation could be applied as an alternative disinfection method. An example of such alternative disinfection treatment for swimming pools does not provide for a residual in the swimming pool, and consists of a biological sand filter, coagulation, ultrafiltration and finally UV irradiation.

To determine if it is safe to apply this UV-based concept in public swimming pools, a microbial risk assessment was made. In this risk assessment, the main difference between traditional swimming pools with a residual disinfectant and the proposed concept is that the microorganisms will not be exposed to a disinfectant as soon as they are brought into the pool water. Therefore, they might survive longer in the pool water, increasing the probability of infection of (other) bathers. Although, most microorganisms are harmless, some bacteria, viruses or parasites of faecal origin may cause illnesses such as gastroenteritis (WHO 2003). Gastroenteritis is one of the most common diseases throughout the world and the relation between the presence of faecal contamination and swimming-associated gastroenteritis has been demonstrated before (Prüss 1998, Wade et al. 2003, Zmirou et al. 2003). Therefore, the focus of this microbial risk assessment was based on the presence of the enteric pathogenic bacteria: *Campylobacter jejuni*, *Escherichia coli* O157:H7 and *Salmonella enterica*, known to cause gastroenteritis. Taking into account the objective of assessing the risk of gastrointestinal infection by these pathogens, bathers should swallow pathogens to be exposed and thus the pathogen concentration, swimmer exposure and dose-response relations were investigated to determine the risk of infection during swimming.

7.2 Materials and methods

7.2.1 Bathing parameters

Swimming is a popular activity for a variety of people who visit (indoor) swimming pools for various reasons like recreation, education, exercising and rehabilitation therapy. In this microbial risk assessment, a standard indoor swimming pool was considered. The adopted size of the pool was 25 m × 10 m × 2 m, creating a pool water volume of 5×10^5 L. Based on these pool dimensions and the area needed per bather, which is 2.3 m^2 (NYCRR 2011), the maximum number of bathers in this pool was calculated to be 108. However, because reference assumptions were chosen to be averages, it was assumed that, on average, 40 people swim at the same time. This was based on the averaged assumption of 10 bathers swimming per lane, while a pool of 10 m width has four lanes.

The duration of swimming in a swimming pool is also important, because the longer bathers swim, the longer they are exposed to the microorganisms inside the pool. The duration of bathing in a swimming pool was investigated by Schets et al. (2011) and could be described by a log-normal distribution; the average swimming duration of men, women and children were 68 min, 67 min and 81 min, respectively. Another research about swimming in the Netherlands has found that 9%, 42%, 36% 11% and 1% bathers between 15-79 years went swimming for < 30 min, 30-60 min, 1-2 h, 2-4 h and > 4 h respectively (Van der Werff et al. 2013). In this risk assessment it was assumed that bathers swim on average 1 h. Assumed was that all bathers entered the pool at time t = 0 min and left the pool after 60 min.

To determine how often a Dutch person takes a swim, data of 1999, 2003 and 2007 (Van der Werff et al. 2013) was combined, which resulted in an average swim frequency of 75, 66 and 59 times per year and thus 1.4-1.1 times per week, respectively. Because a decreasing trend could be observed, in this risk assessment the last known average swimming frequency was used, assuming that bathers swim 59 times per year.

During a swim, pool water is ingested and bathers are exposed to the ingested microorganisms. Various research has been conducted to determine the volume of swimming pool water swallowed during a swim. The WHO guidelines assume that 20-50 mL of water is swallowed per hour of swimming activity (WHO 2003). Dufour et al. (2006) found that adults on average swallow 16 mL per swimming event, men 22 mL, women 12 mL and children 37 mL, which was based on cyanuric acid concentrations in urine. Schets et al. (2011) found that men, women and children ingest 34 mL, 23 mL and 51 mL during a swimming event, respectively, based on questionnaires in which the swallowed volume of water consisted of frequencies of four volume classes. The classes were determined by measuring the volume of a mouthful, which was Gamma distributed. Finally, Suppes et al. (2014) found that, on average, people swallow 13.7 mL, adults 3.5 and children 25.7 mL, which was based on cyanuric acid concentrations in urine. In this risk assessment it was assumed that a bather ingests 13.7 mL per swimming event.

7.2.2 Microorganism release in swimming pools

According to Soller et al. (2010), approximately 97% of all non-foodborne illnesses from known (potentially waterborne) pathogens in the US are caused by *Norovirus*, *rotavirus*, *adenovirus*, *Cryptosporidium* spp., *Giardia lamblia*, *Campylobacter jejuni*, *Salmonella enterica*, and *E. coli* O157:H7 (calculated based on data from Mead et al. (1999)). Use of reference pathogens is an accepted practice in the field of quantitative microbial risk assessment (Soller et al. 2006, Soller et al. 2003, WHO 2004). Taking into account the objective of determining the risk on gastroenteritis, bathers should swallow pathogens to be exposed. This risk assessment focused on the bacterial pathogens only and, therefore, the bacterial pathogen indicators *Campylobacter jejuni*, *E. coli* O157:H7 and *Salmonella enterica* were used. It was assumed that the probability of infection from any of the pathogens was independent of others potentially present: co-infection from more than one pathogen was not considered.

Determination of the presence of pathogenic *C. jejuni*, *E. coli* and *S. enterica* in swimming pools was based on literature about gastroenteritis. While in the Netherlands the standardized overall incidence of gastroenteritis was 283 per 1000 person-years in 1999 (de Wit et al. 2001), earlier estimations showed 447 per 1000 person-years (de Wit et al. 2000). Furthermore, in England the overall incidence was lower, 190 per 1000 person-years between 1993-1995 (Wheeler et al. 1999), and in the United States the incidence of gastroenteritis was higher, 790 per 1000 person-years (Mead et al. 1999). A small proportion of 2% of the Dutch cases was caused by bacterial pathogens (de Wit et al. 2001, Mead et al. 1999, Tompkins et al. 1999), with *Campylobacter* (1.3%) as the most prominent causative agent (de Wit et al. 2001). The percentage of positive gastroenteritis cases for *E. coli* (VTEC) and *Salmonella* species were found to be 0.3% and 0.4% respectively (de Wit et al. 2001). To estimate the chance that a Dutch bather carries bacterial pathogens into the swimming pool, the probability that a Dutch person was ill (283/1000) was multiplied with the percentage of infection caused by the specific bacteria, assuming that an infected person always sheds pathogens.

To calculate how many *C. jejuni*, pathogenic *E. coli* and *S. enterica* are released during swimming, first the total microorganism release was determined. As Keuten et al. (2012) described, three steps can be discriminated in the release of anthropogenic pollutants, including microorganisms from skin and body cavities, in swimming pools: by initial, continuous and incidental anthropogenic pollutant release. The initial anthropogenic pollutant release is introduced into the pool water during the first minutes of body contact with the water and can be simulated with standardized shower experiments. The continuous anthropogenic pollutant release is continuously produced during swimming activities and is assumed to mainly consist of sweat, hair and skin cells, but feacal bacteria might be washed off from human bodies as well. The incidental anthropogenic pollutant release is the result of human excreta such as urine, vomit or faecal material entering the pool water, either accidentally or on purpose. In this risk assessment it was assumed that all bathers showered before entering the swimming pool and behave hygienically so initial and incidental anthropogenic pollutant release were excluded and only the continuous anthropogenic pollutant release was simulated. Furthermore, it was assumed that the microorganisms present in an indoor swimming pool were only introduced by bathers. The continuous anthropogenic pollutant release has been researched during submerged exercises under laboratory conditions (chapter 2). The number of intact skin cells released per body was measured every 5 min until 30 min of exercise and resulted in an intact cell release rate which decreased in time. These released cell numbers were taken into account and extrapolated from 30 to 60 min in this research, Table 7.1.

After the total microbial release was quantified, the release should be identified to determine whether faecal bacteria are released. Because the composition of a continuous anthropogenic microbial community of bathers (AMCB) is unknown, the characterization results of an initial AMCB were used (chapter 3). 9% of the bacterial population was identified as *Enterobacteriaceae* and thus 9% of the shedded cells could be faecally related. Although, no pathogens were identified in the initial AMCB, this percentage could contain faecal pathogens which might result in gastroenteritis.

In order to estimate how many faecal pathogens are shed during swimming, the known pathogen concentration in faeces was taken into account. Therefore, the number of continual released faecal bacteria was translated to grams of released feaces by dividing the calculated concentration of faecal *Enterobacteriaceae* by the number of faecal coliform bacteria found in faeces according to Gerba (2000). Feacal coliform bacteria occur in the faeces of all humans at fairly constant concentrations (Gerba 2000) of $10^{7.4}$ faecal coliform bacteria per gram faeces (Feachem et al. 1983). Multiplication of the continual released mass of faeces by the concentration of pathogens in faeces, the pathogen release could be calculated from an infected person. However, most literature on *Campylobacter*, *E. coli* or *Salmonella* has only determined whether a pathogen was present in faeces and not in which concentration. Fukushima and Tsunomori (2005) quantified the concentration of diarrheagenic *E. coli* in faeces which was found to be between 10^4-10^8 bacteria. However, Feachem et al. (1983) found that infected persons excrete 10^8 EIEC and 10^8-10^9 ETEC bacteria per g faeces, while the excreting time is typically 3-5 days. Furthermore they found that *C. jejuni* infected persons with diarrhea excrete 10^6-10^9 bacteria per g faeces for, on average, 15 days, while *Salmonella* infected persons would excrete between 2.5×10^5-1.0×10^9 bacteria per gram faeces of which 5-10% of the infected persons excrete still *Salmonella* after 2 months. Based on the findings reported by Feachem et al. (1983), it was assumed that an infected bather sheds 10^8 bacteria per g faeces. The total pathogen release was calculated with EQ 7-1.

$$\text{pathogen release} = \frac{\textit{Enterobacteriaceae}\ \text{release}}{\text{coliforms per g faeces}} \times [\text{pathogen}]\ \text{in faeces} \times \text{infection probability} \times \text{pathogen presence} \times \#\ \text{bathers} \qquad \text{(EQ 7-1)}$$

Where

Enterobacteria release = the intact cell microorganism release per bather in time as determined in chapter 2, multiplied with 9% of possible faecally related bacteria (*Enterobacteriaceae*) according to chapter 3

Coliforms per g faeces = faecal coliform bacteria present per g faeces (Feachem et al. 1983)

[pathogen] in faeces = the concentration of pathogens per gram faeces

Infection probability = probability being ill (283/1000 according to de Wit et al. 2001)

Pathogen presence = the percentage of the specific pathogen causing gastroenteritis (Soller et al. 2010)

bathers = number of bathers in the pool

Table 7.1 *Assumptions used to calculate the risk of infection.*

Parameter	Value	
Pool volume	5×10^5 L (based on the pool dimensions 25×10×2 m)	
Average number of bathers	40	
Swimming time	1 h	
Number of swimming events	59 times per year	
Ingested pool water	13.7 mL	
Cumulative microorganism release over time	*Time (min)*	*Intact cells released*
	0	0
	5	2964
	10	5697
	15	7143
	20	8463
	25	8904
	30	9304
	40	9603 (based on extrapolation)
	50	9753 (based on extrapolation)
	60	9791 (based on extrapolation)
% *Enterobacteriaceae* released by bathers	9%	
Number of faecally coliform bacteria per g faeces	$10^{7.4}$	
Number of pathogens per g faeces	10^8	
Probability an ill bather releases pathogens in the pool	Change of being ill 283/1000	
Pathogen presence in positive gastroenteritis cases	*Campylobacter jejuni*	1.3%
	Escherichia coli O157:H7	0.3%
	Salmonella enterica	0.4%
UV-based treatment efficiency	5-log	
Recirculation time (time needed to treat all pool water)	240 min	
Dose response model	*Campylobacter jejuni* (Hypergeometric)	α=0.024 / β=0.011
	Escherichia coli O157:H7 (Beta-Poisson)	α=0.4 / β=45.9
	Salmonella enterica (Beta-Poisson)	α=0.3126 / β=2884

7.2.3 Pathogen removal

Once the pathogens are released in the pool, they will be removed when the water is recirculated through the treatment. The UV-based swimming pool treatment consists of a biological sand filter, coagulation, ultrafiltration and disinfection with UV-light at 400 J m^{-2}. To determine the overall treatment efficiency, the log removal of the disinfection techniques were taken into account. The log removal of a sand filter is usually 1-2 log, for ultrafiltration log reductions for these bacteria are normally at least 5.5-log (AWWA 2005), while a 4-log removal by UV irradiation of these bacteria could already be achieved at lower doses than the 400 J m^{-2} applied (Hijnen et al. 2006). Pilot scale experiments with a UV-based treatment showed that overall only a 1.3-log reduction was measured due to detection limits (data not shown). Because the high theoretical log reductions were not observed during practical experiments, the average of a 5-log removal was used in this risk assessment, taking into account possible failure of one of the treatments as well.

Besides the treatment efficiency, also the recirculation time is important to remove pathogens. The time needed to treat all of the swimming pool water was set on 4 h, based on the rule of thumb used in private swimming pools (Ref 1-4). This is more often than for regular public chlorinated swimming pools in which the entire volume should be recirculated in maximum 6 h (NYCRR 2011).

The total removal of pathogens was calculated with EQ 7-2.

$$\text{pathogen removal} = [\text{pathogen}] \times \text{treatment efficiency} \times \frac{V_{pool}}{\text{recirculation time}} \times \Delta t \qquad \text{(EQ 7-2)}$$

Where
[pathogen] = concentration of pathogens in cells L^{-1} in the pool, calculated by EQ 7-3.
Treatment efficiency = efficiency of the complete treatment
V_{pool} = pool volume in L
Recirculation time = time to treat the complete pool water
Δt = time (1 min) used to calculate the [pathogen] during the 1 h of exposure

$$[\text{pathogen}] = \frac{\text{pathogen release} + \text{pathogen removal}}{V_{pool}} \qquad \text{(EQ 7-3)}$$

Pathogen removal is in a chlorinated swimming pool less of importance because a residual disinfectant is present which reacts with the pathogens inside the pool instead of during treatment. Therefore, modelling the efficiency of a chlorinated swimming pool was based on the disinfection capacity of the residual disinfectant only

Although in chapter 4 the effect of chlorination on an initial anthropogenic community was found to be about a 2-log reduction, according to literature the log reductions of these pathogens are higher. Borgmann-Strahsen (2003) found that under swimming pool conditions more than a 4-log reduction was observed for *E. coli*. Because *E. coli* was found to be a suitable indicator organism for *C. jejuni* (Lund 1996), it might be assumed that also *C. jejuni* will achieve at least a 4-log reduction. However, generally a log reduction of > 5-log CFU L^{-1} of pathogens can be achieved with 0.5-1.0 mg L^{-1} free available chlorine during a

minimum of 30 s exposure time (Luo et al. 2011, Shen et al. 2013, Van Haute et al. 2013), also for the chlorinated swimming pool a disinfection efficiency of 5-log was chosen.

7.2.4 Dose response

After the concentration of pathogens in time was determined by EQ 7-3, the exposure to pathogens in pool water was assumed to be the average concentration to which bathers were exposed during their swimming event of 1 h.

The dose-response relationship for *E. coli* and *S. enterica* can be simulated with a beta-Poisson distribution (EQ 7-4), and the α and β parameters are α=0.4 / β=45.9 for *E. coli* O157:H7 (Soller et al. 2010) and α=0.3126 / β=2884 for *Salmonella enterica* (Soller et al. 2010).

$$P(\text{inf}) = 1 - \left(1 + \frac{\text{dose}}{\beta}\right)^{-\alpha} \qquad \text{(EQ 7-4)}$$

Where
Dose = concentration of pathogens in the pool
α and β = constants specified for each microorganism, Table 7.1.

For *C. jejuni* the beta-poisson approximation cannot be used for low cell concentrations since the probability of illness is limited by the probability of infection (Teunis et al. 2005). Therefore, the probability of infection from *C. jejuni* was estimated using the beta-Poisson dose-response distribution in the form of the Kummer confluent hypergeometric function 1F1, EQ 7-5. α=0.024 and β=0.011 for *Campylobacter jejuni* (Soller et al. 2010).

$$P(\text{inf}) = 1 -{}_1 F_1(\alpha, \alpha + \beta, -\text{dose}) = 1 - \sum_{n=0}^{\infty} \frac{(\alpha)_n}{(\alpha+\beta)_n} \frac{(-\text{dose})^n}{n!} \qquad \text{(EQ 7-5)}$$

Calculations were done with the online tool at functions.wolfram.com, in which the Hypergeometric1F1[a,b,z] function was used. The parameters a, b and z were respectively α, (α+β) and (–dose) as described in EQ 7-5.

7.2.5 Risk characterization

The yearly risk of infection was calculated with EQ 7-6, separately for each of the bacterial pathogens.

$$P(\text{yearly infection}) = 1 - (1 - P(\text{inf}))^{\text{Yearly swimming events}} \qquad \text{(EQ 7-6)}$$

7.2.6 Sensitivity analysis

To determine the sensitivity of the risk outcome to the variability or uncertainty of the input parameters, a normal range sensitivity analysis was performed. The reference and worst case values of each of the parameter values were taken into account. The sensitivity ratio was based on the multiplication factors between the maximum risks of infection and values and the reference risks of infection and values.

7.3 Results and discussion

7.3.1 Risks of infection in a swimming pool with UV-based water treatment

A quantitative microbial risk assessment was made for a swimming pool with a UV-based treatment, consisting of a biological sand filter, coagulation, ultrafiltration and disinfection with UV irradiation. To calculate the yearly risk of infection caused by *C. jejuni*, *E. coli* or *S. enterica* by ingestion, different assumptions were made as described in the Materials and Methods section (Table 7.1). With these reference assumptions the pathogenic cell concentration in a pool during the exposure time was calculated (Figure 7.1). The increase of the concentration of pathogenic cells was caused by the cell release from bathers in time (chapter 2). However, from 20 min onwards, the pathogenic cell concentration decreased as a result of (i) the number of cells released per body became less in time and (ii) disinfection of the cells, which is related to the recirculation time and treatment efficiency. Overall the average calculated cell concentration during the first hour of exposure time was 7.5×10^{-4}, 1.7×10^{-4} and 2.3×10^{-4} cells L^{-1} for *C. jejuni*, *E. coli* and *S. enterica,* respectively, based on the reference assumptions (Table 7.1). With this cell concentrations, the dose, risk of infection and finally the yearly risk of infection could be calculated with EQ 4, 5, and 6, of which the outcome is given in Table 7.2. The yearly risk of infection was thus calculated to be 4.2×10^{-4}, 4.3×10^{-6} and 8.5×10^{-8} for *C. jejuni*, *E. coli* and *S. enterica,* respectively.

Table 7.2 *Results of the calculated risk assessment of a swimming pool with UV-based treatment.*

	C. jejuni	***E. coli***	***S. enterica***
Pathogen concentration (cells L^{-1})	7.5×10^{-5}	1.7×10^{-4}	2.3×10^{-4}
Dose (# cells)	1.0×10^{-5}	2.4×10^{-6}	3.2×10^{-6}
P(inf)	7.1×10^{-6}	7.3×10^{-8}	1.4×10^{-9}
P(yearly infection)	4.2×10^{-4}	4.3×10^{-6}	8.5×10^{-8}

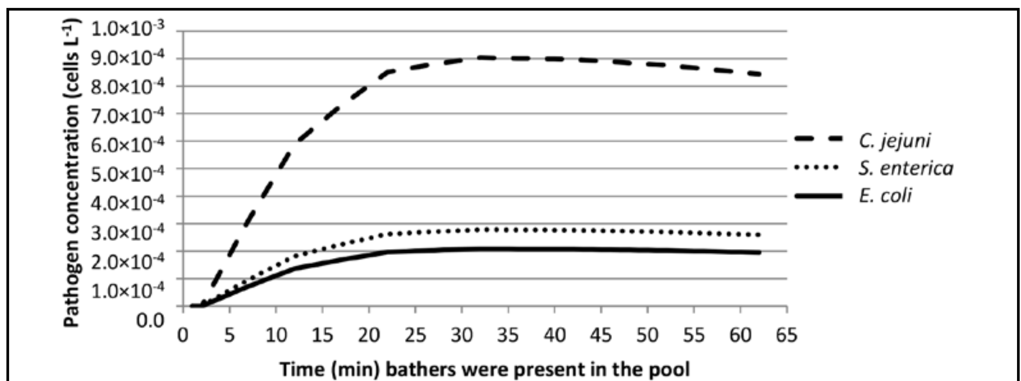


Figure 7.1 *The concentration of pathogenic bacteria in a swimming pool with UV-based treatment, including biological sand filter, coagulation, ultrafiltration and UV irradiation, calculated according to the assumptions made in Table 7.1.*

7.3.2 Sensitivity analysis

To determine the sensitivity of the risk outcome to the variability or uncertainty of the input parameters, a normal range sensitivity analysis was performed. A few parameters were assumed to be constant and were not varied in the sensitivity analysis: pool volume, microorganisms released in time, percentage of *Enterobacteriaceae*, the dose-response model parameters and the swimming time (Table 7.1). The other parameters influenced the concentration of pathogenic cells in the pool water, or the probability of exposure to pathogens via swimming. Since most of those parameters were similar for *C. jejuni*, *E. coli* and *S. enterica*, the influence of them was only varied for *E. coli*.

Number of bathers releasing pathogens: This was related to the number of bathers present in the pool and the chance that they release pathogens. Increasing the number of infected bathers from 40 to 108, the maximum bathing capacity of this pool, caused a linear increase of the risk of infection and was the most influencing parameter on the yearly risk of infection, Table 7.3.

Swimming events: The average number of swimming events per person was simulated to be 59 times per year. However, a fanatic swimmer exercises more than 1.1 times per week. Assuming a person would every working day go for a swim of one hour, the yearly risk of infection increased linearly. Also the number of swimming events had an impact on the yearly risk of infection, Table 7.3.

Ingestion volume: The yearly risk of infection increased linearly with increasing ingestion volume. Generally the ingestion volume is between 3.5 mL (adults Suppes et al. 2014) and 51 mL (children Schets et al. 2011), Table 7.3.

Pathogen concentration in faeces: The concentration of pathogens excreted via faeces is linearly related to the yearly risk of infection. Increasing the pathogen concentration with 2-log from 10^8 to 10^{10} bacteria per g faeces, resulted in a 2-log increase of the yearly risk of infection. Table 7.3.

Presence of pathogen in gastroenteritis cases: Also the pathogen presence in positive gastroenteritis cases was varied. Again, an increase of pathogen presence resulted in a linear increase of the risk of infection by that pathogen. The presence of *E. coli* in percentages influenced the yearly risk of infection, Table 7.3.

Treatment efficiency: In this risk assessment was assumed that the overall UV-based treatment reduces the bacteria by a 5-log reduction. However, the minimum log reduction of both ultrafiltration and UV irradiation is 4-log (AWWA 2005, Hijnen et al. 2006) and the overall treatment efficiency is thus probably higher. Assuming a worst case scenario of a 1-log reduction, the yearly risk of infection was calculated to be 4.330×10^{-6}, which decrease to 4.291×10^{-6} at a 2-log reduction and 4.287×10^{-6} from a log reduction of 3-log onwards, Table 7.3. However, the influence of the treatment efficiency is related to the recirculation time and should therefore be higher when recirculation times are shorter.

Recirculation time: Observed was that the yearly risk of infection logarithmically increased with increasing recirculation time. In this research the recirculation time was set on 240 min. Varying the recirculation time showed that from 210 min (3.5 h) onwards, the differences between the yearly risk of infection were less than 2% difference every 30 min, Table 7.3.

The simulated yearly risk of infection was thus most sensitive for the number of bathers releasing pathogens.

***Table 7.3** Sensitivity analysis for E. coli.*

	Reference assumption		Worst case		Value Max / Value Ref.	P(inf) Max / P(inf) Ref.	P(inf) / Value
	Parameter Value	P(inf)	Parameter value	P(inf)			
Recirculation time (min)	240	4.3×10^{-6}	360	4.3×10^{-6}	1.5	1.0	0.7
Treatment efficiency	5-log	4.3×10^{-6}	1-log	4.3×10^{-6}	0.9	1.0	1.1
Number of swimming events per year	59	4.3×10^{-6}	260 (5× per week)	1.9×10^{-5}	4.4	4.4	1.0
Number of infected bath-ers releasing pathogens		4.3×10^{-6}		1.2×10^{-5}	27.0	2.7	0.1
Ingested pool water (mL)	13.7	4.3×10^{-6}	51	1.6×10^{-5}	3.7	3.7	1.0
Presence of pathogen in positive gastro-enteritis cases	0.3%	4.3×10^{-6}	100%	1.4×10^{-3}	333.3	333.1	1.0
Pathogen concentration per g faeces	10^{8}	4.3×10^{-6}	10^{10}	4.3×10^{-4}	100.0	99.6	1.0

7.3.3 Moment of exposure

Another parameter that should be taken into account is the moment of exposure. In the Netherlands, swimming pools are generally open for public between 7:00 – 22:00 h. In this risk assessment, the risk of infection was only determined during the first opening hour, assuming 0 pathogens were present in the pool at time t = 0 min. However, as the pathogens concentration was increased in time, the pathogen concentration after one hour was higher than 0 cells L^{-1} (Figure 7.1) and therefore, bathers entering the pool after the first hour might suffer from higher pathogen concentrations and thus from higher risks of infection.

In order to determine this effect, the pathogen concentration during 20 h was simulated for *E. coli* (Figure 7.2). Every time bathers entered the pool, similar patterns were observed during their exposure time. Furthermore, because the pathogen concentration did not decrease until 0 cells L^{-1}, the average pathogen concentration increased every hour from 1.7×10^{-4} cells L^{-1} during the first opening hour (7:00-8:00), until 9.2×10^{-4} cells L^{-1} during the last opening hour (21:00-22:00h). After the pool was closed (22:00 h) and no new bathers entered the pool, the pathogen concentration decreased until 9.8×10^{-5} cells L^{-1}, where after the pool was opened again (7:00 h). Since the average cell concentration during a swimming

event increased in time, the yearly risk of infection increased as well; from 4.3×10^{-6} during the first opening hour to 2.3×10^{-5} during the last opening hour.

Taking this variation into account, the averaged pathogen concentrations over the day, during opening hours, should be used to calculated the yearly risk of infection, instead of the pathogen concentration during the first opening hour. The average calculated cell concentration during opening hours was 3.1×10^{-3}, 7.2×10^{-4} and 9.5×10^{-4} cells L^{-1} for *C. jejuni*, *E. coli* and *S. enterica,* respectively. The corresponding yearly risk of infections were calculated to be 1.7×10^{-3}, 1.8×10^{-5} and 3.5×10^{-7} for *C. jejuni*, *E. coli* and *S. enterica,* respectively.

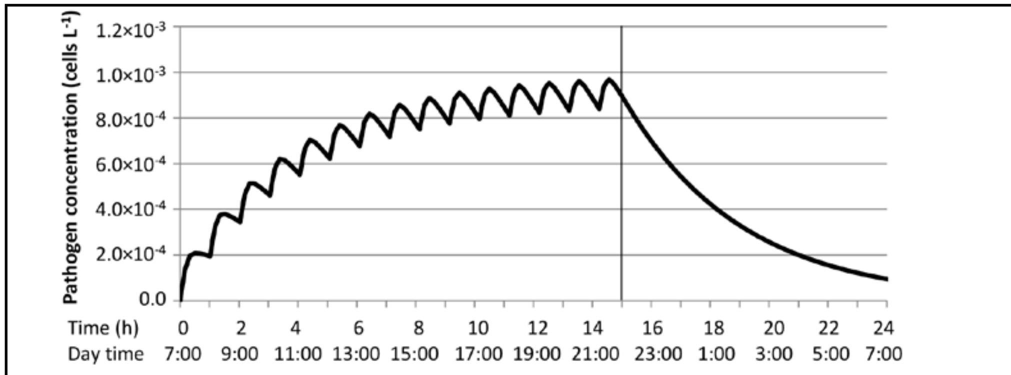


Figure 7.2 *The concentration of E. coli calculated during the day in a UV-based treated swimming pool, following the assumptions made in Table 7.1. At 0 min (7:00 h), the swimming pool was opened and 40 bathers entered the pool to swim for 1 h. Every hour 40 bathers entered the pool till 22:00 h, the assumed closing time. From this time on, the concentration of pathogenic cells decreased until the pool was opened again.*

7.3.4 UV-based treatment versus chlorinated swimming pools

To be able to compare the microbiological risks of a UV-based treated swimming pool with a chlorinated swimming pool, similar conditions were used as described in Table 7.1, except for the treatment part. In a chlorinated swimming pool, a residual disinfectant is present resulting in disinfection inside the pool instead of during treatment. Therefore, modelling the efficiency of a chlorinated swimming pool was based on the disinfection capacity of the residual disinfectant only. Assuming a 5-log reduction in time, inactivation of the pathogenic bacteria was calculated by multiplication of the pathogen cell concentration (cells L^{-1}) with the pool volume (L^{-1}) and the log reduction.

The pathogenic *E. coli* concentration was calculated and plotted during the day (Figure 7.3). Observed was that the cell concentrations in a chlorinated pool were similar for every opening hour during the day and on average 4.0×10^{-6}, while the averaged cell concentration during the day in a UV-based treated swimming pool was a factor of 180 higher (7.2×10^{-4}). The effect of direct disinfection by an residual disinfectant as soon as the microorganisms are released into the pool water was thus calculated to be more effective compared to the UV-based treatment where cells are only inactivated when they pass through the treatment. Based on the average pathogen concentration during opening hours, the yearly risk of infection was calculated to be 9.8×10^{-8} for the chlorinated swimming pool and 1.8×10^{-5} for the UV-based swimming pool treatment. Although the yearly risk of infection was lower for

chlorinated pools, in this model the cells in the chlorinated pool were only inactivated and not removed. Removal should be done in the treatment which, in a standard chlorinated swimming pool, consists of a sand filter, pH correction and chlorination. Based on a pilot study, the removal efficiency in such a treatment, were found to be lower (0.3-log) than removal by the UV-based treatment (1.3-log), although higher log reductions were difficult to measure because of detection limits (Keuten et al. in preparation).

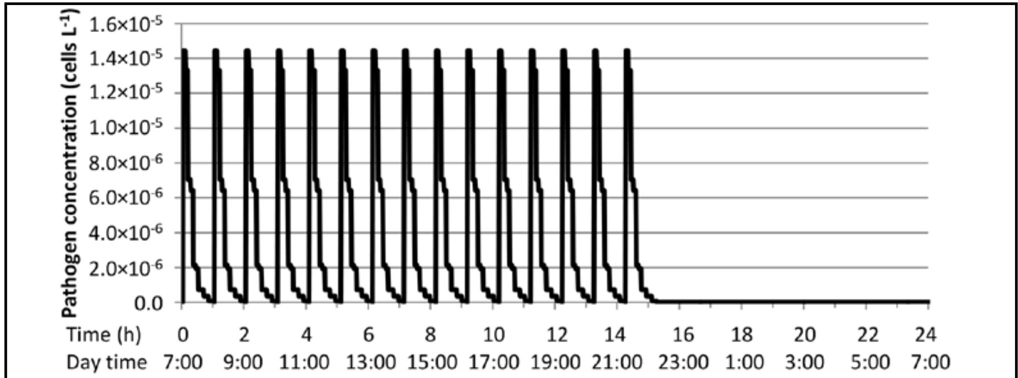
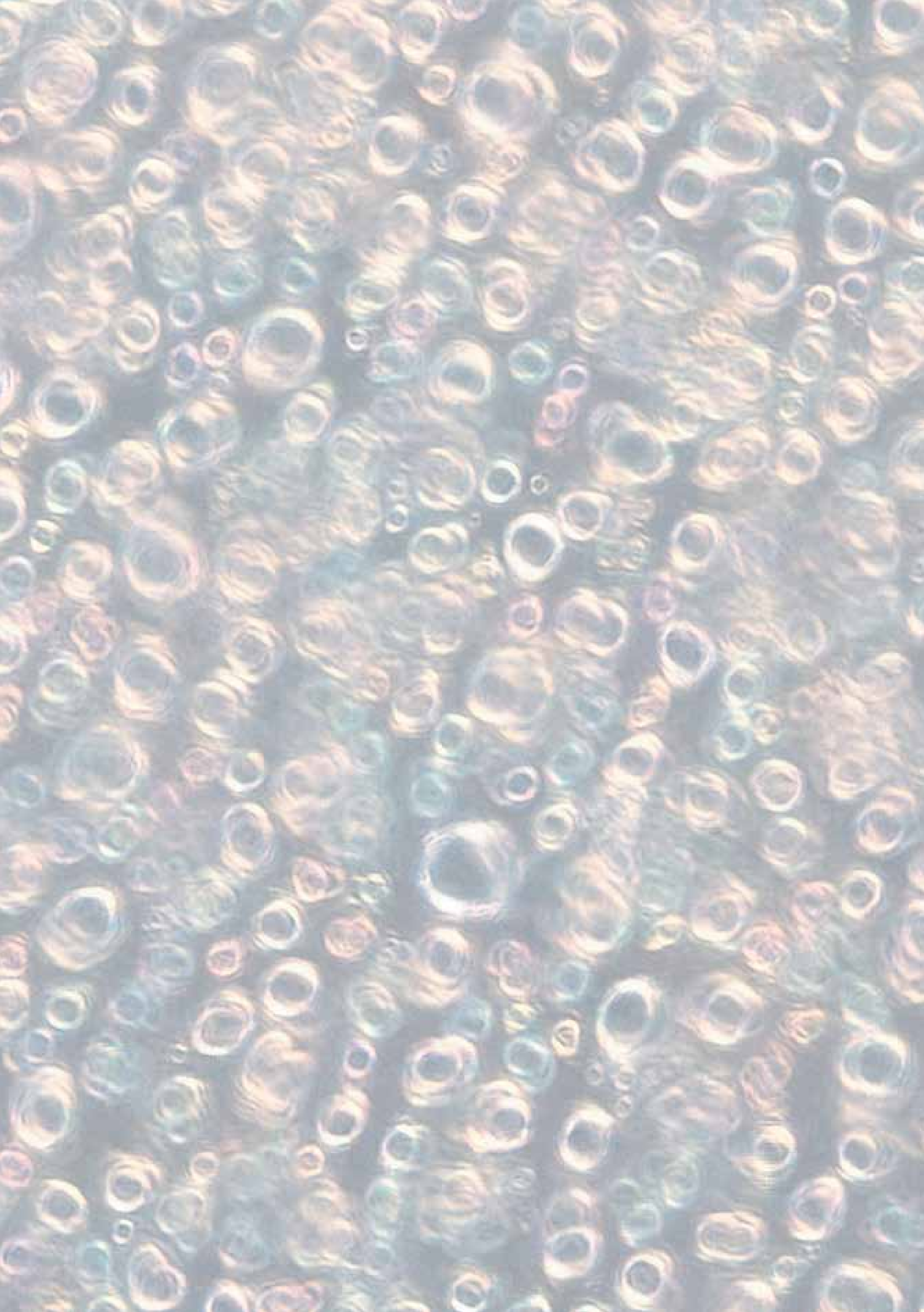


Figure 7.3 *The concentration of E. coli calculated during the day in a chlorinated swimming pool.*

7.4 Conclusion

In this research a microbial risk assessment was made for an indoor swimming pool with chlorination and a UV-based treatment without use of a residual disinfectant, but with use of a biological sand filter, coagulation, ultrafiltration and UV irradiation. Overall the calculated pathogen concentrations, averaged during opening hours, in a UV-based swimming pool were 3.1×10^{-3}, 7.2×10^{-4} and 9.5×10^{-4} cells L^{-1} for *C. jejuni*, *E. coli* and *S. enterica* respectively, whereas the yearly risks of infection were 1.7×10^{-3}, 1.8×10^{-5} and 3.5×10^{-7} respectively. Comparing these results to a chlorinated swimming pool, the pathogenic *E. coli* concentrations and risk were a factor of 180 lower, 4.0×10^{-6} and 9.8×10^{-8} respectively. Still, the yearly risks of infection of *E. coli* and *S. enterica* in a UV-based treated swimming pool were lower than the drinking water guidelines (10^{-4}). Besides, the advantage of the UV-based treatment over chlorination is not microbiologically but chemically related, since no chlorinated DBPs are formed which might affect human health. Therefore, in future research a complete health risk assessment is recommended balancing both microbiological and chemical aspects.



8 General conclusions

This thesis focused on the microbiology in a swimming pool, studying how many microorganisms and which bacteria are released by bathers and how such an anthropogenic community responds to chlorination and UV irradiation. Besides, microorganisms which survived the pool water disinfection might start growing in biofilms and since there is no residual disinfectant present in a UV-based treated swimming pool, biofouling is more likely to occur than in a chlorinated swimming pool. To prevent any suspended or attached pathogens remaining in the pool, the probability of biofilm formation should be as low as possible and controlled. Therefore, it should be investigated which swimming pool materials have a low biofouling potential as well as how biofilms can be removed without any chemical disinfectants present in a UV-based treated swimming pool. Finally, taking all this into account, a quantitative microbial risk assessment should be made to determine what the yearly risks of infection are when bathers swim in a UV-based treated swimming pool.

Conclusions are drawn by dividing this research into three subjects:

1. The anthropogenic microbial community of bathers
2. Biofouling
3. Quantitative microbial risk assesment

In this chapter, the findings of every individual chapter ware placed in a broader perspective for every subject. This will lead to a final conclusion at the end of this chapter.

8.1 The anthropogenic microbial community of bathers

In order to know whether the anthropogenic microbial community released by bathers (AMCB) in swimming pools has a health impact, (i) the number of microorganisms released, (ii) which microorganisms are released and (iii) (iii) their disinfection efficiency was investigated. The release of microorganisms during a swimming activity can be described as the initial, continuous, and incidental anthropogenic pollutant release. The initial anthropogenic pollutant release is introduced into the pool water during the first minutes of body contact with the water and consists of the residue of evaporated sweat, microorganisms and pollutants on the swimmer's skin, as well as any cosmetics on the skin. The initial anthropogenic pollutant release has been quantified by the use of standardized shower experiments. The continuous anthropogenic pollutant release is continuously produced during swimming activities and has been assumed to mainly consist of sweat and skin cells. The incidental anthropogenic pollutant release however, is the result of human excreta such as urine, vomit or faecal material entering the pool water, either accidentally or on purpose. The anthropogenic pollutant release was influenced by the temperature and level of exercise, although the particle and intact cell release was less influenced by these parameters. The release of the microbial components (cATP and intact cells) was observed to drop over time. Whereas in the first 10 min about 61% of the intact cells were released, between 10-20 min 30% was released and between 20-30 min only 9%. Nevertheless, the release of microbiological components was assumed to continue after 30 min of exercise, but at a reduced rate. Although decreasing the temperature below 29 °C reduced total continual anthropogenic pollutant release (sweat), the release of microorganisms will continue. Therefore, the only way to lower the released microorganisms in the pool is to limit the number of bathers.

The initial AMCB was used to determine which microorganisms were released during a swimming activity. More than 99% of the characterised bacteria were found to be Gram-negative. However, it was expected that an initial AMCB would contain mainly skin bacteria, which are mostly Gram-positive. This suggests that Gram-negative bacteria are more easily removed by rinsing the body than Gram-positive bacteria. This would also suggest that in swimming pools, Gram-negative bacteria are dominant over Gram-positive bacteria, although it is unknown if the supposedly more chlorine resistant Gram-positive bacteria are released in a later stage of swimming. Characterisation of a continual anthropogenic community released during a swimming activity is therefore recommended as future research.

Characterisation of the total initial AMCB showed that the bacterial families with a relative abundance of ≥ 10% were *Flavobacteriaceae* (24%), *Xanthomonadaceae* (23%), *Moraxellaceae* (12%) and *Pseudomonadaceae* (10%). To identify which of these families could survive in a chlorinated swimming pool, the community was chlorinated. In the Netherlands the free available chlorine concentration is required to be between 0.5 and 1.5 mg Cl_2 L^{-1}, which is based on a 4-log removal of *Pseudomonas aeruginosa* at 1 mg Cl_2 L^{-1} within 30 s contact time. After chlorination of the initial AMCB with 1 mg Cl_2 L^{-1} for 30 s, the relative abundance of *Pseudomonadaceae* was increased to 22%, while the other relative abundances remained similar (±3%). Furthermore, the relative abundance of *Pseudomonadaceae* increased with longer contact times at 1 mg Cl_2 L^{-1}. This suggests that *Pseudomonadaceae* were more chlorine resistant than the other identified bacteria within the total initial AMCB.

Characterizing the intact cell community of the initial AMCB reflects another way to determine chlorine resistance. The bacterial families present for ≥10% of the intact (non-chlorinated and chlorinated) communities were *Xanthomonadaceae* (21-17%) and *Moraxellaceae* (48-57%).

Moraxellaceae were thus suggested to be more chlorine resistant than the other identified intact bacteria present. Within both families of *Pseudomonadaceae* and *Moraxellaceae*, (opportunistic) pathogens were found, which are therefore likely to be released in swimming pools as well. Because these families were more chlorine resistant than others, there could be a potential health risk.

Determination of the microbial water quality to estimate the health risk is normally done with indicator organisms. One of the used indicator organisms is *Pseudomonas aeruginosa*, which should not be detectable in 100 mL according to Dutch legislation. *P. aeruginosa* was detected in the initial AMCB and seems therefore a logical indicator organisms. Besides, since *Pseudomonadaceae* was found to be relatively more chlorine resistant that the other identified bacteria, absence of *P. aeruginosa* therefore suggests absence of other bacteria as well.

To verify this preliminary conclusion, the response of *Pseudomonadaceae*, *P. fluorescens*, and the faecal indicator *Escherichia coli* were compared to the response of an initial AMCB after subjecting to different chlorine doses (0-15 mg Cl_2 min L^{-1}). The corresponding log reduction was measured with heterotrophic plate count (HPC) and flow cytometry, live/dead staining (FCM) whereby the intact cell concentration was analysed. After chlorination, no colony forming units (CFU) of the indicator organisms were detected with HPC, while they were found for the AMCB. This suggests that the microorganisms in the initial AMCB were more chlorine resistant than the indicator organisms. However, measuring with FMC showed similar log reductions of the initial AMCB and *P. fluorescens*, while *E. coli* showed higher log reductions. Because the concentration of *E. coli* was more easily reduced by chlorination than the AMCB, *E. coli* might underestimate the effectiveness of chlorination in swimming pools and is therefore not a useful indicator organism of chlorinated systems.

Although most swimming pools are chlorinated, an alternative treatment based on biological sand filtration, coagulation, ultrafiltration and UV irradiation was investigated. Therefore, the response to UV irradiation was also researched with *P. fluorescens*, *E. coli* and the AMCB. After UV irradiation, the intact cell concentration remained constant, probably because of DNA damage instead of membrane damage. The corresponding log reductions based on HPC results were similar for the initial AMCB and *E. coli* at similar UV doses higher than 4.7 mJ cm^{-2}. *P. fluorescens* showed higher log reductions already at lower UV doses. This suggests that *E. coli* was a more suitable indicator organism for UV irradiated water than *P. fluorescens*. Clearly, the use of indicator organisms was dependent on the use of disinfection technique, which should be included in regulation.

The use of two measurement techniques resulted in different observations of log reduction. The same sample showed different log reductions when measured with HPC or FCM after chlorination. Intact cells were detected in all non-disinfected and chlorinated samples. However, the culturability of the indicator organisms was below the detection limit, suggesting that the intact cells of the indicator communities should be considered viable but non-culturable (VBNC). VBNC microorganisms might still pose a threat for human health as ingestion creates different conditions that might trigger repair, a phenomenon called resuscitation. Determination of microbial levels of indicator organisms in chlorinated swimming pools with HPC might therefore not reflect the actual microbial levels, resulting in an overestimation of the disinfection efficiency and thus an underestimation of the health risks.

Besides, both HPC and FCM are measurement techniques that determine the concentration of viable cells. Therefore, one could expect that in time the methods would give the same results, although this was not the case after one day of response time. This may have been caused by the fact that cells can survive longer when they do not have to adapt to the environment.

8.2 Biofouling

Besides planktonic cells, cells can form aggregates or attach to and grow on a surface forming a biofilm. Different methods have been used to prevent biofouling, such as use of materials with biofouling limiting characteristics and disinfection water containing planktonic microorganisms. Part of this thesis was to investigate biofilm development on 11 swimming pool materials (PVC on stainless steel, PVC, stainless steel, rough foil, smooth foil, concrete, rough tiles, smooth tiles, pultrusion polyester, polyester and polypropylene) in a laboratory-scale set-up. Non-chlorinated tap water was used as inoculum, and nutrients were dosed simulating highly occupied swimming pool conditions with a UV-based treatment. The biofilm development on the different materials was quantified over time (4-28 days). After 14 of the 28 days, the highest biofilm quantity was observed; therefore, the biofilm quantity on the different materials was compared on day 14. The lowest average biofilm quantity was found on polypropylene, while the highest average quantity was measured on stainless steel and concrete. Therefore, this research suggests that polypropylene would be a good material to use in order to prevent large quantities of biofilm in highly occupied swimming pools.

The degree of bacterial adhesion on materials depends on various material characteristics such as surface charge, surface roughness and hydrophobicity. It was observed that biofouling increases as the surface roughness increases, probably since shear forces at the direct surface of the material diminish (microorganisms can be shielded) and the absolute surface area is higher when surfaces are rougher (Donlan 2002). However, in this research it was found that the extent of biofilm formation on these swimming pool materials was not related to their surface roughness. Furthermore, it was expected that biofilm formation occurred more rapidly on hydrophobic materials than on hydrophilic ones. After 4 days, the biofilm quantity seemed to be slightly decreasing with increasing cohesion energy, meaning that the more hydrophilic materials showed lower biofilm quantities. This suggests that in the beginning, cells attach more easily onto hydrophobic materials, which is in line with literature. However, from day 7 onward, the relation between the biofilm quantity and the cohesion energy became less clear, while after 14 days, the biofilm quantity seemed to be increasing with the cohesion energy, indicating that a higher biofilm quantity was observed on the more hydrophilic materials. Increased biofouling over time on hydrophilic materials has been previously observed. As an exception, after 14 days, a high biofilm quantity was also observed on the hydrophobic stainless steel. Since the biofilm growth on both hydrophilic (concrete) and hydrophobic (stainless steel) materials was similar at day 14, the cohesion energy might not have been the sole determining factor for growth at this point. This effect may be explained by the adhesion of cells to a surface occurring in a few hours instead of days. More research is suggested on the phenomenon of submerged biofouling and the influence of material properties in time.

Besides the influence of material properties on the extent of biofouling, the influence of nutrient addition was also investigated. The biofilm quantity was approximately a factor of 11 higher when high bathing load conditions were applied than application in plain tap water conditions, while the difference between concrete and polypropylene was about a factor of 6. This suggests that bathing load has a stronger influence on biofilm growth than material properties in swimming pools. While in this research two extremes were tested, namely high bathing load conditions and low nutrient conditions, the expected biofilm growth in a UV-based treatment swimming pool on these materials will be somewhere in between. To determine the biofilm extent in reality, pilot scale research is suggested.

Although the first step would be to prevent biofouling, when a biofilm has been formed, the biofilm should also be disinfected and removed. Similar disinfection techniques as for planktonic cells could be used to disinfect biofilms, although lower log reductions have been observed for chlorination. The rate limiting factor for disinfection is the diffusion of chlorine into the biofilm (Chen and Stewart 1996), which is related to physicochemical interactions between the disinfectant and the EPS. Therefore, microorganisms in the deeper regions of the biofilm might be exposed to a lower concentration of disinfectant. As an alternative disinfection technique, UV irradiation was applied. The penetration of UV light through a biofilm was found to be linearly decreasing as well. It was determined that in total, UV light could pass maximal through a biofilm of 104 µm thick.

To study the effect of disinfection and mechanical removal of biofilms, the biofilm was subjected to (i) UV irradiation at varying doses and frequencies, (ii) brushing and (iii) brushing followed by UV irradiation. Daily UV irradiation doses ≥ 50 J m^{-2} lowered the cellular ATP concentration of biofilms developed on concrete plates, while 250 J m^{-2} stabilised the biofilm growth and 1500 J m^{-2} reduced the active biofilm amount. The biofilm reduction of the different treatments determined with total and cellular ATP was for (i) only brushing 9-7%, (ii) only 50 J m^{-2} UV irradiation 36-48% and (iii) brushing followed by 50 J m^{-2} UV irradiation 45-57% respectively, indicating that the combination of subsequent brushing and UV irradiation showed to be the most effective strategy for biofilm control. To optimize the treatment, more experiments should be performed on both brushing and UV irradiation as well as on the applied frequency. Examples for optimization could be electric brushing and higher UV doses.

Once these parameters have been optimized, a device with a brush and UV lamp should be developed that can perform in water. Together with the company UV van Remmen Technieken, the outline for such a device has been made. In order to use UV light under water, the UV lamp should be protected with Quartz glass, and the power connection should be waterproof. Next the brush should be placed in front of the UV lamp in a device that can roll and stick to the walls. Therefore, a circuit board should be made and a protocol designed which allows the device to irradiate the complete swimming pool with a rate that applies a sufficient UV dose on the walls. In this way after brushing, the remaining biofilm is disinfected. In addition, the removed biofilm aggregates should not be released into the pool water as there is a chance that the aggregates will settle again, forming a biofilm elsewhere. Therefore, the biofilm aggregates should be collected in a large filter bag.

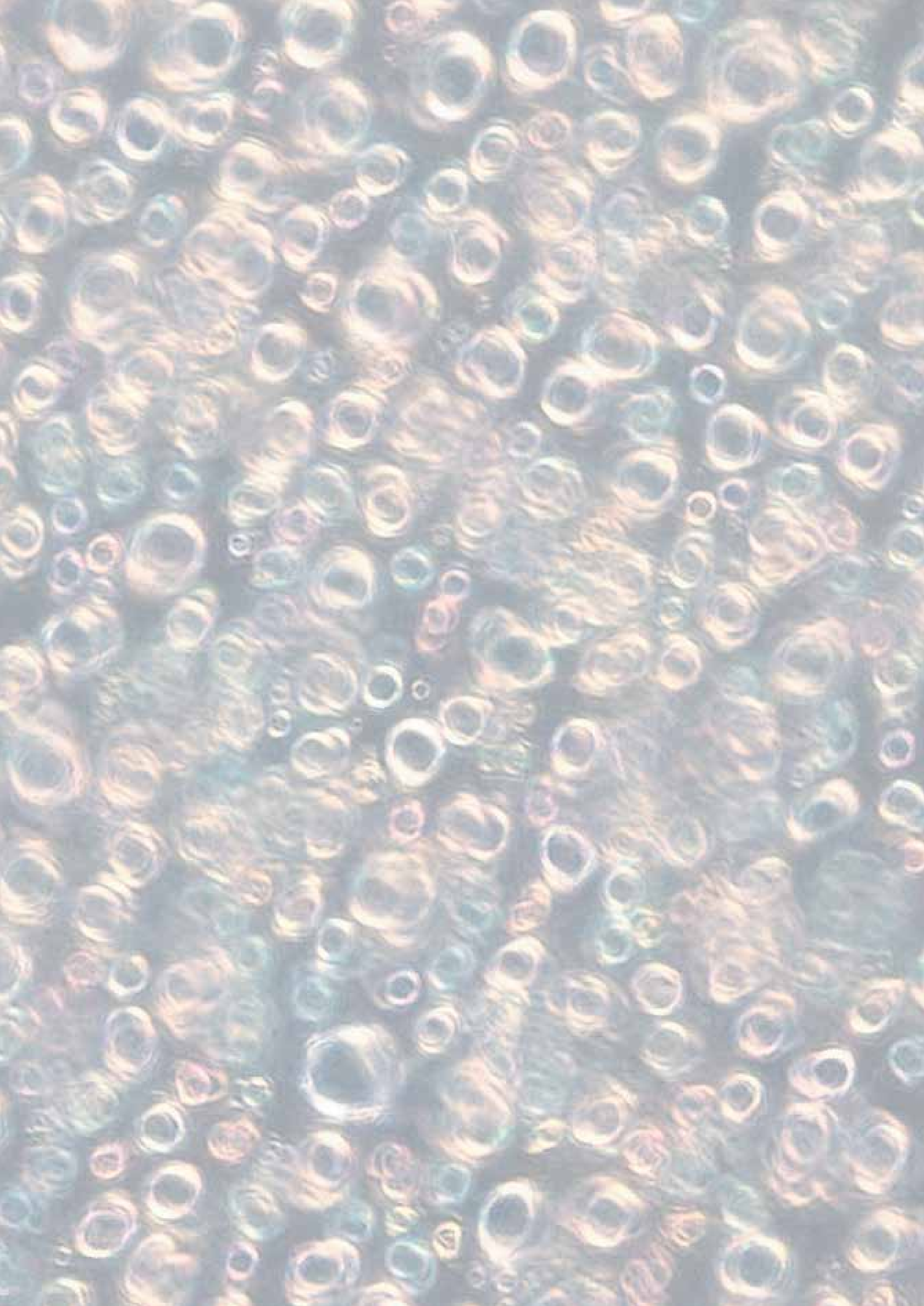
8.3 Quantitative microbial risk assessment

Finally, all results were combined to make a microbial risk assessment of an indoor swimming pool with a UV-based treatment without the use of a residual disinfectant, but instead using a biological sand filter, coagulation, ultrafiltration and UV irradiation. A risk model was built on the following reference assumptions; an indoor swimming pool of 25 m × 10 m × 2 m, the total pool volume was treated every 240 min, with a microorganism removal rate of 5-log, data on microorganism release by bathers and exposure by ingesting 13.7 mL of pool water during 1 h of swimming for 59 times per year. The chance of gastroenteritis, one of the most common diseases throughout the world, was determined as the relation between the presence of faecal contamination, and swimming-associated gastroenteritis has been demonstrated before. Therefore, the focus of this microbial risk assessment was based on the presence of enteric pathogenic bacteria: *Campylobacter jejuni*, *Escherichia coli* O157:H7 and *Salmonella enterica*.

Overall the average bacterial cell concentrations during the bathers exposure time, averaged during opening hours, were 3.1×10^{-3}, 7.2×10^{-4} and 9.5×10^{-4} cells L^{-1} for *C. jejuni*, *E. coli* and *S. enterica* respectively, while the concentrations were calculated to be 4.0×10^{-6} in a chlorinated swimming pool. Although less than 0.012 bathers of the 1000 bathers per year will get gastroenteritis from one of these pathogens by swimming in a UV-based treated swimming pool, about 180 times fewer bathers will become ill after swimming in a chlorinated swimming pool. The yearly risks of infection of these pathogens in a UV-based treated swimming pool were 1.7×10^{-3}, 1.8×10^{-5} and 3.5×10^{-7} for *C. jejuni*, *E. coli* and *S. enterica* respectively. Since the yearly risks of infection of *E. coli* and *S. enterica* were lower than the drinking water guidelines (10^{-4}), the UV-based swimming pool treatment seems promising. However, besides microorganisms, also other pollutants will be excreted during swimming, which could form disinfection by-products (DBPs) in chlorinated swimming pools. Since some of those DBPs are unhealthy, a determination of the chemical health risks should made to make a complete (microbiological and chemical) health risk assessment. Finally, comparing the outcome of the complete health risk assessment of a swimming pool treated with a biological sand filter, coagulation, ultrafiltration and UV irradiation with the complete health risk assessment of a chlorinated pool, would indicate whether this UV-based treatment concept would be a healthier option, increasing the health effects of swimming.

8.4 Overall conclusion

This thesis describes the research about microbiological aspects of chlorinated swimming pools and a new UV-based treatment concept in which no residual disinfectant is present, but where the treatment instead consists of a biological sand filter, coagulation, ultrafiltration and UV irradiation. However, without residual disinfectant, the microorganisms released during bathing are not directly treated and might form a biofilm on the swimming pool surfaces. Therefore, biofilm formation should be minimized. Based on the results of this research, polypropylene was found to have the lowest biofilm development under high bathing load conditions, suggesting that polypropylene would be the best material to use on walls and floor of the pool. Furthermore, biofouling could be controlled with UV irradiation and brushing, although this treatment should be further optimized. The microbiological safety was assessed via a quantitative microbial risk assessment. To do so, the anthropogenic microbial community released by bathers was quantified and identified. The yearly risks of infection on gastroenteritis by the bacterial pathogens *C. jejuni*, *E. coli* and *S. enterica* averaged during the day, were calculated to be 1.7×10^{-3}, 1.8×10^{-5} and 3.5×10^{-7} respectively, while the yearly risk of infection by *E. coli* in a chlorinated swimming pool is a factor of 180 lower (9.8×10^{-7}). However, the estimated risks for *E. coli* and *S. enterica* turned out to be lower than used for the drinking water guidelines (10^{-4}). This suggests that the new treatment concept is promising and should be investigated further at pilot scale to verify the results of this study in practice. Moreover, new guidelines should be developed for this concept, also taking into account indicator organisms with similar log reductions as an AMCB, as it has been shown that this depends on the disinfection method. Finally, application of a UV-based treated swimming pool without (chlorine) residual disinfectant reduces the formation of DBPs, leading to a healthier way of enjoying this sport activity.



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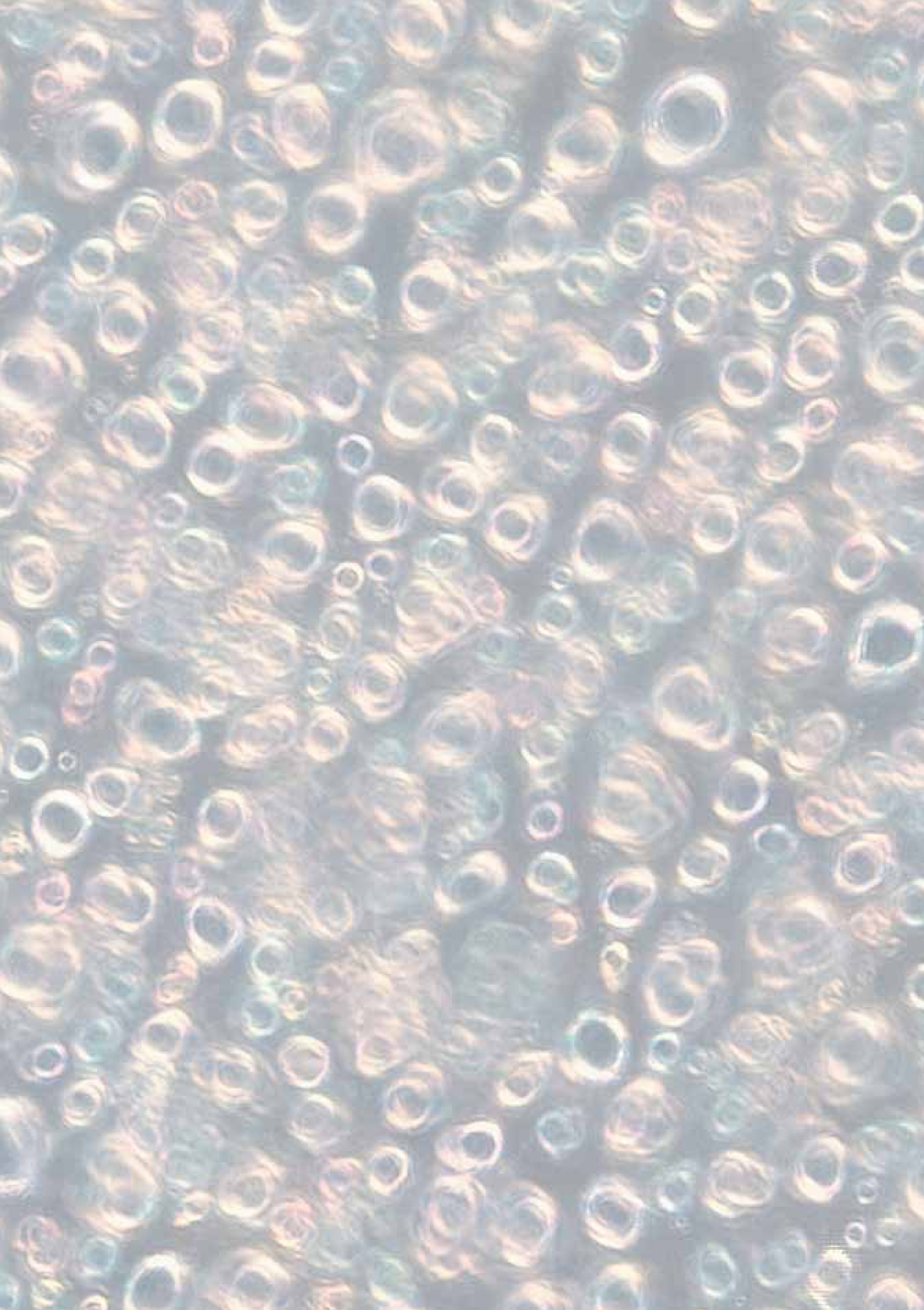
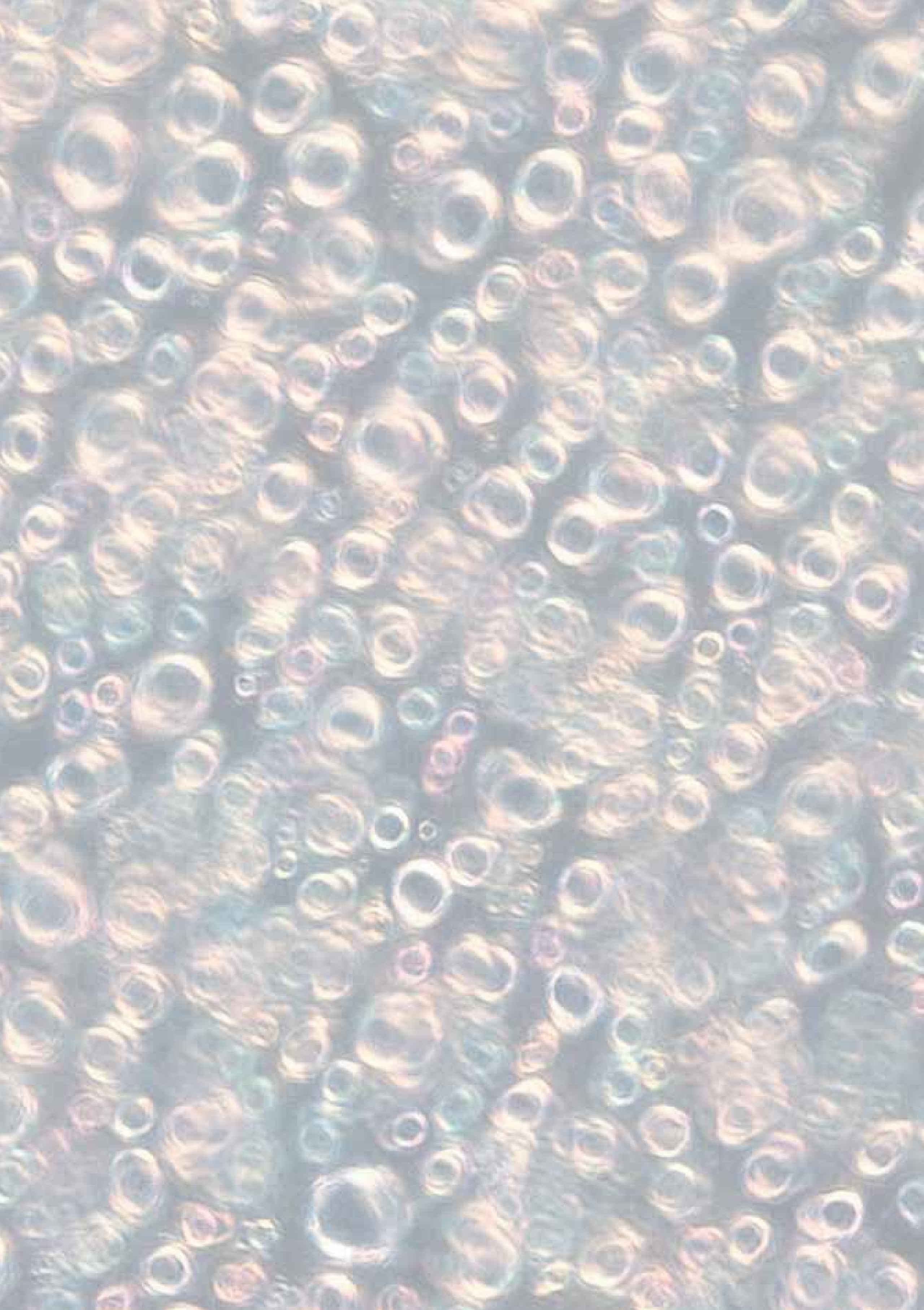


List of Abbreviations

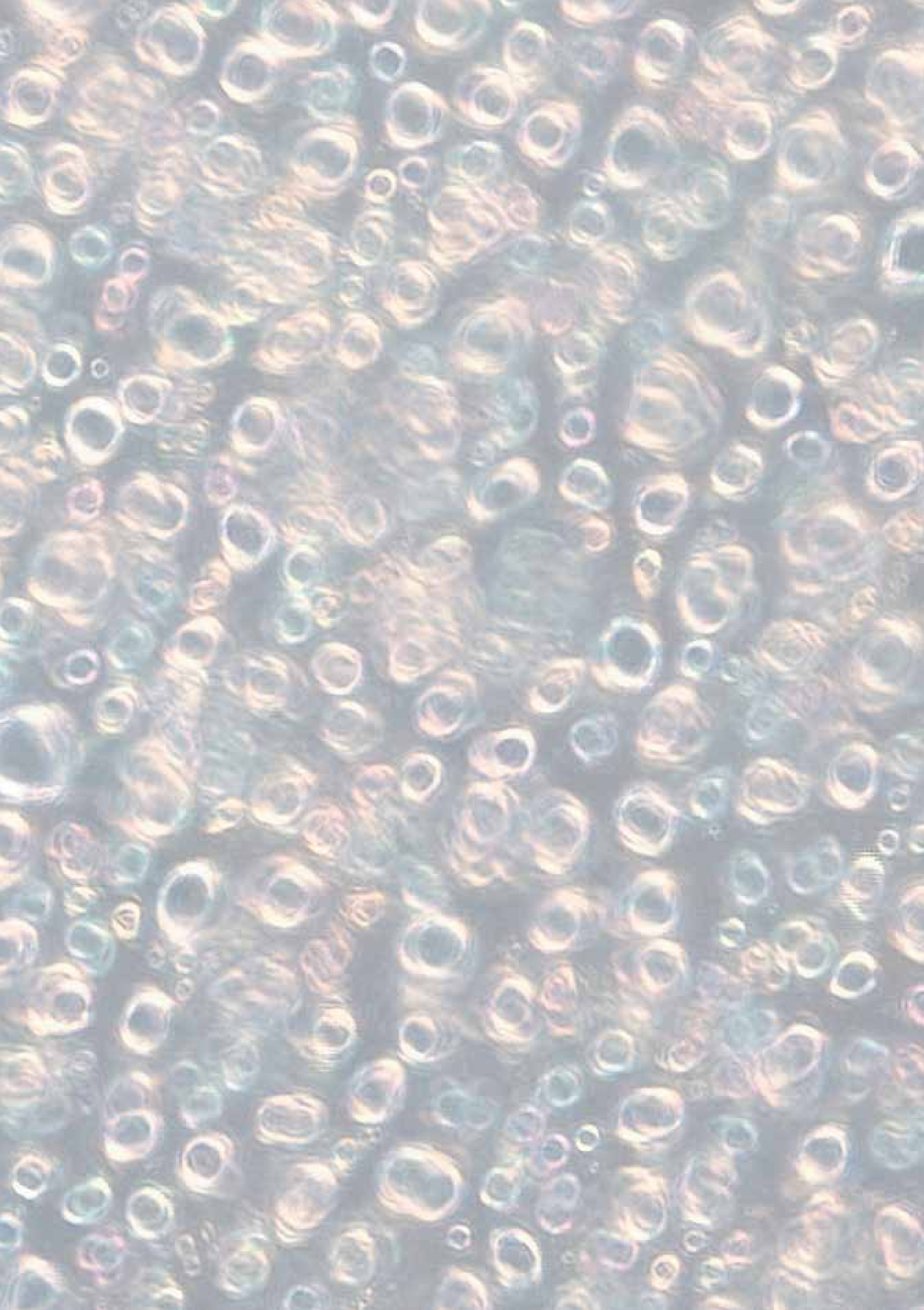
AMCB:	anthropogenic microbial community of bathers
ATP:	adenosine triphosphate
	tATP: total
	cATP: cellular
BSA:	body surface area
CFU:	colony forming units
DNA:	deoxyribonucleic acid
DPBs:	disinfection by-products
EDTA:	ethylenediaminetetraacetic acid
EPS:	extracellular polymeric substances
FCM:	flow cytometry, live/dead staining
HES:	high energy sonification
HPC:	heterotrophic plate count
NPOC:	non-purgeable organic carbon
OTU:	operational taxonomic unit
PBS:	phosphate buffer saline
PI:	propidium iodide
PVC:	polyvinyl chloride
qPCR:	quantitative polymerase chain reaction
RLU:	relative light units
RMS:	rooth mean square roughness
rRNA:	ribosomal ribonucleic acid
TN:	total nitrogen
UV:	ultraviolet
VBNC:	viable but non-culturable
VO_2:	energy consumption

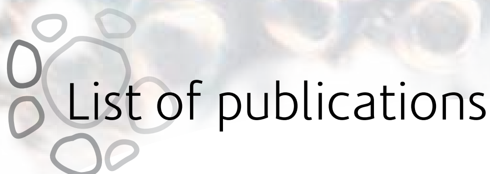


Curriculum vitae

Marjolein Peters was born on the 5th of June 1988 in Leidschendam, the Netherlands. After obtaining her VWO diploma at the Veurs Lyceum in Leidschendam in 2006, she started studying Life Science and Technology at Delft University of Technology and Leiden University. During this study the basic understanding of molecular biology, biotechnology and thermodynamics were educated. Her bachelor thesis was about Liquid-liquid demixing of butanol and water. During her master specialisation, Marjolein studied the tracks Biochemical Engineering and Cell Factory. The courses included modelling and optimisation of different treatment processes and behaviour of various microorganisms. Her master thesis was about the anaerobic ammonium oxidation (anammox) bacteria, which is used in waste water treatment to reduce the ammonium and nitrite concentration. In order to determine the characteristics of the anammox cells, free anammox cells were enriched. Furthermore, during an internship the microbial dune population of dunes used during the drinking water treatment of Dunea were characterised.

After obtaining her M.Sc degree in 2011, Marjolein started her PhD research on the microbiological aspects in a swimming pool under supervision of promotor Luuk Rietveld. She investigated the microorganism release by bathers and the formation of biofilms under non-chlorinated swimming pool conditions. Besides, she was involved in many activities like education, guiding of (international) students who wanted to do an internship within our section, as well as those who wanted to participate within her own PhD project.





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Keuten, M.G.A., Peters, M.C.F.M., Daanen, H.A.M., de Kreuk, M.K., Rietveld, L.C., van Dijk, J.C. (2014) Quantification of continual anthropogenic pollutants released in swimming pools. Water research 53 (2014) 259-270

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Peters, M.C.F.M., Keuten, M.G.A., Knezev, A., van Loosdrecht, M.C.M., Vrouwenvelder, J.S., Rietveld, L.C., de Kreuk, M.K. (submitted) Impact of chlorination and UV irradiation on an anthropogenic microbial community from bathers, *Escherichia coli* and *Pseudomonas fluorescens*.

Peters, M.C.F.M., Keuten, M.G.A., Zuiddam, M.R., Verliefde, A.R.D., Vrouwenvelder, J.S., Rietveld, L.C., de Kreuk, M.K. (submitted) Biofouling in swimming pools: role of material characteristics and nutrients.

Peters, M.C.F.M., Keuten, M.G.A., Vrouwenvelder, J.S., Rietveld, L.C., de Kreuk, M.K. (in preparation) Biofilm disinfection by UV irradiation and removal by brushing.

Peters, M.C.F.M., Keuten, M.G.A., de Kreuk, M.K., Vrouwenvelder, J.S., Rietveld, L.C., Medema, G. (in preparation) Quantitative microbial risk assessment for an indoor swimming pool with chlorination and UV-based treatment.

Other publications

Marjolein Peters (2014) The development of a chlorine free swimming pool. Druppel, February 2014, Volume 24

Marjolein Peters (2016) Making a splash (with robots!). Delta, September 2016

Oral and poster presentations at international conferences

6th International Conference Swimming Pool & Spa (2015) Amsterdam, the Netherlands

- Response of an anthropogenic population versus indicator organisms after chlorination. Oral and poster.
- Biofilm growth on swimming pool material. Oral and poster.

PAO Zwembad symposium (2014), Nootdorp, the Netherlands

- Desinfectie zwembaden – Minimale vrij chloor concentratie. Oral

Young Water Professionals BeNeLux (2013)

- Chlorine cell disinfection determination with flow cell cytometry and plate count. Poster.
- Quantification of continual anthropogenic pollutant release in swimming pools. Poster.

5th International Conference Swimming Pool & Spa (2013) Rome, Italy

- Minimum chlorine concentration to ensure disinfection – Chlorine inactivation of the mixed population versus indicator microorganism. Oral and poster.
- Chlorine inactivation of mixed population versus indicator microorganism. Poster.

Pools'12 (2012) Delft, the Netherlands

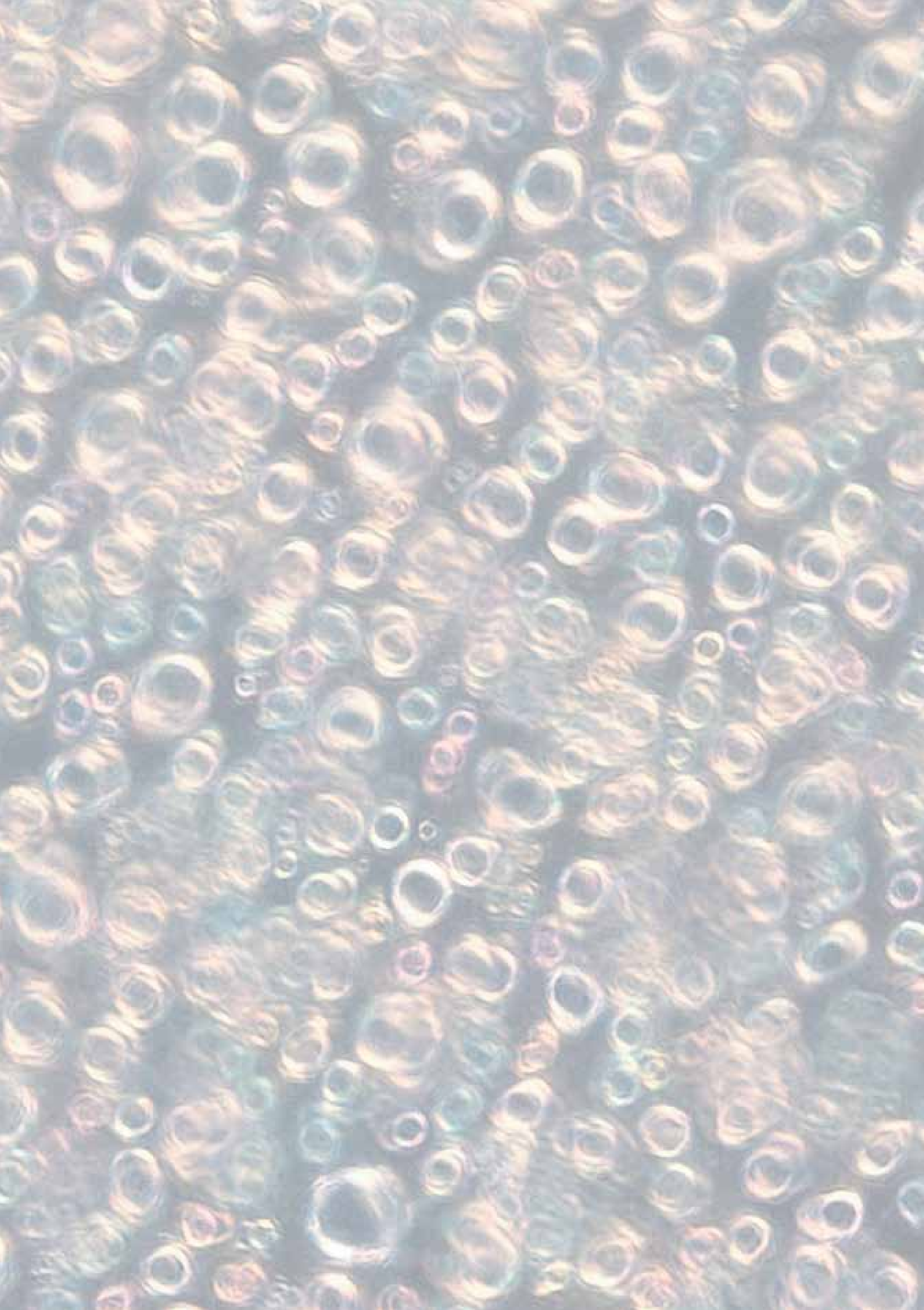
- Microbial population in swimming pools – Filtration, DNA extraction, (q)PCR and DGGE. Oral.

Workshop Biological stability (2012), Delft, the Netherlands

- Microbial population in swimming pools – Filtration, DNA extraction, (q)PCR and DGGE. Oral.

Young Water Professionals BeNeLux (2011)

- Microbiological safe swimming pools without chlorine. Poster



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