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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 and therefore could form a potential health risk. The total community before and after chlorination (1 mg Cl_2 L^{-1} for 30 s) was characterized. More than 99% of the bacteria in the shower water were Gram-negative. The dominant bacterial families with a relative abundance of ≥10% of the total (non-chlorinated and chlorinated) communities were *Flavobacteriaceae* (24–21%), *Xanthomonadaceae* (23–24%), *Moraxellaceae* (12–11%) and *Pseudomonadaceae* (10–22%). The relative abundance of *Pseudomonadaceae* increased after chlorination and increased even more with longer contact times at 1 mg Cl_2 L^{-1}. Therefore, *Pseudomonadaceae* were suggested to be relatively more chlorine resistant than the other identified bacteria. To determine which bacteria could survive chlorination causing a potential health risk, the relative abundance of the intact cell community was characterized before and after chlorination. The dominant bacterial families in the intact community (non-chlorinated and chlorinated) were *Xanthomonadaceae* (21–17%) and *Moraxellaceae* (48–57%). *Moraxellaceae* were therefore more chlorine resistant than the other identified intact bacteria present.

Key words | bacterial population, characterization, chlorine resistance, grey water

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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 (FAC) 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 (Ministerie van Infrastructuur en Milieu 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).

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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). These incidents indicate that some microorganisms could be resistant to disinfectants (Hingst *et al.* 1995), such as the (opportunistic) pathogens *Pseudomonas aeruginosa, Staphylococcus aureus* and *Klebsiella pneumoniae* (Papadopoulou *et al.* 2008).

In the Netherlands, swimming pool water is made of tap water, which is pH adjusted and chlorinated. The pool water circulates continuously through water treatment which traditionally consists of sand filtration. 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. To determine which bacteria might be released in swimming pools, the composition of an anthropogenic bacterial wash-off community released by bathers was characterized and the impact of chlorination on the community was investigated.

METHODS

Shower procedure

The initial anthropogenic pollutant release is introduced into the pool water during the first few 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. Specific factors like age, location, and sex contribute to the variability of the microbial flora of the skin (Grice & Segre 2011), therefore an average anthropogenic community was obtained by collecting all shower water of 10 western European adults (five male and five female) between 20 and 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 tap water in triplicate and drained until only a few drops of water came out.

The water used for showering was non-chlorinated tap water (Ministerie van Infrastructuur en Milieu 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}. As 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.

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 characterization 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).

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 FAC dose; and (ii) the impact of different FAC 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 1.

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 FAC 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 FAC concentrations probably reduced during the contact time because of disinfection and oxidation of organics. Therefore, the FAC 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 characterize 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

Table 1 | Overview of the chlorination experiments performed with the anthropogenic community, before bacterial community characterization

No. of samples	Initial FAC[a] concentration (mg Cl_2 L^{-1})	Chlorine incubation time	Total/intact community characterized
1	0		Intact
1	1	30 s	Intact
2	0		Total
2	1	30 s	Total
1	1	5 min	Total
1	1	20 min	Total

[a]FAC = free available chlorine.

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.

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 & Zhang 2013).

Subsequently, the extracted DNA samples were characterized 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 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 (next generation sequencing) 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 1,000 sequences per sample. For all samples, rarefaction curves were plotted (see supplementary data: Figure S1, Table S1) and the inverse Simpson's diversity index and Good's coverage were calculated (Table S2) (the supplementary data are available with the online version of this paper). The inverse Simpsons's diversity index was between 2.4 and 4.1 and the Good's coverage was ≥97.5% for all samples.

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.

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 ethylenediaminetetraacetic acid (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}.

RESULTS

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 2. Characterization 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 characterized. 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%).

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 2). 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 2). 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

Table 2 | The relative abundance of bacteria present in the anthropogenic community from bathers based on sequencing results

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%

Notes: Chlorination was performed with an initial FAC concentration of 1 mg Cl_2 L^{-1} for 30 s; the intact cell community was observed after removal of extracellular DNA; <1 is considered to be background sequences and not exclusively referring to the sample; nd = not detected.

were similar (Table 2). 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 2). 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.

Cell counts combined with deep sequencing results

Evaluation of the anthropogenic community with flow cytometry live/dead staining revealed a total cell concentration of 4.15×10^7 total cells mL^{-1} and an intact cell concentration of 9.63×10^6 intact cells mL^{-1} for the non-chlorinated sample. This means that only 23% of the cells in filtered shower water were intact. 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 intact cell families with a relative abundance of ≥5% (Table 2), the most chlorine resistant were *Moraxellaceae* and *Sphingomonadaceae*, with a chlorine resistance of 59–60% (Table 3). Although the chlorine resistance of both *Moraxellaceae* and *Sphingomonadaceae* are similar, the relative abundance, and thus the quantification results, of *Moraxellaceae* are about 8 times higher than those of *Sphingomonadaceae*. The impact of chlorination on *Moraxellaceae* is therefore larger, confirming the chlorine resistance of *Moraxellaceae*.

Impact of varying initial FAC doses

The effect of changing the contact time (0 s, 30 s, 5 min and 20 min) was investigated, while the initial FAC concentration was kept at 1 mg Cl_2 L^{-1}, complying with the guideline for Dutch swimming pools (Ministerie van Infrastructuur en Milieu 2011). After characterizing the total community, an impact of chlorine contact time on the community presence was observed (Figure 1). 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.

DISCUSSION

The bacterial anthropogenic community

In this research, characterization of a bacteria anthropogenic community was performed to determine: (i) which

Table 3 | Quantification of the intact cell community by combination of deep sequencing results (Table 2) and cell counts, whereafter the chlorine resistance was determined

	Non-chlorinated sample No. of intact cells ml^{-1}	Chlorinated sample No. of intact cells ml^{-1}	Intact cells ratio Chlorinated/non-chlorinated
Xanthomonadaceae	2.07×10^6	8.04×10^5	0.39
Moraxellaceae	4.57×10^6	2.70×10^6	0.59
Burkholderiales_incertae_sedis	7.44×10^5	2.87×10^5	0.39
Caulobacteraceae	6.78×10^5	2.02×10^5	0.30
Sphingomonadaceae	5.79×10^5	3.48×10^5	0.60

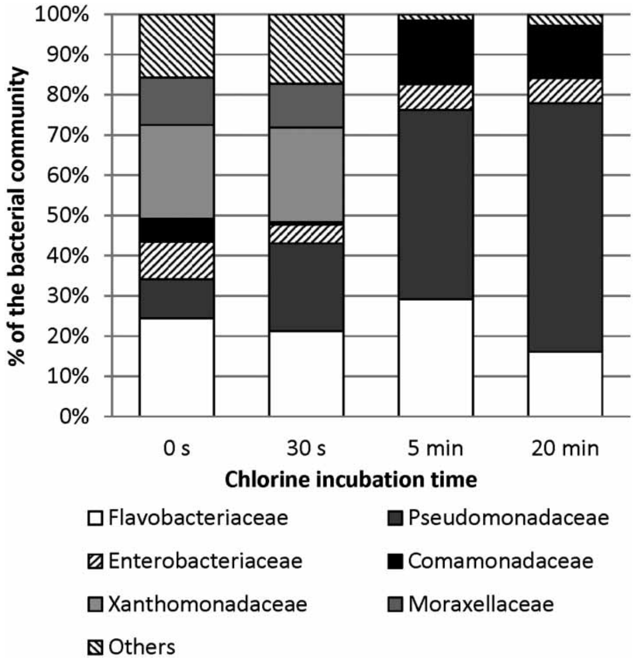

Figure 1 | The difference in anthropogenic bacterial communities from shower water after disinfection with an initial FAC concentration of 1 mg Cl_2 L^{-1} at different contact times.

bacteria are present in a wash-off community for which both the total and intact cell community are relevant; and (ii) which of the bacteria present could cause a potential health risk in swimming pools and therefore, the intact (possibly living) cell community should be characterized.

The 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}. Due to 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* and *Pseudomonadaceae*, which had a relative abundance of ≥10%. Of the intact cell community however, only *Xanthomonadaceae* and *Moraxellaceae* have a higher relative abundance than 10% (Table 2). These results indicate that there is a big difference between the total and intact cell community composition.

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 & Segre 2011). Many skin-related bacteria found after swabbing the skin (Human Microbiome Project Consortium 2012, Supplementary), like *Staphylococcaceae*, *Propionibacteriaceae*, *Corynebacteriaceae* or human-mouth related, like *Streptoccaceae*, are known to be Gram-positive (Human Microbiome Project Consortium 2012). Gram-negative bacteria are not often found on human skin (Marples 1965) and assumed to be contaminants from the gastrointestinal tract (Roth & James 1988; Chiller *et al.* 2001). As 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. 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. However, whether the supposedly more chlorine-resistant Gram-positive bacteria (Le Chevallier *et al.* 1980) are released in a later stage of swimming is unknown.

The effect of chlorination

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 & Lebaron 2000; 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.

Ramseier *et al.* (2011) found a 0.15-log removal for chlorinated drinking water from the tap, based on intact cell counts at 0.5 mg Cl_2 L^{-1} min^{-1}. 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 (Ministerie van Infrastructuur en Milieu 2011) determined by plate counts from the research of Fitzgerald & 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 & 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 FAC could oxidize the organic material and therefore reduce the effective dose.

In addition to the general effect of chlorination on cells, the effect on the bacterial families present in the anthropogenic community was also determined. 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 & Lyman 1961; Whiteman *et al.* 1997). DNA denaturation has been described in the literature to occur from a chlorine dose of 1,500 mg Cl_2 L^{-1} (Prütz 1996; Suquet *et al.* 2010; Van Aken & 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. Characterization of a total chlorinated community 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. Therefore, determination of the living and potential (opportunistic) pathogens in chlorinated water should be done by characterization of the intact cell community. Combination of this technique with intact cell counts helps to quantify the concentration of intact, and thus possible living, cells to determine the number of (opportunistic) pathogens which can be used in a risk assessment.

Bacterial origin and (opportunistic) pathogens

Most of the bacterial families found in the wash-off community are related to aquatic environments or have been detected as part of the human skin community. For example, bacteria which have been found in shower areas, e.g. in a biofilm on shower curtains, were *Cytophaga*, *Flavobacteria*, *Bacteroides*, *Sphingomonas* spp. and *Methylobacterium* spp. (Kelley *et al.* 2004). The genus *Cloacibacterium*, of the large family *Flavobacteriaceae*, has been found in shower water (Oh *et al.* 2013), while *Flavobacterium* has also been detected in swimming pools (Favero & Drake 1966).

Furthermore, *P. aeruginosa*, belonging to the family of *Pseudomonadaceae*, is one of the best-known opportunistic pathogens related to swimming pools. Also, faecally derived *Enterobacteriaceae* contain human pathogens. Both *E. coli* (O157) and *Shigella* spp. are considered to be responsible for faecally derived microbial hazards in swimming pool water (WHO 2006). 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*.

To accurately determine the human health risks during swimming, sequencing should be performed at species level, since not all species in human-related bacterial families represent human pathogens. Therefore, further characterization was performed and the families in which human pathogens were found and where they have been found in the environment are shown in Table 4. Since human (opportunistic) pathogens have been found in this wash-off community, they could also be present in swimming pools. In order to cause a potential health risk, they should be present in the intact community in which *S. aureus* was not found (Table 2) and after chlorination, *P. aeruginosa* was not present either. *S. maltophilia*, *A. baumannii* and *S. paucimobilis* were found as intact cells after chlorination with 1 Cl_2 mg L^{-1} for 30 s. Further

Table 4 | Found bacterial family, genus and corresponding human pathogen (species) and their natural environment

Bacterial family name, genus	Human (opportunistic) pathogens	Environment
Xanthomonadaceae, *Stenotrophomonas*	*S. maltophilia* (Brooke 2012)	*Stenotrophomonas* has been found in water, soil and petroleum (Timmis 2010), moist places such as shower heads (Crossman *et al.* 2008), sporadically in Dutch drinking water (Van der Wielen & Van der Kooij 2011) and on the foreheads of people (Dekio *et al.* 2005).
Moraxellaceae, *Acinetobacter*	*A. baumannii* (Seifert & Dijkshoorn 2008)	*Acinetobacter* is often found in water, soil, living organisms and on human skin (Seifert *et al.* 1997; Berlau *et al.* 1999; Fournier & Richet 2006).
Pseudomonadaceae, *Pseudomonas*	*P. aeruginosa* (Papadopoulou *et al.* 2008)	*Pseudomonas* is present in insects, humans, soil, plants and water (Nikel *et al.* 2014). *P. aeruginosa* is commonly found in the human mouth, nose and throat (Eriksson *et al.* 2002) and surrounding the swimming pool (Jacobson 1985).
Sphingomonadaceae, *Sphingomonas*	*S. paucimobilis* (Hsueh *et al.* 1998)	*S. paucimobilis* has been found in fluids in humidifiers and in tap water in hospitals (Hsueh *et al.* 1998).
Staphylococcaceae, *Staphylococcus*	*S. aureus* (Papadopoulou *et al.* 2008)	*S. aureus* has been detected on the anterior nasal mucosa and skin as well as in the faeces of a substantial portion of healthy individuals (WHO 2006) and released by bathers under all swimming conditions (Robinton & Mood 1966).

research is needed to identify whether these cells were also viable and thus can cause a health risk during swimming.

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 $\geq$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. As these families were more chlorine resistant than others, there could be a potential health risk in swimming pools, even though chlorination is applied.

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REFERENCES

Berlau, J., Aucken, H., Malnick, H. & Pitt, T. 1999 Distribution of Acinetobacter species on skin of healthy humans. *Eur. J. Clin. Microbiol. Infect. Dis.* **18**, 179–183.

Biesbroek, G., Sanders, E. A., Roeselers, G., Wang, X., Caspers, M. P., Trzcinski, K., Bogaert, D. & Keijser, B. J. 2012 Deep sequencing analyses of low density microbial communities: working at the boundary of accurate microbiota detection. *PLoS One* **7**, e32942.

Brooke, J. S. 2012 Stenotrophomonas maltophilia: an emerging global opportunistic pathogen. *Clin. Microbiol. Rev.* **25**, 2–41.

Campbell, T. W. & Lyman, D. J. 1961 Chlorinated isotactic polyhydrocarbons. *Journal of Polymer Science* **55**, 169–180.

Centers for Disease Control and Prevention (CDC) 2000 Pseudomonas dermatitis folliculitis associated with pools and hot tubs – Colorado and Maine, 1999–2000. *MMWR* **49**, 1087–1091.

Chiller, K., Selkin, B. A. & Murakawa, G. J. 2001 Skin microflora and bacterial infections of the skin. *J. Investig. Dermatol. Symp. Proc.* **6**, 170–174.

Crossman, L. C., Gould, V. C., Dow, J. M., Vernikos, G. S., Okazaki, A., Sebaihia, M., Saunders, D., Arrowsmith, C., Carver, T., Peters, N., Adlem, E., Kerhornou, A., Lord, A., Murphy, L., Seeger, K., Squares, R., Rutter, S., Quail, M. A., Rajandream, M. A., Harris, D., Churcher, C., Bentley, S. D., Parkhill, J., Thomson, N. R. & Avison, M. B. 2008 The complete genome, comparative and functional analysis of Stenotrophomonas maltophilia reveals an organism heavily shielded by drug resistance determinants. *Genome Biology* **9**, R74. doi: 10.1186/gb-2008-9-4-r74.

Dekio, I., Hayashi, H., Sakamoto, M., Kitahara, M., Nishikawa, T., Suematsu, M. & Benno, Y. 2005 Detection of potentially novel bacterial components of the human skin microbiota using culture-independent molecular profiling. *J. Med. Microbiol.* **54**, 1231–1238.

Dziuban, E. J., Liang, J. L., Craun, G. F., Hill, V., Yu, P. A., Painter, J., Moore, M. R., Calderon, R. L., Roy, S. L. & Beach, M. J. 2006 Surveillance for waterborne disease and outbreaks associated with recreational water – United States, 2003–2004. *MMWR Surv. Summ.* **55**, 1–30.

Edgar, R. C., Haas, B. J., Clemente, J. C., Quince, C. & Knight, R. 2011 UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* **27**, 2194–2200.

Eriksson, E., Auffarth, K., Henze, M. & Ledin, A. 2002 Characteristics of grey wastewater. *Urban Water* **4**, 85–104.

Favero, M. S. & Drake, C. H. 1966 Factors influencing the occurrence of high numbers of iodine-resistant bacteria in iodinated swimming pools. *Applied Microbiology* **14**, 627–635.

Fitzgerald, G. P. & Der Vartanian, M. E. 1969 Pseudomonas Aeruginosa for evaluation of swimming pool chlorination and algicides. *Applied Microbiology* **17**, 415–421.

Fournier, P. E. & Richet, H. 2006 The epidemiology and control of Acinetobacter baumannii in health care facilities. *Clinical Infectious Diseases* **42**, 692–699.

Grice, E. A. & Segre, J. A. 2011 The skin microbiome. *Nat. Rev. Microbiol.* **9**, 244–253.

Guo, F. & Zhang, T. 2013 Biases during DNA extraction of activated sludge samples revealed by high throughput sequencing. *Appl. Microbiol. Biotechnol.* **97**, 4607–4616.

Hingst, V., Klippel, K. M. & Sonntag, H. G. 1995 Investigations concerning the epidemiology of microbial resistance to biocides. *Zentralblatt Fur Hygiene Und Umweltmedizin* **197**, 232–251.

Hsueh, P. R., Teng, L. J., Yang, P. C., Chen, Y. C., Pan, H. J., Ho, S. W. & Luh, K. T. 1998 Nosocomial infections caused by sphingomonas paucimobilis: clinical features and microbiological characteristics. *Clinical Infectious Diseases* **26**, 676–681.

Human Microbiome Project Consortium 2012 Structure, function and diversity of the healthy human microbiome. *Nature* **486**, 207–214.

Jacobson, J. A. 1985 Pool-associated Pseudomonas aeruginosa dermatitis and other bathing-associated infections. *Infect. Control* **6**, 398–401.

Joux, F. & Lebaron, P. 2000 Use of fluorescent probes to assess physiological functions of bacteria at single-cell level. *Microbes and Infection* **2**, 1523–1535.

Kelley, S. T., Theisen, U., Angenent, L. T., St Amand, A. & Pace, N. R. 2004 Molecular analysis of shower curtain biofilm microbes. *Appl. Environ. Microbiol.* **70**, 4187–4192.

Keuten, M. G., Schets, F. M., Schijven, J. F., Verberk, J. Q. & van Dijk, J. C. 2012 Definition and quantification of initial anthropogenic pollutant release in swimming pools. *Water Res.* **46**, 3682–3692.

Le Chevallier, M. W., Seidler, R. J. & Evans, T. M. 1980 Enumeration and characterization of standard plate-Count bacteria in chlorinated and raw water-supplies. *Appl. Environ. Microbiol.* **40**, 922–930.

Lowbury, E. J. 1969 Gram-negative bacilli on the skin. *Br. J. Dermatol.* **81**, 55–61.

Marples, M. J. 1965 *The Ecology of the Human Skin*. Thomas, Springfield.

Ministerie van Infrastructuur en Milieu 2011 *Besluit Hygiëne en Veiligheid Badinrichtingen en Zwemgelegenheden (Government Regulations About Hygiene and Safety in Bathhouses and Swimming Halls), No. BWBR0003716*, http: wetten.verheid.nl. Ministerie van Infrastructuur en Milieu, The Netherlands (In Dutch).

Ministerie van Infrastructuur en Milieu 2013 *De kwaliteit van het drinkwater in Nederland in 2013 (Drinking Water Quality in the Netherlands in 2013). Report*. Inspectie Leefomgeving en Transport – Ministerie van Infrastructuur en Milieu, Utrecht, The Netherlands (In Dutch).

Nikel, P. I., Martinez-Garcia, E. & de Lorenzo, V. 2014 Biotechnological domestication of pseudomonads using synthetic biology. *Nat. Rev. Microbiol.* **12**, 368–379.

Oh, J., Freeman, A. F., Program, N. C. S., Park, M., Sokolic, R., Candotti, F., Holland, S. M., Segre, J. A. & Kong, H. H. 2013 The altered landscape of the human skin microbiome in patients with primary immunodeficiencies. *Genome Res.* **23**, 2103–2114.

Papadopoulou, C., Economou, V., Sakkas, H., Gousia, P., Giannakopoulos, X., Dontorou, C., Filioussis, G., Gessouli, H., Karanis, P. & Leveidiotou, S. 2008 Microbiological quality of indoor and outdoor swimming pools in Greece: investigation of the antibiotic resistance of the bacterial isolates. *International Journal of Hygiene and Environmental Health* **211**, 385–397.

Prest, E. I., Hammes, F., Kotzsch, S., van Loosdrecht, M. C. & Vrouwenvelder, J. S. 2013 Monitoring microbiological changes in drinking water systems using a fast and reproducible flow cytometric method. *Water Res.* **47**, 7131–7142.

Prest, E. I., El-Chakhtoura, J., Hammes, F., Saikaly, P. E., van Loosdrecht, M. C. & Vrouwenvelder, J. S. 2014 Combining flow cytometry and 16S rRNA gene pyrosequencing: a promising approach for drinking water monitoring and characterization. *Water Res.* **63**, 179–189.

Prütz, W. A. 1996 Hypochlorous acid interactions with thiols, nucleotides, DNA, and other biological substrates. *Arch. Biochem. Biophys.* **332**, 110–120.

Ramseier, M. K., von Gunten, U., Freihofer, P. & Hammes, F. 2011 Kinetics of membrane damage to high (HNA) and low (LNA) nucleic acid bacterial clusters in drinking water by ozone, chlorine, chlorine dioxide, monochloramine, ferrate(VI), and permanganate. *Water Res.* **45**, 1490–1500.

Robinton, E. D. & Mood, E. W. 1966 A quantitative and qualitative appraisal of microbial pollution of water by swimmers – a preliminary report. *Journal of Hygiene-Cambridge* **64**, 489–499.

Roth, R. R. & James, W. D. 1988 Microbial ecology of the skin. *Ann. Rev. Microbiol.* **42**, 441–464.

Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., Lesniewski, R. A., Oakley, B. B., Parks, D. H., Robinson, C. J., Sahl, J. W., Stres, B., Thallinger, G. G., Van Horn, D. J. & Weber, C. F. 2009 Introducing *Mothur*: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl. Environ. Microbiol.* **75**, 7537–7541.

Seifert, H. & Dijkshoorn, L. 2008 Overview of the Microbial Characteristics, Taxonomy, and Epidemiology of Acinetobacter. In: *Acinetobacter Biology & Pathogenesis* (E. Bergogne-Bérézin, H. Friedman & M. Bendinelli, eds). Springer, Berlin, pp. 19–45.

Seifert, H., Dijkshoorn, L., Gerner-Smidt, P., Pelzer, N., Tjernberg, I. & Vaneechoutte, M. 1997 Distribution of Acinetobacter species on human skin: comparison of phenotypic and genotypic identification methods. *Journal of Clinical Microbiology* **35**, 2819–2825.

Shannon, M. A., Bohn, P. W., Elimelech, M., Georgiadis, J. G., Marinas, B. J. & Mayes, A. M. 2008 Science and technology for water purification in the coming decades. *Nature* **452**, 301–310.

Suquet, C., Warren, J. J., Seth, N. & Hurst, J. K. 2010 Comparative study of HOCl-inflicted damage to bacterial DNA ex vivo and within cells. *Arch. Biochem. Biophys.* **493**, 135–142.

Timmis, K. N. (ed.) 2010 *Handbook of Hydrocarbon and Lipid Microbiology*. Springer-Verlag, Berlin, Heidelberg, pp. 1805–1811.

Van Aken, B. & Lin, L.-S. 2011 Effect of the disinfection agents chlorine, UV irradiation, silver ions, and TiO_2 nanoparticles/near-UV on DNA molecules. *Water Science & Technology* **64** (6), 1226–1232.

Van der Wielen, P. & Van der Kooij, D. 2011 *Opportunistisch ziekteverwekkende micro-organismen in drinkwater. Report BTO 2011.035 (Oppertunistic Pathogens in Drinking Water in the Netherlands)*. KWR, Water Cycle Research Institute, Nieuwegen, The Netherlands.

Venkobachar, C., Iyengar, L. & Rao, A. V. S. P. 1977 Mechanism of disinfection – effect of chlorine on cell-membrane functions. *Water Res.* **11**, 727–729.

Villarreal, J. V., Jungfer, C., Obst, U. & Schwartz, T. 2013 DNase I and Proteinase eliminate DNA from injured or dead bacteria but not from living bacteria in microbial reference systems and natural drinking water biofilms for subsequent molecular biology analyses. *J. Microbiol. Methods* **94**, 161–169.

Whiteman, M., Jenner, A. & Halliwell, B. 1997 Hypochlorous acid-induced base modifications in isolated calf thymus DNA. *Chemical Research in Toxicology* **10**, 1240–1246.

WHO 2006 *Guidelines for Safe Recreational Water Environments. 2: Swimming Pools and Similar Environments.* World Health Organization, Geneva.

Yang, S., Lin, S., Kelen, G. D., Quinn, T. C., Dick, J. D., Gaydos, C. A. & Rothman, R. E. 2002 Quantitative multiprobe PCR assay for simultaneous detection and identification to species level of bacterial pathogens. *Journal of Clinical Microbiology* **40**, 3449–3454.

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