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Bacteria in Public Swimming Pools – Inactivation Kinetics, Prevalence of Pathogens and Value of Indicators

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Summary

Swimming pools and other recreational areas (e.g. hot tubs, saunas) enjoy worldwide popularity. Although procedures like disinfection are mandatory, swimming pools are commonly inhabited by diverse microorganisms potentially hazardous to human health, including viruses, bacteria, protozoans and fungi. Yet, there is a lack of comprehensive knowledge on the inactivation of microbes by disinfectants, the prevalence of pathogens in swimming pool water and the value of indicators in assessing the dangers associated.

One common class of disinfecting agents used in swimming pools are chlorine compounds, both because of their effectiveness in inactivating microbes and the accompanied low costs. Using chlorine, inactivation of microbes relies on the powerful oxidizing features of these compounds. Furthermore, as chlorine remains stable in water, these compounds may be used as residual agents. However, this common practice is accompanied by considerable disadvantages. Chlorine compounds readily react with a broad spectrum of potential partners, of which microbes are only a fraction. As a result, a large variety of disinfection by-products are released, some of which are significantly unhealthy. Considering the lack of knowledge on microbial hazards, the question arises, which concentrations of chlorine are necessary and preferable in public swimming pools.

The present study addresses several aspects of environmental hygiene associated with the use of chlorine in swimming pools. Two main tools in maintaining hygienic conditions are assessed: chlorine disinfection and evaluation of pool water quality using indicator bacteria. Routine data was analyzed for the occurrence of bacterial indicators. Applying MALDI-TOF techniques, it was determined which cultivable and potentially hazardous bacteria may be found in (German) public swimming pools. In order to include also less accessible and therefore less controllable parts of the microbial ecosystem of swimming pools, sand filter material was sampled and examined. Furthermore, inactivation kinetics of bacteria by hypochlorous acid were evaluated based on experiments using a setup mimicking swimming pool conditions and three exemplary test strains (*Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*). Raw data was fitted with several mathematical models taken from literature. In a parallel approach, chlorine consumption during the disinfection process was examined.

To date, regulations on swimming pool maintenance mainly rely on practical experience rather than on empiric data from scientific studies. The present thesis is motivated by the intention to fill this gap by providing information on the topics of disinfection and monitoring. Inactivation kinetics followed comprehensible trends comparable to the results achieved in other fields of research. Chlorine consumption during this process proceeded quite fast, which presumably influenced the outcome. The results on indicators and pathogens imply that the practice of using the first to assess the presence of the latter is questionable. Especially *E. coli* proved very susceptible to chlorine inactivation, making it a weak indicator. For *P. aeruginosa*, the occurrence of survival states (small colony variants, viable but non-culturable states) is assumed. Since the assessment of hygiene relies on culture-dependent methods, this has implications for swimming pool maintenance. The Gram-positive species *S. aureus* was considerably more resistant to chlorine than its Gram-negative counterparts, raising the question whether it would be more reasonable to use this species as an indicator. Also, the question arises if it is acceptable that normally only bacteria are used as indicators.

Zusammenfassung

Schwimmbäder und vergleichbare Erholungseinrichtungen (wie z.B. Whirlpools, Saunen) erfreuen sich weltweiter Beliebtheit. Obwohl Maßnahmen wie Desinfektion Pflicht sind, werden sie für gewöhnlich von einer Vielzahl von Mikroorganismen besiedelt, von denen manche Krankheitserreger sind, wie zum Beispiel Viren, Bakterien, Protozoen und Pilze. Dennoch gibt es wenig übergreifendes Wissen bezüglich der Inaktivierung von Mikroben durch Desinfektionsmittel, das Auftreten von Pathogenen in Schwimmbadwasser und den Wert von Indikatoren zur Ermittlung der verbundenen Gefahr.

Chlorverbindungen vereinen eine hohe Effizienz gegenüber Mikroben mit vergleichsweise niedrigen Kosten und werden daher gerne als Desinfektionsmittel in Schwimmbädern eingesetzt. Die Inaktivierung von Mikroben beruht dabei auf einer stark oxidativen Wirkung. Chlor bleibt in Wasser stabil, weshalb diese Verbindungen für eine anhaltende Behandlung verwendet werden können. Diese verbreitete Praxis hat jedoch auch deutliche Nachteile. Chlorverbindungen reagieren mit vielen potenziellen Partnern, von denen Mikroben nur eine Teilmenge darstellen. Entsprechend wird eine Vielzahl von Desinfektionsnebenprodukten freigesetzt, von denen einige sehr ungesund sind. Betrachtet man das fehlende Wissen in Bezug auf mikrobielle Gefahren, so stellt sich die Frage, welche Konzentrationen an Chlor in Schwimmbädern nötig und wünschenswert sind.

Die vorgestellte Studie befasst sich mit einigen Aspekten der Umwelthygiene. Zwei der Schritte, die unternommen werden, um die Hygiene in Schwimmbädern zu gewährleisten, werden untersucht: die Chlordesinfektion und die Überwachung mittels Indikatorbakterien. Routinedaten wurden auf das Auftretens von Überschreitungen bezüglich einiger Indikatorbakterien hin untersucht. MALDI-TOF wurde verwendet, um zu bestimmen, welche kultivierbaren und potenziell schädlichen Bakterien in (deutschen) Schwimmbädern zu finden sind. Um dabei auch wenig zugängliche und somit schlechter zu kontrollierende Teile des Ökosystems in Schwimmbädern zu berücksichtigen, wurden außerdem Sandfilterproben untersucht. Des Weiteren wurde die Kinetik der Inaktivierung dreier exemplarischer Teststämme (*Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*) durch hypochlorige Säure untersucht. Hierbei kam ein Versuchsaufbau zum Einsatz, der die Bedingungen in Schwimmbädern nachstellen sollte. Die Rohdaten wurden im Anschluss mit verschiedenen mathematischen Modellen gefittet, die aus der bestehenden Literatur ausgewählt wurden.

Derzeitige Standards zur Wartung von Schwimmbädern basieren hauptsächlich auf praktischen Erfahrungen und nicht auf empirisch erhobenen Daten. Die vorgestellte Studie hat zum Ziel diese Lücke zu schließen, indem sie Informationen zu den Themen der Desinfektion und der Überwachung beiträgt. Die erhaltenen Desinfektionskinetiken sind vergleichbar zu den Ergebnissen aus anderen Forschungsfeldern und zeigen nachvollziehbare Abläufe. Der Chlorverbrauch während der Desinfektion verlief allerdings recht schnell, was die Ergebnisse beeinflusst haben könnte. Die Ergebnisse bezüglich des Auftretens von Indikatorbakterien und pathogenen Keimen in Schwimmbadwasser stellen die Nützlichkeit von Indikatoren in Frage. Die Ergebnisse bezüglich Indikatoren und Pathogenen lassen es fragwürdig erscheinen erstere zu verwenden, um letztere nachzuweisen. Insbesondere *E. coli* erwies sich als sehr empfindlich gegenüber Chlor, was diese Art zu einem schwachen Indikator macht.

Für *P. aeruginosa* wiederum wird das Auftreten von Überlebensstadien angenommen (small colony variants, viable but non-culturable states). Dies hat Konsequenzen für die Schwimmbadpflege, da die Ermittlung des Hygienestatus auf Kultivierungsmethoden basiert. Die Gram-positive Art *S. aureus* erwies sich als resistenter gegenüber Chlor als die Gram-negativen Arten, was die Frage aufwirft ob es nicht besser wäre diese Art als Indikator einzusetzen. Außerdem stellt sich die Frage, ob es unterstützenswert ist, dass normalweise nur Bakterien als Indikatoren verwendet werden.

Chapter 1

Context of the Present Thesis [1)]

The present study deals with topics from the field of environmental hygiene - or more precisely - swimming pool water hygiene. Main topics are the inactivation of bacteria by residual chlorine in the pool water and the use of indicator bacteria for assessment of the hygienic state of swimming pools.

1.1 Bacteria in Swimming Pools

Swimming pools enjoy enormous popularity all around the world. Yet, although subject to maintenance procedures like disinfection, swimming pools commonly are nevertheless inhabited by a variety of microorganisms [1, 2]. Some of these are potentially hazardous for human health. Respective microbial communities may be composed of viruses, bacteria, protozoans and fungi [2-4]. Especially through contaminations from human sources, human pathogens may be introduced to swimming pool water [2, 5-7].

Generally, microbes may enter recreational waters by several routes [1]. These include migration from environments in contact (e.g. the ventilated air system) as well as the addition of fresh water. Regarding the introduction of microbes by bathers, the two major routes are fecal contaminations and non-fecal shedding (e.g. of mucus, saliva, sweat, skin particles or vomit) [2]. Fecal matter may be introduced either by washing-off of material residing on bather bodies (e.g. due to inadequate showering behavior), or by accidental fecal release (AFR). However, aside from human sources, contaminated water sources and introduction by animals (e.g. water birds or rodents) can also play a role.

Once introduced to a swimming pool, the survival of bacteria depends on diverse environmental factors such as temperature, pH, exposure to light, the content of organic matter in the water, and the presence of other organisms [8]. With respect to individual pathogenicity, most agents of relevance belong to the taxonomic groups of viruses and fungi [2]. The group of bacteria includes pathogenic agents, although most are opportunistic rather than primary pathogens (meaning that these species only become a health hazard, if a potential host has a preliminary condition allowing for an infection). However, the ability of some bacterial species to form biofilms enhances their capability to persist under the conditions given in swimming pools (e.g. *Pseudomonas aeruginosa* and *Legionella pneumophila*) [9]. Consequently, these species also more likely infect bathers than other bacterial species. Furthermore, there are some species of free-living bacteria which are able to grow under the environmental conditions given in swimming pools [10]. These species potentially multiply up to a point at which infectious concentrations are reached (e.g. causing respiratory, dermal or even central nervous system infections) [2].

[1)] Some of the explanations in this chapter were also published elsewhere:

Schlosser T., Kreuter L., and L. Erdinger, *Disinfection in Recreational Areas*, in *Disinfectants: Properties, Applications and Effectiveness*, Eds. Cardoso A.S., Martins Almeida C.M., Costa Cordeiro T., and de Jesus Gaffney, Nova Science Publishers, pp. 171-200.

Next to several viruses (e.g. Norovirus, Enterovirus), protozoans (e.g. *Giardia* spp., *Cryptosporidium* spp.), and fungi (e.g. *Trichophyton* spp.), the WHO lists the following bacteria associated with infections after bathing activity: *Shigella* spp. and *E. coli* O157, *Legionella* spp., *Leptospira interrogans sensu lato*, *Mycobacterium* spp., *Pseudomonas aeruginosa*, and *Staphylococcus aureus* [2].

1.2 Disinfection of Swimming Pools

Disinfectants are antimicrobial agents, used to inactivate microorganisms. Disinfection does not necessarily mean that all present microbes are destroyed and is less effective than sterilization. In order to be qualified as a disinfecting agent, a chemical compound needs to fulfill several criteria. These include the effectiveness of a given compound in inactivating microbes, applicability in a given medium (e.g. air, water, surfaces), toxicity and other hazards associated with use of the respective agent, and, last but not least, also operating costs [11].

In order to maintain a hazard-free environment for bathers, swimming pool water is disinfected through chemical and/or physical means. In doing so, the risk of an infection acquired during bathing is minimized, as microbial contaminations are reduced below infectious doses.

Chlorine compounds are one class of disinfecting agents combining high effectiveness with comparably low costs. Therefore, chlorine disinfectants are quite popular [12], i.e. being used in swimming pools all around the world [13]. The inactivation of microbes by chlorine compounds relies on their powerful oxidizing features. Chlorine biocides may be used as residual agents [2] because they remain stable in water for a considerable time [14].

Commonly, molecular chlorine (Cl_2), hypochlorous acid and hypochlorite are summarized as 'free available chlorine' [2, 14, 15]. Of these three, the main contributor to the inactivation of microbes is hypochlorous acid (HOCl). Dependent on the pH value of the medium, hypochlorous acid dissociates into hydrogen (H^+) and hypochlorite (OCl^-) ions [2].

There are diverse factors influencing the efficiency of disinfection. For example, the type of reactor is of crucial importance for the outcome in any setup designed to examine inactivation kinetics. In swimming pool basins and experimental setups associated with them, especially hydraulic characteristics need to be considered.

Like chemical reactions, microbial inactivation is strongly determined by temperature. The underlying relationship is expressed by the Arrhenius equation [11, 16], where the rate constant k is given by [17]

$$k = A\, e^{-\frac{E_a}{RT}} \tag{1}$$

and where A is the pre-exponential factor (a constant for each given reaction; the frequency of collisions in the correct orientation), R is the universal gas constant [8.314 kg m^2/s^2 mol K], T is the absolute temperature [K], and E_a is the activation energy for the reaction (units as RT). With increasing temperature, inactivation rates also increase. Additionally, changes in temperature may have effects on solubility and decomposition of the disinfectant.

Regarding disinfection with chlorine compounds, next to temperature, also pH is of considerable influence [18]. The dependence of chlorine speciation on pH is expressed by the following equilibrium [11]:

$$H_2O + Cl_2 \leftrightarrow HOCl + Cl^- + H^+ \leftrightarrow OCl^- + Cl^- + 2H^+ \tag{2}$$

At pH values higher than 3, elemental chlorine (Cl_2) is completely hydrolyzed to hypochlorous acid (HOCl). If, again, the pH increases above 4, HOCl dissociates into hypochlorite (OCl^-) and hydrogen (H^+) ions. If pH values higher than 10 are reached, hypochlorous acid is completely dissociated. Hypochlorous acid is approximately 80 to 200 times more effective in inactivating microbes than hypochlorite [2, 11, 14]. Furthermore, the pH may affect the stability of inactivation agents and the resistance of some microbial species [11].

Accordingly, the pH value of swimming pool water has a strong impact on disinfection efficiency and is commonly considered in standards on swimming pool maintenance [2, 19]. The same applies for water temperature [15, 20], a parameter also strongly determined by bather comfort.

1.3 Indicator Bacteria

In addition to disinfection, another major step in the prevention of waterborne infection is routine control for indicator bacteria [2, 19]. Instead of examining the presence of pathogenic species, concentrations of indicator bacteria are determined. Several reasons speak for the determination of indicators instead of pathogens, of which feasibility is only the most prominent. For example, the large variety of pathogenic agents potentially present in swimming pool water complicates the decision as to which parameters should be tested on a regular basis. Also, concentrations of pathogens are typically comparably low, hence impeding reliable detection.

Indicator bacteria are representatives of groups of microbes occurring under defined circumstances (e.g. after a fecal incident or after process failure). According to the WHO, a microbial fraction or species has to match diverse criteria to be applicable as an indicator [2]. First, the fraction needs to occur in the medium of interest (e.g. swimming pool water) in concentrations exceeding those of pathogenic microbes. Furthermore, the indicator should be easy to isolate, identify and enumerate. Tests for the indicator should be inexpensive, allowing for application in high numbers of samples. Moreover, to provide useful information, the indicator should be absent in unpolluted environments, while being present if the source of contamination is present (e.g. fecal or shed material). Additionally, the indicator should not multiply in the examined environment (e.g. chlorinated water). Finally, the indicator needs to respond to the given conditions and to treatment in a manner comparable to pathogens of concern.

Both research on bacterial contaminations in swimming pools and proposal of indicator species for measurement of associated health risks date back about one century [21]. Today, bacterial indicators include the heterotrophic plate count (HPC), a parameter providing a general measure of microbial numbers, and several specific indicators such as *Escherichia coli* and coliforms (indicating fecal contaminations), and *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Legionella* spp. (indicating non-fecal contaminations or process failures) [2, 19, 22]. According to the strict definitions of the WHO, of these, only HPC, coliforms and *E. coli* are considered true indicators [2]. Yet, also the other indicators listed are employed in the field. This is because, traditionally, indicators were used solely for identification of fecal contaminations. Today, however, three types of microbial indicators are used [23]: Process indicators are administered to test the efficacy of processes such as cleaning and disinfection routines. Fecal indicators are used to verify that no fecal contaminations (and associated pathogens) are present. Finally, index and model organisms indicate the presence of related pathogens.

1.3.1 Heterotrophic Plate Count

The term heterotrophic plate count (HPC) refers to several culture dependent microbial fractions which are recovered from water samples by varying methods. All versions of the HPC have in common that they cover a broad range of microbes. Hence, the heterotrophic plate count always provides general information on the overall presence of microbes, without giving information, however, on the individual species participating. The only criterion considered is, that the respective microbes are heterotrophs, meaning that they require organic carbon for their growth [24]. Apart from bacteria, this potentially also includes molds and yeasts. As there is no universal HPC test method, results may differ significantly depending on which approach was selected for examination [24]. For example, between different approaches, incubation temperatures range from 20 °C to 40 °C, incubation times cover a few hours to several weeks, and nutrient contents of growth media are specified from low to high [24].

The use of HPC indicators has a long tradition in water microbiology [24]. In the 19th century, HPC variants were used as process indicators (e.g. for testing the proper functioning of sand filtration). However, with the introduction of more specific indicators (e.g. for fecal contaminations), the use of HPC tests declined during the 20th century. Nonetheless, until today, HPC measurements are included in regulations on water safety in many countries [24].

For the purposes of this study, the test method described by German DIN 38411 was used [25]. Accordingly, nutrient agar, a growth medium designed for the qualitative and quantitative analyzation of waters (e.g. drinking water, mineral water, and well water) was used to recover bacteria. Table 1 lists the components of nutrient agar.

Table 1: Composition of nutrient agar (DEV) [26].

Component	Grams per Liter Purified Water
Meat Peptone	10.0
Meat Extract	10.0
Sodium Chloride	5.0
Agar	18.0

1.3.2 Coliform Bacteria / *Escherichia coli*

The group of bacteria known as coliforms is an artificial class of Gram-negative, rod-shaped, and non-spore forming bacteria. The members of the group not necessarily are directly related. Especially the subcategory of fecal coliforms is composed of suitable indicators for fecal contamination in swimming pools. These bacteria are only able to reproduce if located in the intestines of animals. However, as coliform bacteria may survive outside the intestines of animals for several days, they may be isolated from media like swimming pool water for increased periods after their introduction. Further benefits of coliforms as indicators are, that they are both non-pathogenic and easy to culture, making them an ideal indicator for fecal contaminations.

Before 1994, coliforms were classified by their ability to ferment lactose at temperatures of 36 ± 2 °C and the production of acid or gas during this process [27]. At this time, the group included the genera *Escherichia*, *Enterobacter*, *Klebsiella* and *Citrobacter*. In 1994, the definition was changed for the first time. Now, coliforms were classified by their ability to acidify lactose [28]. Coliforms additionally included the genera *Hafnia*, *Pantoea*, *Serratia* and *Yersinia*. The newest approach states that the presence of a lacZ gene [29] is the crucial criterion to include a

bacterial species in this group. Two subcategories have been introduced: primary environmental coliforms (e.g. *Cedecea*, *Ewingella*, *Leclercia*, *Moellerella*, *Pantoea*, *Rahnella*) and primary fecal coliforms (e.g. *Citrobacter*, *Enterobacter*, *Hafnia*, *Klebsiella*, *Serratia*, *Yersinia*).

While commonly included in the group of fecal coliforms, the species *Escherichia coli* is considered an even more specific indicator of fecal pollution than other members of this group. Accordingly, apart from examination of coliforms, this species is used individually as a parameter for the assessment of water quality [2, 19, 30].

For the present study, the Colilert Test (IDEXX) was used to detect coliform bacteria and *Escherichia coli*. This test uses two nutrient-indicators, ONPG and MUG, which are metabolized by the coliform enzyme β-galactosidase and the *E. coli* enzyme β-glucuronidase, respectively. Metabolization by β-galactosidase results in a color change towards yellow. Metabolization by β-glucuronidase results in fluorescence. By counting fields in a tray which show these changes, the most probable number of bacteria per 100 ml of sample [MPN/100 ml] is calculated.

1.3.3 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a Gram-negative inhabitant of moist habitats. This species is used as a process indicator in Germany [19], meaning that it is employed as a parameter to identify failures in procedures such as regular cleaning, monitoring and maintenance of disinfectant concentrations and pH [2]. Presumably, shedding from infected bathers is the major source for *P. aeruginosa* in swimming pools and related environments [31]. The warm and moist conditions of swimming pools provide an ideal climate for growth [2]. After initiating action to remove a contamination with *P. aeruginosa*, the occurrence of recontaminations is quite common. Such recontaminations may go back to reintroductions from areas not entirely reached by hydraulics and maintenance procedures (e.g. parts of the filter system) [2], as *Pseudomonas aeruginosa* is known for its ability to form persistent biofilms. Furthermore, although several types of illnesses are associated with the presence of *P. aeruginosa* in pools and similar environments, the absolute incidence of such infections is difficult to determine as, normally, symptoms are comparably mild and self-limiting, therefore not causing patients to seek immediate medical attention [2].

For the present study, *P. aeruginosa* was recovered from water samples by filtration of 100 ml and subsequent growth on cetrimide agar (BD Pseudosel agar, Becton Dickinson). Table 2 lists the components of cetrimide agar.

Table 2: Composition of cetrimide agar [32].

Component	Grams per Liter purified water
Pancreatic Digest of Gelatin	20.0
Magnesium Chloride	1.4
Potassium Sulfate	10.0
Glycerol	10.0 ml
Cetrimide	0.3 g
Agar	13.6

1.3.4 *Staphylococcus aureus*

Staphylococcus aureus is a Gram-positive, non-spore forming species of bacteria. According to the current state of research, humans are the only existing reservoir [2]. However, this species is found only in a minority of healthy individuals [33].

S. aureus has frequently been isolated from swimming pool water [34]. During swimming, clusters of *S. aureus* cells are washed-off from the bodies of human bathers [35]. Water contaminations have been associated with detrimental effects on human health (e.g. skin rashes, wound and eye infections) [2, 36, 37]. Presumably, inactivation of *S. aureus* is achieved by maintaining concentrations of residual chlorine higher than 1 mg/L [37, 38].

The World Health Organization does not particularly recommend routinely performed monitoring for *Staphylococcus aureus* [2]. Nonetheless, the WHO states that concentrations in swimming pools should be kept lower than 100/100 ml [2]. Although *S. aureus* is also not listed as an indicator in German regulations, there are studies supporting the use of this species as an indicator for process failures in swimming pools [39-41].

1.4 Main Objectives of the Present Study

As outlined above, there are two major steps in providing a non-hazardous environment in swimming pools with respect to the presence of pathogenic bacteria: disinfection of bathing water (e.g. using residual chlorine) and routine monitoring for microbial contaminations (e.g. using indicator bacteria).

The present study investigated both these steps; each chapter deals with another aspect of these two main topics. In chapter 2, routine data on the occurrence of indicator bacteria in public swimming pools is evaluated for the incidence of exceedance. Limits for the individual parameters were adopted from German standards on swimming pool maintenance. Chapter 3 addresses the inactivation kinetics of three representative bacterial strains of chlorine under conditions as commonly found in (German) public swimming pools. For the examination, a setup was designed using a large ratio of test volume to inoculum volume. In chapter 4, the occurrence of small colony variants, a phenomenon encountered in the experiments described in chapter 3, is highlighted and interpreted in detail. The phenomenon has also been described in the context of clinical studies and may be of relevance for public health considerations. Chapter 5 deals with chlorine consumption during the process of bacterial inactivation. Chlorine consumption could have influenced the outcome of the experiments performed for chapter 3. Furthermore, it plays a role in swimming pool maintenance. Finally, chapter 6 lists information on which bacteria can be recovered from water samples taken in (German) public swimming pools. The focus lies on such species potentially hazardous for human health. In total, the study provides information on several aspects of swimming pool maintenance.

Chapter 2

Analysis of Routine Data for the Incidence of Indicators

Chapter Abstract

Routine examination of indicator bacteria is an important tool in maintaining hygienic conditions in swimming pools. In Germany, the standard DIN 19643 determines which indicator bacteria have to be examined on a regular basis [19]. These include the process indicator *Pseudomonas aeruginosa,* the fecal indicator *Escherichia coli* and the heterotrophic plate count at 36 °C. In analogy to regulations on drinking water [22], for each of these parameters, specific limit values have been defined (0 cfu/100 ml for the specific indicators and 100 cfu/100 ml for the heterotrophic plate count). Swimming pool maintenance requires compliance with these requirements. Respectively, exceedance of these limits leads to the necessity to perform responsive steps such as cleaning and use of increased concentrations of disinfectants. Apart from these microbiological parameters, diverse chemical parameters are examined (e.g. chlorine concentration and pH). However, of these, only chlorine concentration was included in the considerations made for this chapter.

A dataset was analyzed statistically for the incidence of cases of exceedance regarding the parameters mentioned. The respective dataset was extracted from a database generated during routine control. The dataset contained information on the examined parameters, determined on-site or subsequent to sampling. Exceedance of the selected parameters was defined in accordance with German regulations. Subsequent to extraction, data was analyzed for the occurrence of patterns e.g. in the chronological distribution of exceedance events and the severity of exceedance respectively.

Generally, cases of exceedance occurred only at low frequencies for all indicator bacteria examined. However, the achieved results still indicate an annual rhythm in the occurrence of exceedance events. Contaminations most commonly occurred during the summer months. Regarding the severity of these contaminations, a predominance of cases with low concentrations of bacteria was determined. Within the range of chlorine concentrations applied in accordance with German regulations (0.3 to 0.6 mg/L of free chlorine), chlorine concentrations did not have a measurable effect on the occurrence of contaminations with indicator bacteria. However, considering the broader total range of chlorine concentrations used in the swimming pools examined, there was a detectable effect on the incidence of contaminations with indicator bacteria. Of all indicators examined, apparently *Escherichia coli* was most susceptible to the exposure to chlorine. This is in line with the results achieved in other chapters of the present thesis and has implications discussed in the overall conclusions.

2.1 Introduction

Swimming pools are inhabited by a large variety of microorganisms. As outlined before, microbial communities in swimming pools may include such species of pathogenic potential for human bathers. Thus, to avoid health risks associated with contact with these pathogens, routine control for microbial contaminations is an integral part of pool maintenance. Estimation of the level of contamination with microbes is commonly achieved by examination for indicator bacteria. For instance, *Escherichia coli* is used as an indicator for fecal contaminations and *Pseudomonas aeruginosa* to assess the general hygienic state of swimming pools.

For the present chapter, data collected during routine control was statistically analyzed for the incidence of cases of contamination with several indicator bacteria. Positive cases were assessed for the exceedance of the limits defined in German DIN 19643 [19]. Apart from those indicator bacteria mentioned, namely *P. aeruginosa*, *E. coli* and the heterotrophic plate count at 36 °C, also coliform bacteria and the heterotrophic plate count at 22 °C were included in the examinations. The selected additional parameters were added, as they were part of the routine examinations performed to create the database and, therefore, information on them was available. Including them was beneficial for getting a general idea of bacterial contaminations in the swimming pool water samples examined.

2.2 Materials and Methods

2.2.1 Dataset Used for Assessment

The examined dataset was provided by an inspection laboratory (Medical Microbiology and Hygiene, University Hospital Heidelberg) accredited for the examination of bathing water by the German accreditation body (Deutsche Akkreditierungsstelle GmbH, DAkkS) at the time. All data has been produced during accredited laboratory work. The dataset was extracted from a local library and subsequently analyzed for cases of exceedance regarding several indicator bacteria. In adaption to German regulations on swimming pool hygiene [19], the following parameters were included: free chlorine concentrations [mg/L], heterotrophic plate count at 22 °C (HPC 22 °C), heterotrophic plate count at 36 °C (HPC 36 °C), *Escherichia coli*, coliform bacteria, and *Pseudomonas aeruginosa*. Data collected between January 2013 and December 2017 was considered in the analysis.

2.2.2 Requirements According to German Standard DIN 19643

As explained, the limits for the selected parameters were set as defined in German standard DIN 19643 [19]. According to this standard, the limits for chlorine concentrations in public swimming pools are 0.3 mg/L to 0.6 mg/L of free chlorine. Requirements regarding microbial indicators are indicated in table 3. Since there are no limits defined for the two parameters coliform bacteria and HPC at 22 °C, for these parameters the limits defined for *Escherichia coli* and HPC at 36 °C respectively were used. (As described in chapter 1, in experiments performed for the present study, instead of the growth medium defined by DIN EN ISO 6222, DEV agar was used as determined by the German standard on drinking water [22]).

Table 3: Requirements for swimming pool water according to German regulations [19].

Parameter	Requirement	Verification procedure
Escherichia coli	0 cfu/100 ml	DIN EN ISO 9308-1
coliform bacteria	0 cfu/100 ml [1)]	DIN EN ISO 9308-1
HPC 22 °C	100 cfu/ml [2)]	DIN EN ISO 6222
HPC 36 °C	100 cfu/ml	DIN EN ISO 6222
Pseudomonas aeruginosa	0 cfu/100 ml	DIN EN ISO 16266

[1)] not part of routine examinations; adapted from regulations for *E. coli*; [2)] not part of routine examinations; adapted from regulations for HPC 36 °C

2.3 Results

2.3.1 Chronological Distribution

In total, 6,789 sample drawings were performed in the examination period. In figure 1, the distribution of the frequencies of sample drawings over the examined period of five years is presented. Graph (a) shows the results broken down into the frequencies per month. In graph (b) mean values calculated from the results of all five years are shown.

Within this dataset, 394 cases of insufficient concentrations of free chlorine (< 0.3 mg/L) were identified (5.8% of total cases). Regarding indicator bacteria, several cases of exceedance were determined. These comprised 27 cases of contamination with *E. coli* (0.4%), 121 cases of contamination with other coliform bacteria (1.7%), 54 cases of presence of HPC 22°C (0.8%) at concentrations exceeding 100 cfu/ml, 118 cases of presence of HPC 36 °C (1.7%) at concentrations exceeding 100 cfu/ml, and 66 cases of contamination with *P. aeruginosa* (1%).

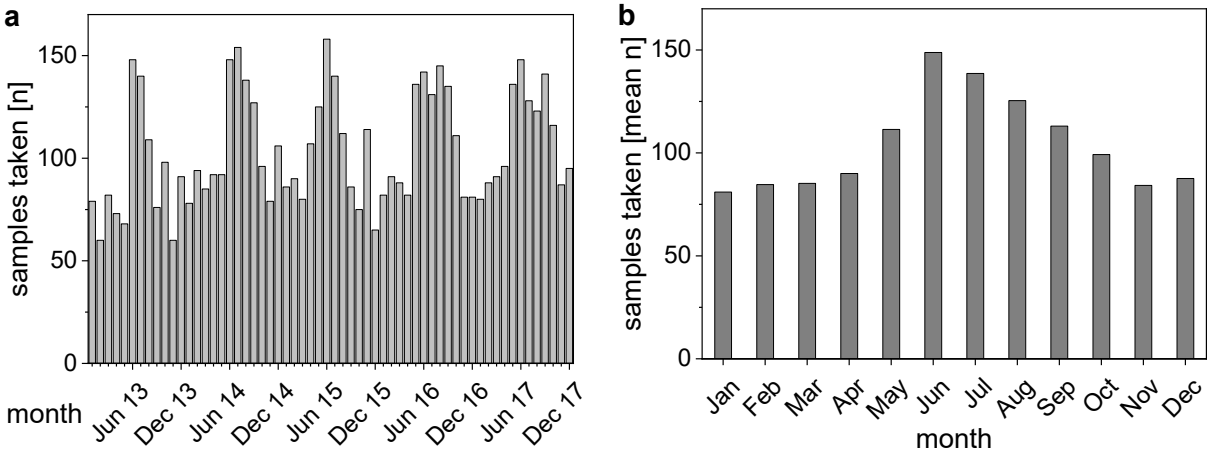

Fig. 1: Wave pattern in the chronological distribution of the frequencies of sample drawings in public swimming pools per month over the course of five years (2013 to 2017): **(a)** sample drawings [n] per month; **(b)** mean values of sample drawings [n] per month (calculated from the results of 2013 to 2017). As can be seen, there are variations in the frequencies of sample drawings over the course on one year.

In figure 2, the chronological distribution of cases of objections is presented for the individual parameters. Apart from indicator bacteria, also chlorine concentrations below the limit of 0.3 mg/L were recorded and were included in the graphs. Data is expressed as numbers of cases related to the number of sample drawings in the respective month. In doing so, the percentage of objections in total cases was calculated, as a check for the influence of the total number of sample drawings. In order to identify potential patterns, data was aggregated by month of sample drawing. In figure 3, data is expressed as total numbers of cases per month, representing the aggregated results of five years (2013 to 2017). As indicated by the mean values presented in figure 3, frequencies of the incidence of objections regarding the selected parameters varied between the months of one year. In order to quantify these differences, the range of occurrence frequencies was examined. The respective results are presented in figure 4 in the form of box plots. In doing so, the individual years can be compared to each other, regarding the respective distributions of positive cases for each parameter.

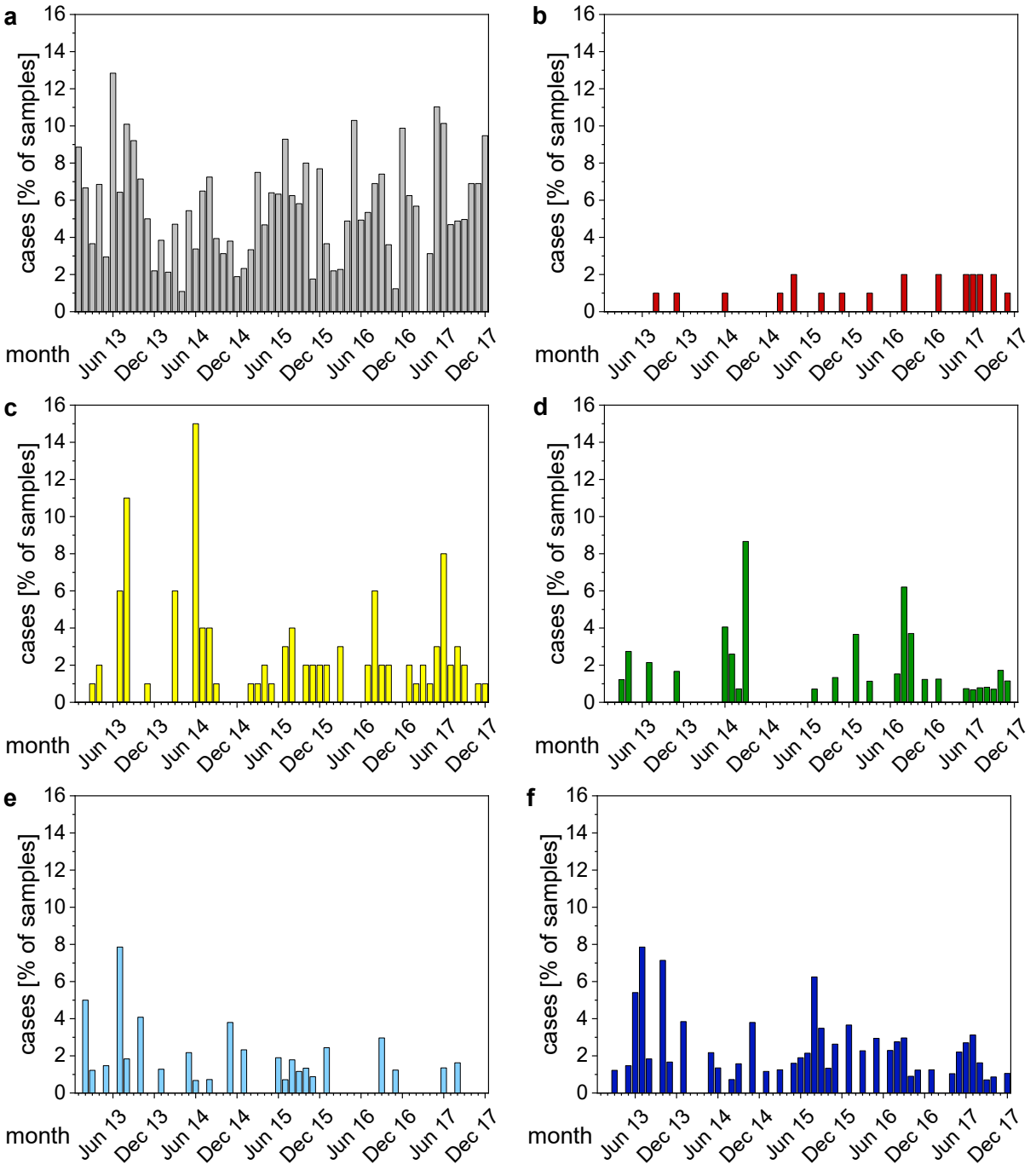

Fig. 2: Chronological distribution of the frequencies of objections [% of total samples per month] over the course of five years (2013 to 2017) regarding chlorine concentrations and indicator bacteria. As can be seen, there commonly were higher incidences of low chlorine concentrations than of contaminations with indicator bacteria: **(a)** free chlorine concentrations below 0.3 mg/L; **(b)** cases of detection of *Escherichia coli* (>0 cfu/100 ml); **(c)** cases of detection of coliform bacteria (>0 cfu/100 ml); **(d)** cases of detection of *Pseudomonas aeruginosa* (>0 cfu/100 ml); **(e)** cases of detection of HPC counts at 22 °C exceeding the limits of the German standard [22] (bacteria count ≥100/ml) per month; **(f)** cases of detection of heterotrophic plate counts at 36 °C exceeding the limits of the German standard [22] (bacteria count ≥100/ml) per month.

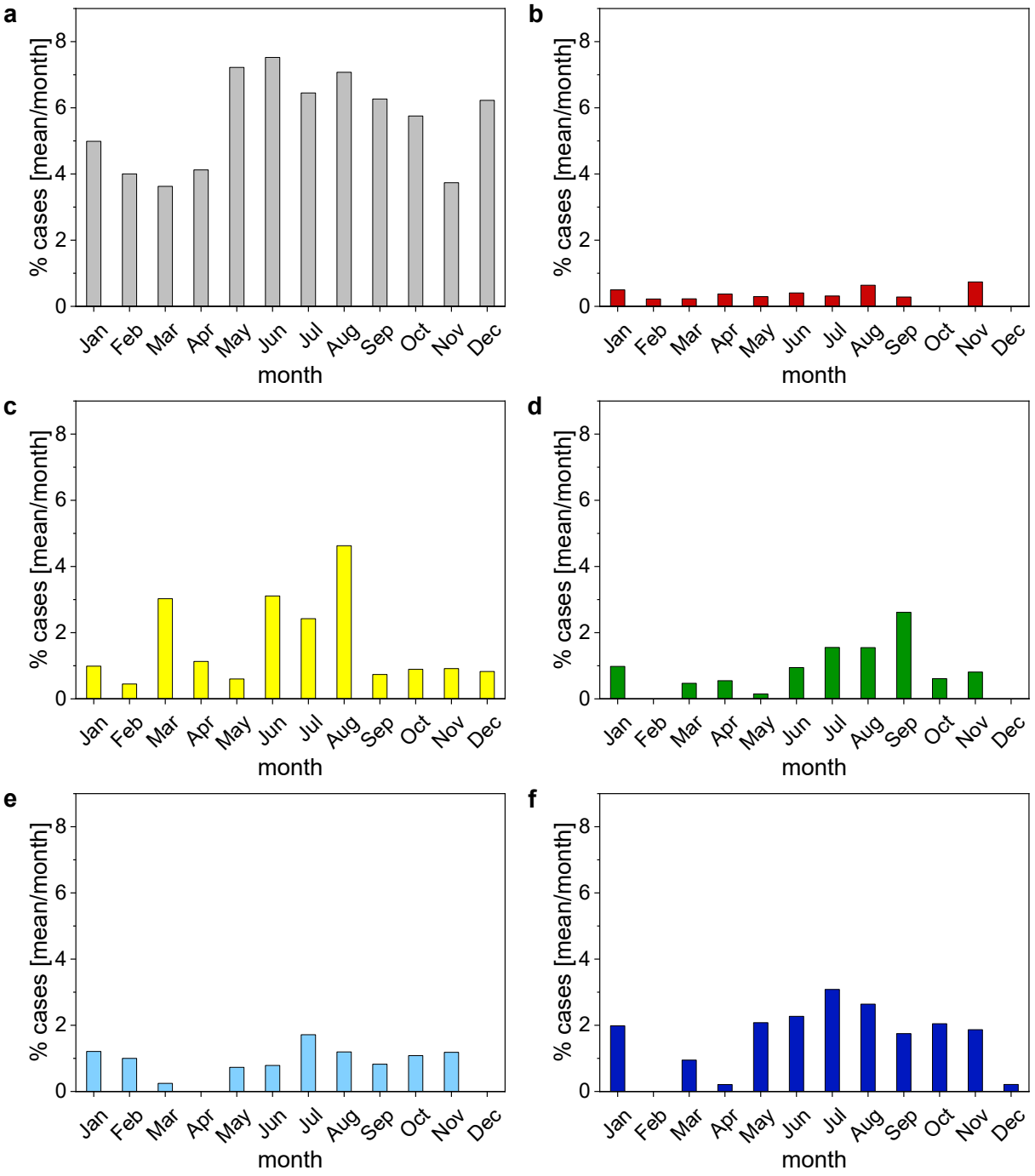

Fig. 3: Mean values of cases of objection [% of total samples] per month calculated from the results of 2013 to 2017. As can be seen, there commonly were higher incidences of low chlorine concentrations than of contaminations with indicator bacteria: **(a)** free chlorine concentrations below 0.3 mg/L; **(b)** cases of detection of *Escherichia coli* (>0 cfu/100 ml); **(c)** cases of detection of coliform bacteria (>0 cfu/100 ml); **(d)** cases of detection of *Pseudomonas aeruginosa* (>0 cfu/100 ml); **(e)** cases of detection of HPC counts at 22 °C exceeding the limits of the German standard [22] (bacteria count ≥100/ml) per month; **(f)** cases of detection of heterotrophic plate counts at 36 °C exceeding the limits of the German standard [22] (bacteria count ≥100/ml) per month.

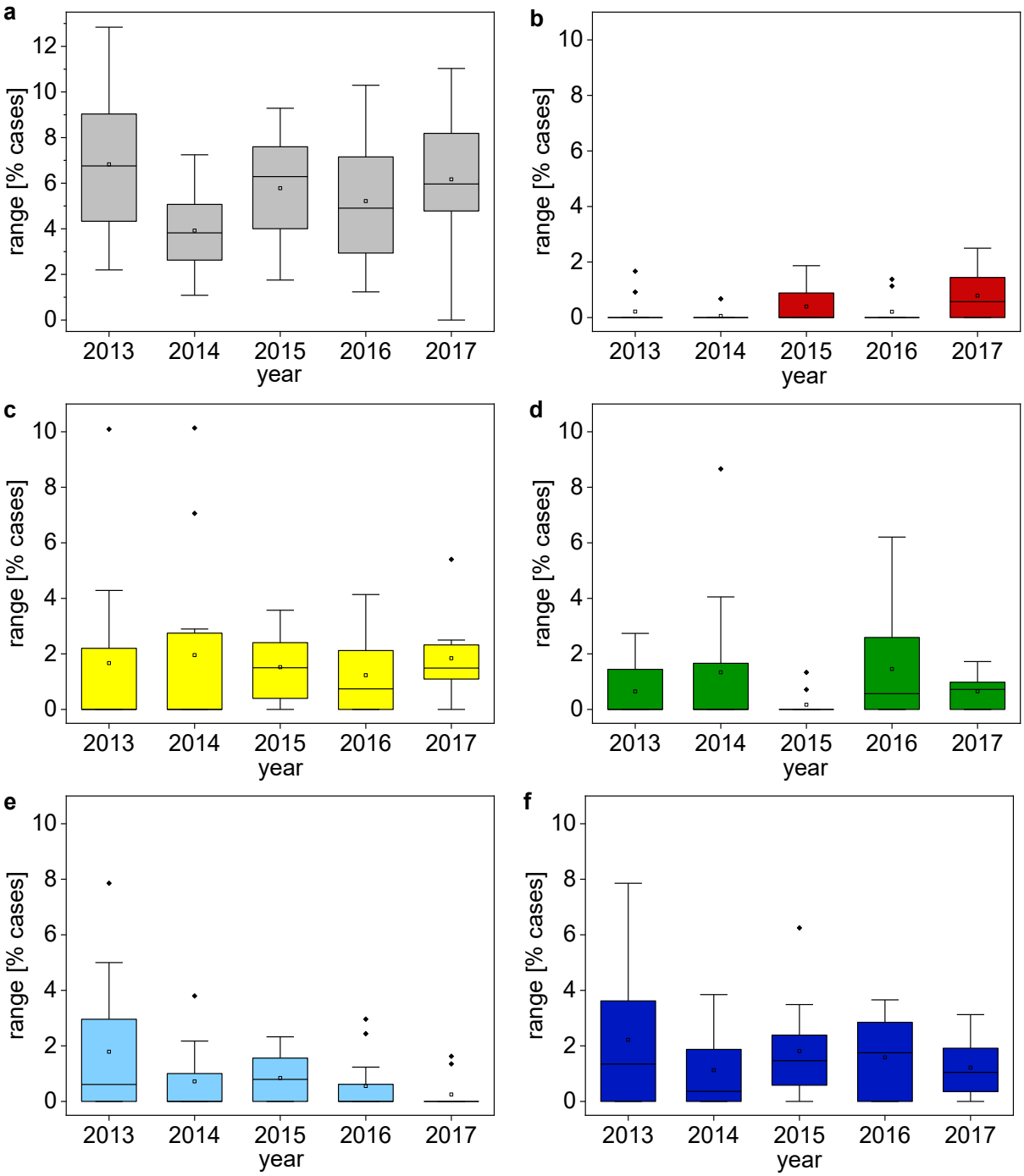

Fig. 4: Range of the determined frequencies of cases in % of total samples. As shown, the range of frequencies for the parameters did not vary much between the five years of examinations. Results express calculated data for one year each (mean values of counts of the individual months): **(a)** low free chlorine concentration (<0.3 mg/L); **(b)** *Escherichia coli* (>0 cfu/100 ml); **(c)** coliform bacteria (>0 cfu/100 ml); **(d)** *Pseudomonas aeruginosa* (>0 cfu/100 ml); **(e)** HPC at 22 °C (≥100/ml); **(f)** HPC at 36 °C (≥100/ml).

2.3.2 Concentrations of Bacteria

Recorded cases of exceedance were sorted into severity groups dependent on the respective concentrations of recovered bacteria. The results are presented in figure 5 and 6.

The limits of the severity groups were set as follows: For the specific indicators (*E. coli*, coliforms and *P. aeruginosa*) the limits were 1 to 9, 10 to 49, 50 to 99, 100 to 149, 150 to 199 and ≥ 200 [cfu/100 ml]. Here, every case of detection was considered an exceedance. For HPC 22 °C and HPC 36 °C respectively, the limits were 1 to 9, 10 to 49, 50 to 99, 100 to 199, 200 to 299, and ≥ 300 [cfu/ml]. Here, only cases with concentrations ≥ 100 cfu/ml were considered as exceedance. Groups with cfu counts of ≥ 200 cfu/100 ml and ≥ 300 cfu/ml respectively where included since concentrations higher than 200 cfu/100 ml and 300 cfu/ml commonly were recorded in this way in the data set (detection limits).

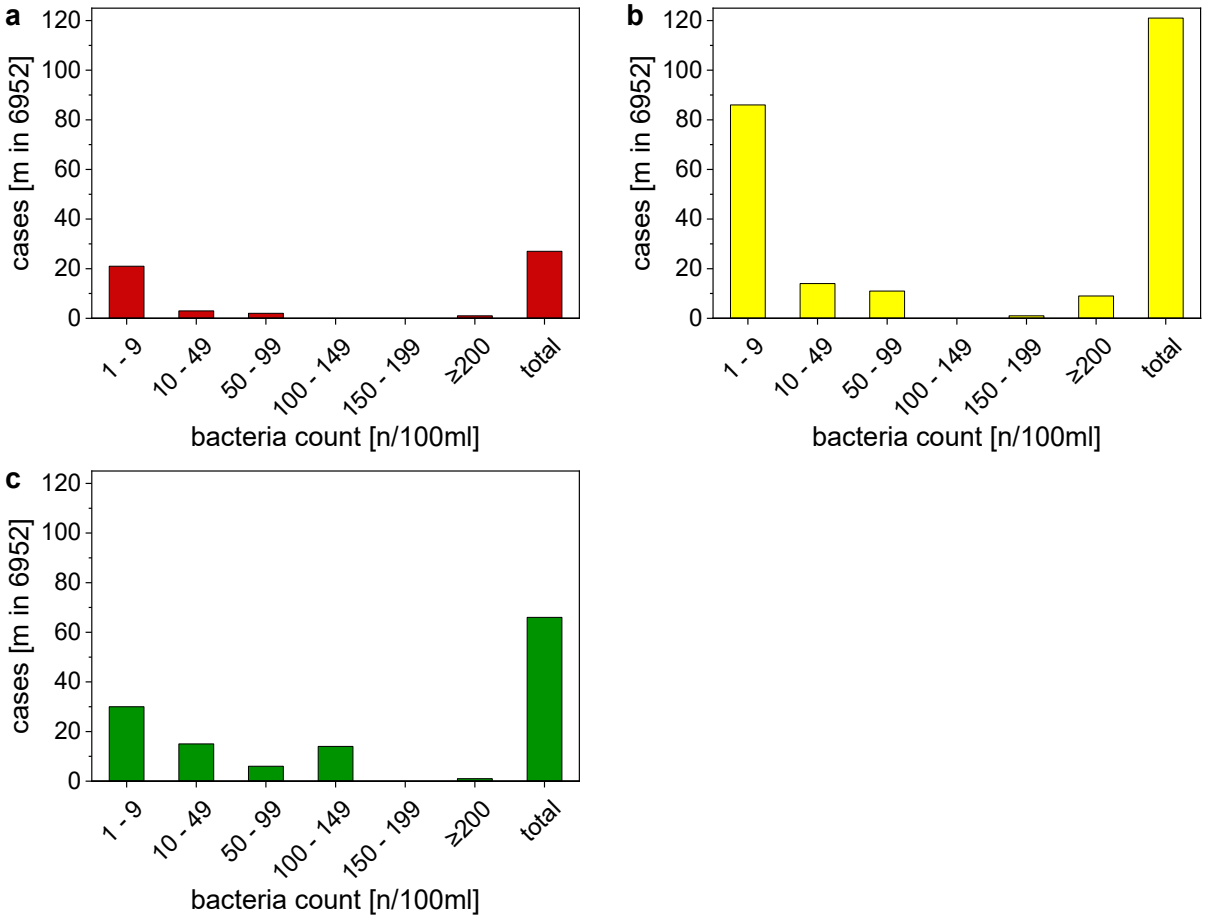

Fig. 5: Numbers of cases of contamination with specific indicator bacteria related to the respective concentrations of bacteria in individual samples. With increasing individual bacteria counts, the number of cases decreases for all three examined indicator bacteria: **(a)** *Escherichia coli*, **(b)** coliform bacteria, and **(c)** *Pseudomonas aeruginosa*. Total numbers of cases of contamination were 27 for *E. coli*, 121 for coliform bacteria and 66 for P. *aeruginosa*.

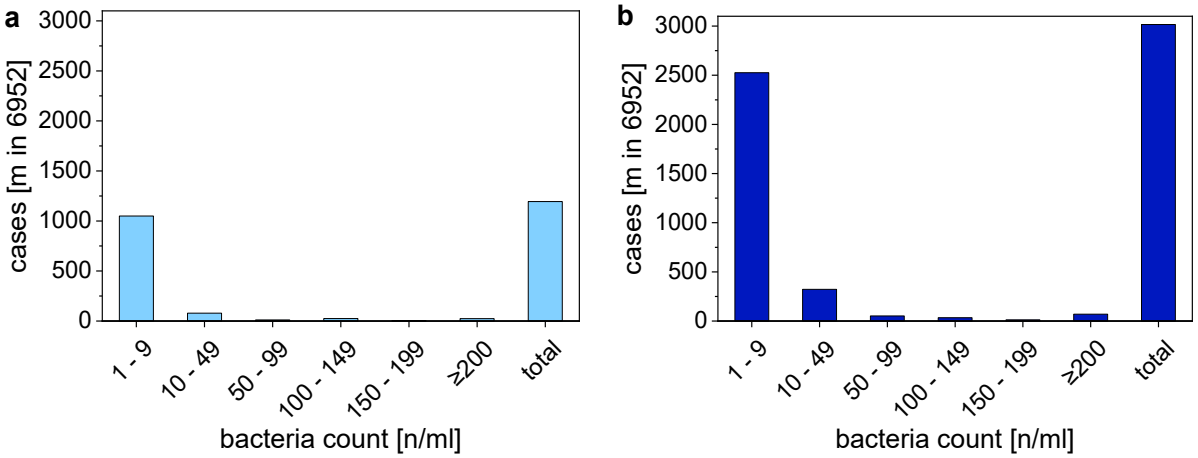

Fig. 6: Numbers of cases of contamination with HPC related to the respective concentrations of bacteria in individual samples. With increasing individual bacteria counts, the number of cases decreases for both parameters: **(a)** HPC at 22 °C, **(b)** HPC at 36 °C. Total numbers of cases of contamination were 1,229 for HPC 22°C and 3,073 for HPC 36°C. Cases up to 100 cfu/ml are not considered as exceedance according to the German standard [22]. With increasing individual bacteria counts, the number of cases decreases for all three examined indicator bacteria.

2.3.3 Correlation Chlorine Concentration and Incidence of Bacteria

In order to examine whether free chlorine concentrations had a measurable effect on the occurrence of indicator bacteria in swimming pool water, both these parameters were analyzed for their correlation. The results are presented in figures 7 to 11. For *Escherichia coli* and coliform bacteria, the respective counts were determined in form of the most probable number (MPN). Here, a limit of 201 is set by the examination procedure.

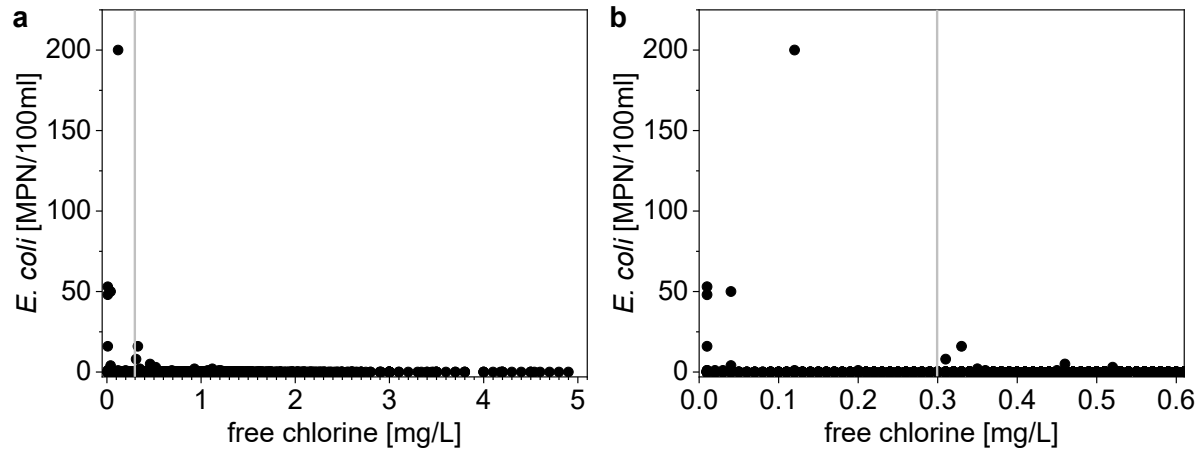

Fig. 7: Correlation of free chlorine concentrations [mg/L] and incidence of *E. coli* [n/100 ml]. The few occurring cases were correlated to low chlorine concentrations: **(a)** scale over the full range of measured concentrations of free chlorine; **(b)** scale over the range of free chlorine concentrations covered by the German standard on swimming pool maintenance [19]. The gray line indicates the respective lower limit as defined by the standard for free chlorine (0.3 mg/L).

For the heterotrophic plate count at 22 and 36 °C respectively, cases higher than 300 cfu/ml were included as 300. For *P. aeruginosa*, cases higher than 100 cfu/100 ml were included as 100.

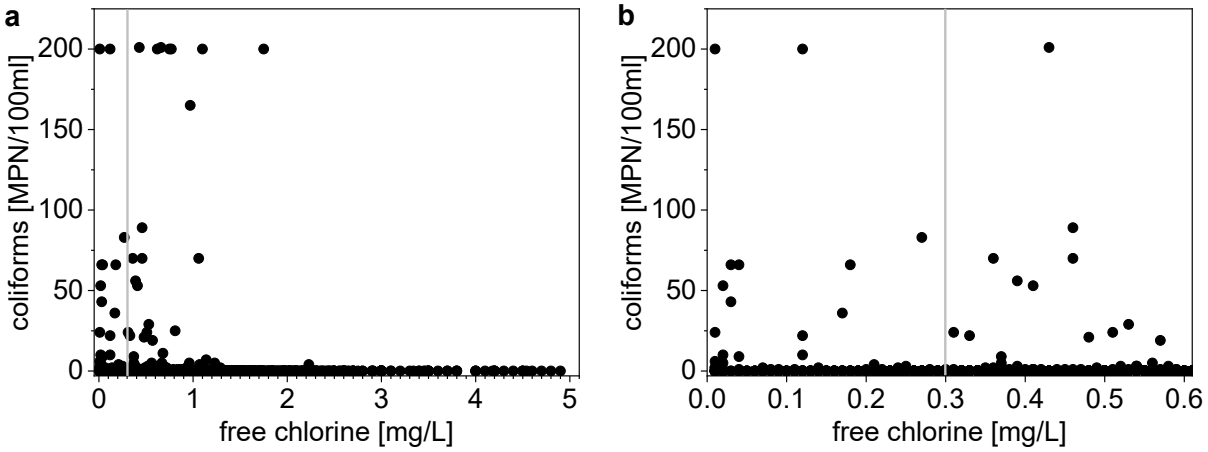

Fig. 8: Correlation of free chlorine concentration [mg/L] and incidence of coliform bacteria [n/100 ml]. The occurring cases were correlated to comparably low chlorine concentrations: **(a)** scale over the full range of measured concentrations of free chlorine; **(b)** scale over the range of free chlorine concentrations covered by the German standard on swimming pool maintenance [19]. The gray line indicates the respective lower limit as defined by the standard for free chlorine (0.3 mg/L). Cases with bacteria counts exceeding 201 MPN/100 ml were frequently stated as `>201´. Hence, they occur as lines of dots in the plots.

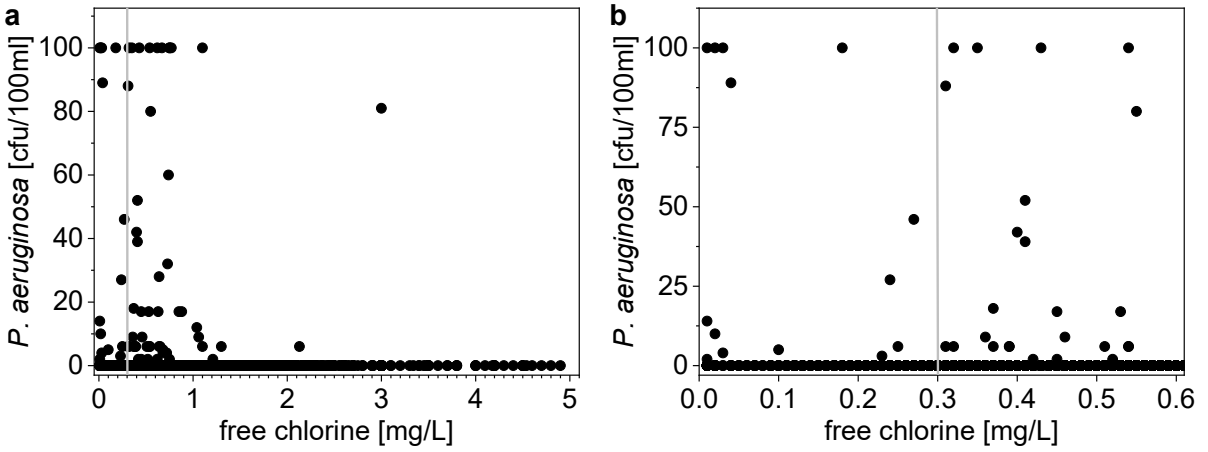

Fig. 9: Correlation of free chlorine concentration [mg/L] and the incidence of *P. aeruginosa* [n/100 ml]. Presumably, within the range of chlorine concentrations used in German public swimming pools, there is no correlation of chlorine concentration and the occurrence of contaminations with *P. aeruginosa*: **(a)** scale over the full range of measured concentrations of free chlorine; **(b)** scale over the range of free chlorine concentrations covered by the German standard on swimming pool maintenance [19]. The gray line indicates the respective lower limit as defined by the standard for free chlorine (0.3 mg/L). Cases with bacteria counts exceeding 100 cfu/ml were frequently stated as `>100´. Hence, they occur as lines of dots in the plots.

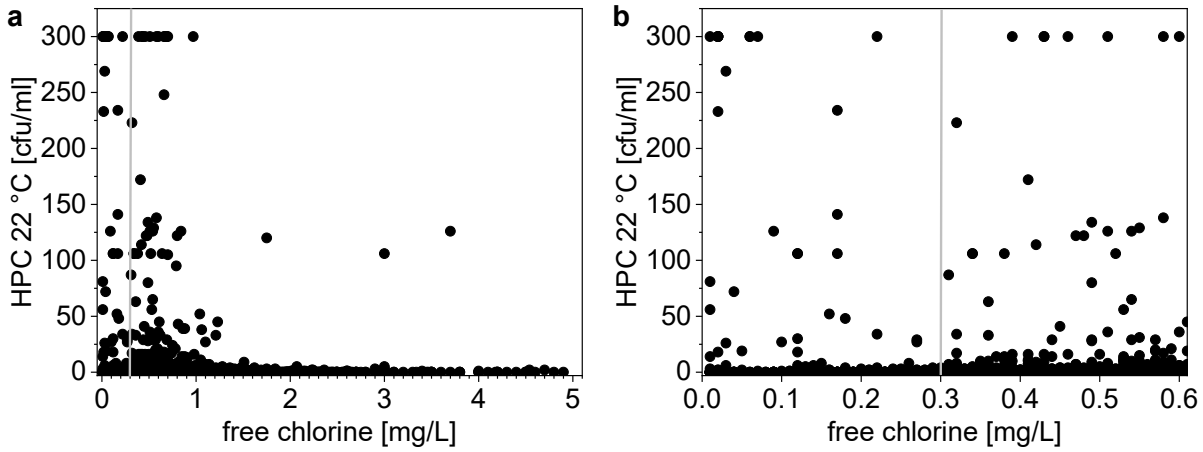

Fig. 10: Correlation of free chlorine concentration [mg/L] and heterotrophic plate count at 22 °C [n/ml]. Apparently, within the range of chlorine concentrations used in German public swimming pools, there is no connection between chlorine concentration and the incidence of objections based on the HPC at 22 °C: **(a)** scale over the full range of measured concentrations of free chlorine; **(b)** scale over the range of free chlorine concentrations covered by the German standard on swimming pool maintenance [19]. The gray line indicates the respective lower limit as defined by the standard for free chlorine (0.3 mg/L). Cases with bacteria counts exceeding 300 cfu/ml were frequently stated as `>300´. Hence, they occur as lines of dots in the plots.

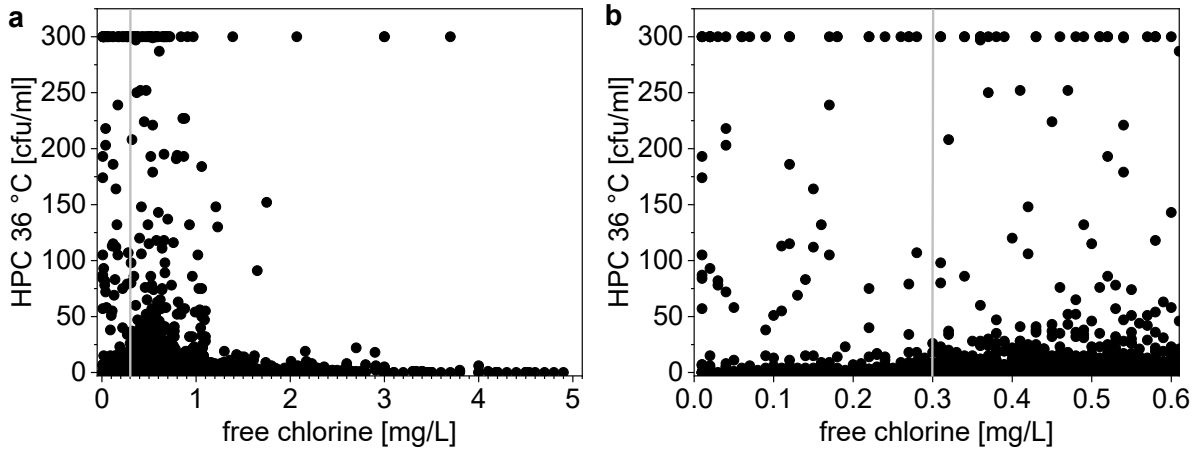

Fig. 11: Correlation of free chlorine concentration [mg/L] and heterotrophic plate count at 36 °C [n/ml]. Apparently, within the range of chlorine concentrations used in German public swimming pools, there is no connection between chlorine concentration and the incidence of objections based on the HPC at 36 °C: **(a)** scale over the full range of measured concentrations of free chlorine; **(b)** scale over the range of free chlorine concentrations covered by the German standard on swimming pool maintenance [19]. The gray line indicates the respective lower limit as defined by the standard for free chlorine (0.3 mg/L). Cases with bacteria counts exceeding 300 cfu/ml were frequently stated as `>300´. Hence, they occur as lines of dots in the plots.

2.4 Discussion

2.4.1 Evaluation of the Selected Methods

Presumably, there are two major extrinsic factors which influenced the outcome of the survey. Firstly, swimming pool water samples were taken at varying frequencies at the individual sites sampled. Therefore, in the resulting dataset, some venues were overrepresented, while other venues were underrepresented. Thus, if a given venue, by any means, had an increased or decreased tendency for contaminations with any of the selected indicator bacteria, this would have influenced the overall result. Secondly, the number of sample drawings varied not only over location, but also over time. For instance, public outdoor swimming pools were sampled only in warm months, as these venues were closed during wintertime. Yet, by relating cases of detection to the total number of sample takings in the respective time period, an influence of this factor on the results should have been minimized. Still, in a nutshell, both local and temporal variations in sample drawings occurred and may have introduced a bias to the results.

2.4.2 Evaluation of the Achieved Results

Chronological Distribution

As indicated, figure 1 shows the chronological distribution of sample drawings. In graph (a) total numbers of sample drawings are applied versus time. As can be seen, the numbers of sample drawings followed a wave pattern. In winter, comparably low numbers of sample drawings were performed. In summer, on the other hand, higher numbers of sample drawings were undertaken. In graph (b) mean values are presented which were calculated using the data of all five years. As shown here, the average number of sample drawings peaked in June.

Supposedly, the determined wave pattern goes back to the following: In the warm months, apart from indoor swimming pools, also open-air swimming pools were open. Therefore, an overall higher number of venues were sampled during summer in comparison to winter. However, apparently, December took an exceptional position. In December, sample drawings were performed at higher frequencies than in the previous and following months. Presumably, this goes back to samplings performed to fulfill legal requirements. These swimming pools would then have had to be sampled in the respective year, but until December did not manage to do so.

In figure 2, the chronological distribution of cases, where bacteria counts in exceedance of the requirements have been determined, are presented. Furthermore, cases where chlorine concentrations dropped below 0.3 mg/L of free chlorine are included to the graph. It becomes apparent, that cases of low chlorine concentrations exceeded cases of contaminations with indicator bacteria. Apparently, these cases where low chlorine concentrations were detected followed a seasonally determined pattern. As explained above, it was checked that the number of sample drawings did not influence the results. To this end, the number of positive cases for any of the selected parameters was related to the total number of samples taken. Hence, the observed seasonal pattern should not have been dependent on the trend described for sample drawings.

In 2013 and 2014, the occurrence of cases of low chlorine concentrations presumably followed a distinct seasonal pattern, with more cases detectable in summer than in winter. However, since the year 2015, this pattern is no longer present in this specificity. From 2015, apart from

peaking numbers of cases in summer, also high numbers of cases were recorded during winter. In spring and fall respectively, comparably lower numbers of cases were recorded.

With respect to the microbial parameters examined, no comparable pattern was identified from the graphs. Cases of contamination with *Escherichia coli* generally occurred only at very low frequencies. Yet, in comparison, cases of contamination with other coliform bacteria occurred more often. Contaminations with *Pseudomonas aeruginosa* occurred at frequencies somewhat in between those of the other specific indictors. Cases of exceedance of the respective limits peaked in the summer months, namely June, July and August. With respect to *P. aeruginosa*, a diversion to fall was detected. Here, even higher frequencies of cases were detected in September than in July and August. Both HPC parameters were distributed more evenly over the course of the years, than the specific indicators. However, the same trend as described above for *P. aeruginosa* was present in the results for the HPC at 36 °C.

In figure 3, the results described above are presented in another way. Mean values of the percent frequency per month were calculated. As there were many negative cases, where there was no exceedance regarding any of the selected parameters, the mean values calculated are accompanied by high standard deviations. Since they did not provide additional information which could be used for the interpretation of the recorded, standard deviations were not included to the respective graphs. Yet, this needs to be kept in mind when following the continuative discussion of this figure. The determined patterns in the distribution of positive cases are only assumed tendencies, but no statistically proved trends.

The results indicate a consistency in the occurrence of cases outside the set limits. Both chlorine concentrations below 0.3 mg/L and bacteria counts exceeding the respective limits occurred at constant frequencies. The median for the percentage of cases of low chlorine concentrations ranged from 4 to 7 percent of the respective total of cases. Exceedance events for indicator bacteria ranged between 0 to approximately 2 percent of total cases. Except for the heterotrophic plate count at 22 °C, no trends became apparent over the course of the years. However, the HPC 22 °C declined between 2013 and 2017, reaching approximately 0% in 2017.

As indicated in figure 3, over the course of one year, total numbers of cases of exceedance correlated well with total numbers of sample drawings. On average, approximately 4 to 7 % of all samples per year had free chlorine concentrations lower than 0.3 mg/L. The occurrence of cases below the limit followed a wave pattern over time. The highest frequencies were reached in the summer months, while in winter, comparably low frequencies were detected. Regarding bacteria counts, the occurrence of exceedance reproduced the wave pattern described for chlorine concentrations. The highest frequencies for cases of contamination with *E. coli* and coliform bacteria respectively were reached in the summer months. Except for the year 2014, counts for HPC 22 °C and HPC 36 °C also peaked in the summer months every year. However, regarding cases of contamination with *P. aeruginosa*, the pattern was broken at several occasions. The results achieved underline those described for figure 2 and can be interpreted in a similar way.

As has been explained, standard deviations have not been included to figures 1 and 3. However, the variance in the frequencies of positive cases is illustrated in the form of box plots in figure 4. As the results demonstrate, there were only minor variances in the results of different months of one year (length of the box plot of one year).

Furthermore, the results indicate that the number of cases did not vary that much between individual years (medians and percentiles of the individual box plots are within the same range). Cases of low chlorine concentrations occurred at reasonably consistent frequencies over the years, with medians lying approximately between 6 and 7 % of total cases per year. According to the box plots, lower and higher incidences of positive cases per month were distributed evenly. The variance in these frequencies was lowest for the year 2014, with the range increasing over the following years.

With respect to specific indicator bacteria, the box plots pinpoint the dominance of cases where colony counts of 0 have been determined. In these cases, the lower percentile and median both lie on the x-axis of the graphs. In general, as there were only very few cases of exceedance in comparison to cases where there were no bacteria detected, the box plots are comparably short in their length in y-direction. However, one may still assume an increase in the variance over the years for all three specific indicators, with box plots increasing in length and the median rising. With respect to the heterotrophic plate count, the variance in positive cases apparently fluctuates over the years. However, as the case for the selected indicator bacteria, also for the HPC at both temperatures, variance occurred only to a comparably low extent.

Concentrations of Bacteria

Figures 5 and 6 respectively correlate the number of positive cases of detection and the respective bacteria counts determined for these cases. Both the results achieved for indicator bacteria (Fig. 5) and those achieved for HPC (Fig. 6) show, that in the majority of cases, only low concentrations of bacteria were detected. Regarding the HPC, these cases have not been recorded as exceedance, if not exceeding 100 cfu/ml. With respect to the specific indicators, every case with colony counts higher than 0/100 ml was recorded as exceedance.

For all classes of indicator bacteria, the same trend becomes apparent. With increasing concentrations of bacteria, the frequency of respective cases declined. In other words, those severity groups including low counts also included most of the detected cases, while severity groups including high counts included only a few cases. This means that exceedance commonly occurred only at moderate concentrations. Interestingly, very high contaminations (higher than 200 cfu/100 ml) were also detected at higher frequencies than contaminations in the middle range. Presumably, exceedance commonly goes back to incidences introducing high concentrations of bacteria. However, presumably, exposed to the oxidative environment of swimming pool water, bacterial concentrations dropped fast. Furthermore, dilution may have played a role in this context.

Correlation of Chlorine Concentration and the Incidence of Bacteria

At first sight, the results presented in figures 7 to 9 indicate a strong impact of free chlorine concentrations on the occurrence of indicator bacteria. Presumably, the effect of chlorine concentration on the incidence of increased counts of bacteria was dependent on the species or fraction examined. *Escherichia coli* and coliform bacteria were the parameters most susceptible to chlorine. Here, contaminations were detected at decreasing rates with increasing concentrations of chlorine.

For the heterotrophic plate count at 22°C and 36 °C, a less distinct pattern was determined. Here, exceedance occurred also at comparably high rates at higher concentrations of chlorine. The same applies for *P. aeruginosa*. Here, high counts of bacteria were determined even at high concentrations of chlorine.

These results are comprehensible. On the one hand, *Escherichia coli* and other coliform bacteria presumably are comparably sensitive to chlorine [42, 43]. On the other hand, the heterotrophic plate count is composed of a broad range of bacteria and yeasts presumably varying in their individual resistance to chlorine. Therefore, it can be assumed that exceedance of the set limit derived from the most resistant species. Exceedance of *Pseudomonas aeruginosa* is considered an indicator of process failures. Considering the results achieved in the present study, occurrence of this germ was not associated to chlorine concentrations. Presumably, here the formation of biofilms played a role. Shed parts of an existing biofilm may have been protected by extracellular layers of slime material. This could explain why *Pseudomonas aeruginosa* apparently was present in all measured chlorine concentrations at approximately the same rates.

At any rate, the influence of chlorine concentrations on the occurrence of bacterial contaminations became apparent only if the whole spectrum of measured concentrations of free chlorine was considered. Considering only the range recommended by German standards, however, cases of contamination were approximately equally distributed over the whole range of measured concentrations of chlorine. This indicates, that with the concentrations selected for German recommendations, there is no significant effect on swimming pool water hygiene. Yet, one needs to keep in mind, that also pH could have played a considerable role here.

2.5 Conclusions

In the context given by the other chapters of this thesis, the results achieved in the present chapter have implications on the use of indicator bacteria in general, and particularly the specific indicator *Escherichia coli*.

According to the results, between 2013 and 2017, there were only very few cases of exceedance regarding the selected indicator bacteria. The incidence of the fecal indicators *Escherichia coli* and coliform bacteria were associated with the use of comparably low concentrations of chlorine. However, this applied far less to the occurrence of contaminations with *Pseudomonas aeruginosa* and of increased concentrations of the heterotrophic plate count.

Escherichia coli and coliform bacteria almost exclusively occurred when chlorine concentrations were low in the respective swimming pools. Presumably, these indicators are comparably sensitive to chlorine disinfection. According to the results, *E. coli* and coliforms were good indicators of inadequate conditions. However, the indication of fecal contaminations in swimming pools presumably would be possible only for short time spans. As these bacteria are inactivated readily if the conditions are within the right range, fecal contaminations could only be detected within short time spans after their introduction to the pool.

The heterotrophic plate count at 22 and 36 °C indicates that with respect to the overall occurrence of bacteria, yeasts and molds, no significant differences are reached over the range of chlorine concentrations applied according to German regulations (0.3 t 0.6 mg/L of free chlorine). However, using higher concentrations, overall counts declined considerably. The heterotrophic plate count may therefore provide information on the overall contamination of swimming pools with potentially hazardous microbes.

Comparable results were achieved for the occurrence of contaminations with *P. aeruginosa*. Although generally linked to chlorine concentrations, over the range of concentrations applied in Germany, this species may be recovered from swimming pool water at approximately constant frequencies.

There is a wave pattern in chlorine concentrations over the course of time, reflected by bacteria concentrations. For both parameters, the highest frequencies of exceedance were reached in the summer months, while in winter, comparably low frequencies were detected. Yet, the occurrence of exceedance stayed within the same range over the five years examined.

Presumably, the occurrence of low chlorine concentrations peaked in summer months, because at this time of the year, dissociation and photolysis introduced by solar radiation were the highest. Furthermore, a consumption of chlorine due to high bather loads may be assumed. Chlorine reacting with the skin of bathers, applied body lotions etc. would no longer have been available for disinfection, which, in turn would explain higher concentrations of bacteria during these months. Yet, higher frequencies of cases of contaminations with bacteria during the summer months could also go back to these same high bather numbers in the respective season of the year. As explained in the first chapter, *P. aeruginosa* is known to form persistent biofilms. According to literature, *P. aeruginosa* may furthermore be considered a normal constituent of human skin flora [4, 44, 45]. Combining both facts, the observed trend in the incidence of cases of contamination with *Pseudomonas aeruginosa* can be interpreted as follows: Like the other indicator bacteria examined, *P. aeruginosa* was introduced during the summer months when many people went to outdoor swimming pools. Over the course of time, an accumulation occurred in the respective pool basins, as the formation of biofilms allowed *P. aeruginosa* to survive under the otherwise lethal conditions. In fall, this accumulation was detectable, e.g. because of shedding of biofilm material. The same may apply to the HPC at 36 °C, which covers a broad range of bacteria, of which some may also be able to form biofilms in swimming pools basins.

Generally, the question arises as to how valuable the use of indicator bacteria is for the assessment of swimming pool water hygiene. The heterotrophic plate count may provide a general measure of microbial contaminations in swimming pool water. Yet, the use of *Escherichia coli* and coliform bacteria appears questionable. In drinking water, these species may indicate contaminations by fecal material (at least in Germany, where drinking water normally is not chlorinated). In swimming pools however, these bacteria may only indicate introduction of fecal material, if the material is present in very high concentrations. However, if this is the case, the incidence will be recognizable also by other means. Tirodimos et al. (2018) achieved comparable results, assessing the incidence of contaminations with *Escherichia coli* in swimming pools and spas in Northern Greece between 2011 and 2016 [46]. The use of *Pseudomonas aeruginosa* as an indicator for process failures appears beneficial. According to the results of this study, at chlorine concentrations as commonly used in German swimming pools, *P. aeruginosa* will occur at roughly constant frequencies, independent of the applied chlorine concentrations.

Therefore, it may be assumed, that if *P. aeruginosa* is detected, this indicates some difficulty in maintaining hygienic conditions. As it may be assumed that *Pseudomonas aeruginosa* survives prolonged periods within a swimming pool or connected environments (e.g. filter systems), this indicator may be consulted at any time without the risk of being only detectable for short timespans after an incidence (as the case with *E. coli*).

Chapter 3

Inactivation of Selected Bacteria Strains by Chlorine [2)]

Chapter Abstract

Chlorine compounds enjoy worldwide popularity as residual disinfectants in swimming pools, due to their high effectiveness accompanied by comparably low costs. However, standards on the used concentrations of free chlorine vary remarkably between individual countries. Defined limits are commonly based on practical experience rather than empirical studies. The same applies to the limits defined for the pH value of swimming pool water, a factor of considerable importance for disinfection success.

On these grounds, an experimental setup was designed, intending to produce empirical and systematic data on the inactivation of bacteria by hypochlorous acid under conditions as given in (German) public swimming pools. Three exemplary species (*Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*) were selected as test organisms. Inactivation kinetics were examined under varying chlorine concentrations and pH regimes. Inactivation kinetics were then evaluated by nonlinear regression, using several established fitting models.

The achieved results confirm that bacterial inactivation by chlorine is dependent on the bacteria species used, chlorine concentration, and pH value. Inactivation kinetics followed non-linear courses and could be fitted with several of the models used. Applying German standards, inactivation of *P. aeruginosa* proceeded at paces borderline to insufficient. *Staphylococcus aureus* was not inactivated at rates as considered appropriate for the inactivation of *Pseudomonas aeruginosa*. By applying the approach described, this study intends to encourage the generation of an empirical and systematic basis for regulations on swimming pool hygiene.

[2)] Parts of this chapter were presented in talks on several occasions:

Kreuter, L., *Inactivation and persistence of bacteria in drinking and bathing water*, Job Application, 29.08.2018, Ludwigshafen (Germany).

Kreuter, L. and L. Erdinger*, Inactivation kinetics of bacteria under swimming pool conditions*, ÖGHMP Annual Conference 2018, Graz (Austria).

Kreuter, L., *"Minimum necessary chlorine concentration" - recent additions*, IntelliPool Project Meeting, 02.11.2016, Southampton (England)

Kreuter. L*, The adaptiveness of P. aeruginosa and its consequences for disinfection,* COS PhD Retreat 2016, 13.10.2016, Stuttgart (Germany).

Kreuter, L., Schlosser T. and L. Erdinger, *Disinfection kinetics in swimming pool water*, Symposium on Improving Pool Water Quality, 30.05.2016, Zell am See (Austria).

Kreuter, L., Schlosser T. and L. Erdinger, *Evaluation of disinfection kinetics in swimming pools, taking into account the occurrence of bacterial subpopulations*, ÖGHMP Annual Conference 2016, Zell am See (Austria).

Kreuter. L, *Exploring the background of swimming pool disinfection - An empirical basis for regulation*, COS PhD Seminar, 09.02.2016, Heidelberg (Germany).

Kreuter, L., *Determination of minimal necessary chlorine concentration for disinfection*, Repeatedly at IntelliPool Project Meetings, 18.01.2016 Ilmenau (Switzerland), 17.09.2015 Barcelona (Spain), 31.03.2015, Heidelberg (Germany)

Kreuter, L., Schlosser T. and L. Erdinger, *Desinfektion und VBNC in Schwimmbädern*, Deutsche Badewasserkommission, 13.10.2015, Berlin (Germany).

3.1 Introduction

Although chlorine disinfectants are used all over the globe, the concentrations used vary remarkably between individual countries. For instance, the German standard determines comparatively low concentrations of 0.3 up to 0.6 mg/L of free chlorine [19], while US codes provide for considerably higher concentrations (e.g. [47, 48]). WHO recommendations provide for values in between these extremes, suggesting concentrations of 1 up to 1.2 mg/L [2].

Generally, the definition of which concentrations of chlorine are appropriate for the disinfection of swimming pool water is based on practical experience rather than on empirically and systematically collected data. Therefore, one objective of the present thesis was to encourage the generation of an empirical and systematic basis, on which examination of microbial safety in swimming pools could rely. Accordingly, this study provides data for the inactivation of bacteria under conditions as commonly found in (German) public swimming pools. Inactivation kinetics of exemplary strains of three bacterial species by hypochlorous acid were determined under several chlorine and pH regimes. For a representation of the situation found in actual swimming pool basins, a large ratio of test volume to inoculum volume was chosen, allowing for the inclusion of effects by dilution to disinfection kinetics as well as preventing errors from the effect of chlorine consumption during the inactivation process.

Several mathematical models were tested for their applicability to match the data obtained. Through comparison, it was determined which of the models provided the best approximations for the inactivation of bacteria by hypochlorous acid in water.

Development of new models for microbial inactivation is a complex undertaking, as various definable and non-definable variables influencing kinetics need to be considered. The associated issues find their expression in the manifold attempts that have been made to explain the shapes of survival curves [49]. Therefore, in the present study, models developed by other researchers were compared, while the researcher deliberately dispensed with the development of new models. Even if considering only such models developed for interpretation of chlorine disinfection, the resulting list is still quite extensive. However, many of these models follow rather complicated approaches. Therefore, some researchers state that respective models lack practical use [50, 51]. Accordingly, most existing studies on inactivation kinetics used either the classical Chick-Watson model [52, 53] or the Hom model [54] respectively [50, 55].

In contrast to the existing literature, the present study applies existing models to a significantly larger test volume in order to simulate the conditions in swimming pools. The scientific contribution of this study is therefore the adaption of existing methods to the requirements of the examination of swimming pool hygiene.

3.1.1 Inactivation of Bacteria as a Kinetic Process

Commonly, interpretation of inactivation kinetics is based on the examination of survival curves. Survival of microbes is represented in semilogarithmic plots of the ratio of the concentration of surviving organisms to the initial number of organisms versus exposure time [11]. Respective curves may appear linear in semi-logarithmic representation, but often express deviations from this linearity, including concave downward, concave upward or even sigmoidal shapes [11]. Examples are illustrated in figure 12.

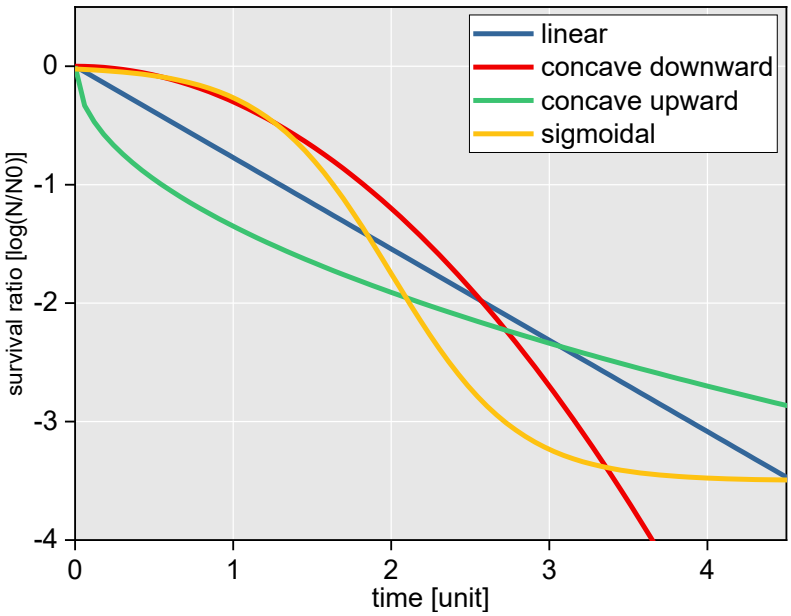

Fig. 12: Typical shapes of microbial survival curves in semi-logarithmic representation – linear, concave downward, concave upward, and sigmoidal. These shapes have been reported for the inactivation of diverse microbes by physical or chemical means in a broad range of applications and fields of research.

Survival curves are the product of fitting experimental data on microbial decline with mathematical models [11]. In doing so, additional information is generated. Trough fitting with mathematical models, observations made in experiments may be explained or at least interpreted [56]. However, since microorganisms will always vary in their reaction, even under similar experimental conditions [11], no model for inactivation kinetics can be applied universally. Nonetheless, once inactivation kinetics are established for a set of conditions, these data may be used for extrapolation and estimation of inactivation kinetics under varying conditions (e.g. disinfectant concentration, pH etc.).

Ideally, the kinetic process of microbial inactivation proceeds similar to a first-order chemical reaction [57]. In this case, the slope of an inactivation curve depends exclusively on disinfectant concentration and the sensitivity of the exposed species. However, in practice, diverse additional factors contribute to disinfection efficiency, including those introduced by the exposed microorganisms (e.g. influence of growth conditions) [57], such factors determined by the disinfectant (e.g. accessibility of the disinfectant, chemical demand), and factors associated with both disinfectant and exposed microbes (e.g. temperature, pH of the medium) [11, 15, 20].

Normally, the antimicrobial activity of chemical compounds goes back to several co-existing modes of action [58]. When microorganisms are exposed to a disinfecting agent, effects on various vital functions occur in parallel. Consequently, it becomes complicated to determine which pathways are ultimately responsible for lethality of the exposure [57]. Accordingly, inactivation kinetics frequently follows non-linear trends. This non-linearity of inactivation has been interpreted in several ways [11]. For example, the multitarget approach assumes that there are diverse vital sites, each of which needs to be hit at least once in order to induce the inactivation of a microbial cell [59]. A different interpretation is offered by the multi-hit approach, which assumes that there is only one sensitive target, which needs to be hit several times before inactivation of the respective cell is achieved [60]. Furthermore, inactivation of

microorganisms may be hampered if microbial cells are not entirely accessible for the biocide used. If, for instance, microbes are not fully suspended in water (e.g. due to aggregation or attachment of cells to debris), this could influence disinfection kinetics [11, 61-64].

For chlorine disinfection, five main factors contributing to efficiency of microbial inactivation have been identified [14]. These are the microbial species exposed, chlorine concentration C [mg/L], contact time t [min], pH and temperature [°C]. Inactivation rates will increase either with increasing contact times t, or with increasing chlorine concentration C. Both the increase of pH and of the temperature will accelerate inactivation of microbes.

3.1.2 Modelling Inactivation Kinetics

Presumably, Kroenig and Paul (1897) were the first who plotted logarithms of surviving organisms against time [50]. They stated that the rules of chemical disinfection could also be applied to disinfection [65]. Later, this was to become known as the mechanistic approach to microbial inactivation [49, 66].

In their study, Kroenig and Paul achieved approximately linear responses [65]. However, later, when Madsen and Nyman (1907) [67] further developed this approach, they encountered deviations from linearity in the resulting inactivation kinetics. Madsen and Nyman therefore hypothesized that their observations were an expression of a variability of resistance within the population exposed to a disinfectant [67]. To distinguish this idea from the mechanistic viewpoint, their approach later became known as the vitalistic hypothesis [49]. The vitalistic concept is based on the idea that individuals in a population may vary in several characteristics [66, 68], e.g. the susceptibility to a disinfecting agent. These differences between individual cells are considered permanent. The variance in microbial susceptibility to biocides was hence described as a “permanent variable population resistance” [50, 68, 69].

According to more recent reviews [49, 50], models on disinfection kinetics may, up to today, be split into these two general classes: mechanistic hypotheses on the one hand and vitalistic hypotheses on the other hand.

3.1.3 Chick-Watson Model

The equation known today as the Chick-Watson model was developed under the assumption that the process of microbial inactivation was analogous to first-order chemical reactions [11, 50, 52]. If this was true, the reaction rate would exclusively depend on the relative concentrations of the disinfectant and the susceptibility of the exposed microorganisms [55].

In her study on disinfection, Chick [70] made the observation that inactivation of *Bacillus paratyphosus* could be described by first-order kinetics. Chick expressed her finding with the equation given by:

$$\frac{dN}{dt} = -kN \tag{3}$$

where dN/dt is the rate for the decrease in viable organisms over time [n/L min], N is the concentration of microorganisms at time t [n/L], and k is the pseudo-first-order inactivation rate constant.

Later, Watson [71] reanalyzed the data generated by Chick and added disinfection concentration to the formula.

The Watson Law is given by:

$$C^n t = k \tag{4}$$

where k is the inactivation rate constant for an organism exposed to a disinfectant under specific conditions (dependent on the used disinfectant, microorganism, and the given environmental conditions), C is the disinfectant concentration [mg/L], t is the time required to inactivate a given percentage of the inoculum [min], and n is the coefficient of dilution.

Integration of equation (4) into equation (3) results in the formula known as the Chick-Watson Law [52, 72, 73] given by:

$$\frac{dN}{dt} = -kC^n N \tag{5}$$

where dN/dt is the inactivation rate [n/L min], N is the number of survivors [n/L], k is the coefficient of specific lethality (pseudo-first-order disinfection rate constant determined experimentally) [L/mg min], C is the concentration of the disinfectant [mg/L], and n is the coefficient of dilution (empirical constant; commonly assumed to be 1 [72]).

Integration results in the relationship [74] given by:

$$\log\left(\frac{N}{N_0}\right) = -kC^n t \tag{6}$$

where N_0 is the concentration of organisms at time = 0 [n/L], and t is the time [min].

The fitting of non-linear curves (e.g. expressing shoulders or tailing) is not possible with this model. As the molecules of the disinfectant and the microbes exposed to them are interpreted as the two reactants of a chemical reaction, such deviations were explained by a distribution of resistances to a given treatment within microbial populations exposed [50, 70].

The Ct Concept

The Ct (concentration * time) concept examines survival ratios as linear functions of biocide concentration and contact time [73]. The higher the Ct value, the more resistant a given test species [11]. The concept thus provides the basis for comparison of disinfectant effectiveness and the relative susceptibilities of different microorganisms to a given disinfectant [75]. The effectiveness is evaluated by comparing concentration - time data at a defined level of microbial inactivation (commonly 99% or 2-log of the initial inoculum) [11, 75]. Accordingly, each data point in a respective plot reflects a combination of concentration and time.

As it comprises a dilution coefficient and a time exponent, the Ct concept presumably was developed based on the Chick-Watson model [11]. Consequently, in its current form, the concept requires a linear slope of log-reduction versus time [56, 76].

If, however, there is a lag-phase or tailing, the concept fails. As a consequence, normally only the log-linear part of survival curves is included for determination of Ct values [56], as was done in the present study. Although initially developed for the examination of thermal disinfection [56, 77], until today, the Ct concept has also frequently been used to assess chemical disinfection (e.g. in chlorinated drinking water [14]). Commonly, pathogenic or otherwise relevant species have been used for examination [56, 73, 76].

Approach Assuming Multiple Linear Phases

Acting on the assumption that inactivation rate and chemical reaction rate are identical, the coefficient of dilution in the Chick-Watson equation reflects the reaction order of an inactivation experiment [56]. If one reactant is vastly excessive to the other (as is commonly assumed in chemical disinfection) reactions will appear like first-order. They have thus been classified as pseudo first-order [56].

However, the Ct concept is only then applicable if the disinfecting agent used has a dilution coefficient of approximately 1 [56, 57, 75]. Accordingly, if n = 1, the C*t product will be constant for a given range of values, meaning the resulting survival curve is log-linear [11]. If n > 1, the factor determining the extent of inactivation is concentration, meaning that the C*t value will decrease with increasing C. If n < 1, the dominating factor is contact time, meaning that the C*t value will increase with increasing C. Consequently, only if the value of n is close to 1, C*t values may be used to compare the efficiencies of different decontaminants in inactivating a given microorganism, or the susceptibility of different microorganisms to a given decontaminant. In experiments, values ranging from 0.34 to 4.74 have been reported for the dilution coefficient of diverse disinfecting agents [11]. Conveniently, because hypochlorous acid is a strong oxidizing agent, this compound is assumed to have a dilution coefficient of ~1 [56, 78].

As will be presented below, survival curves frequently exhibited tailing and, to a lesser extent, also initial lag-phases. Hence, although the Ct concept in general was applicable on the data obtained, sections of the corresponding inactivation curves needed to be excluded from the calculation of Ct values. Bearing in mind that tailing could be a result of the composition of a given population of microbes of several subpopulations, data was assessed for the incidence of multiple log-linear phases. Most frequently, this resulted in one or two separate phases with individual presumed inactivation rates. However, in some cases, even three distinct phases were detected. Both a slow decay followed by comparably faster decay, and fast decay followed by comparably slower decay were observed, as will be described in the results section of this chapter.

3.1.4 Hom Model

In many studies on inactivation kinetics, a change in inactivation rates over time has been observed. The issues accompanying the use of the Chick-Watson model have frequently been overcome by using the Hom model (e.g. by [55]). Originally, Hom developed his model to interpret the inactivation of coliform bacteria in wastewater [54]. Apart from disinfectant concentration, the model introduced contact time as an important controllable variable [11], combining effects of time and concentration relationships.

It is expressed by the equation:

$$\frac{dN}{dt} = -kNt^m C^n \tag{7}$$

where k is the inactivation rate constant [n/ml min], N is the initial concentration of organisms [n/ml], t is the time [min], m is the reaction rate constant, C is the concentration of the disinfectant [mg/L], and n is the coefficient of dilution.

If the equation by Watson (2) is assumed to be correct, the Hom model may be resolved to:

$$\log\left(\frac{N}{N_0}\right) = -\frac{kk't^m}{m} \tag{8}$$

where N_0 is the initial concentration of microorganisms, k is the first-order reaction rate constant, and k' is the Chick-Watson law constant [11].

According to a review, Hom's intention for creating this model was to make sense of non-linear log-survival curves by "altering the dependency of time itself" [50]. As the disinfection rate is dependent on fractional powers of time, non-linear curves are achieved. In other words, disinfection rate either slows down or speeds up over time. If, m = 1 (and n = 1), the Hom model is reduced to the Chick-Watson model. If, m > 1, the resulting curve has a shoulder. If, m < 1, the resulting curve exhibits tailing. There are, however, also limitations to this model. For instance, m, although defined as a constant, decreases rapidly with increasing chlorine concentrations [11]. Furthermore, the model does not provide any explanation for the changes observed in disinfection rate [50].

3.1.5 Intrinsic Quenching (IQ) Model

Contemplating the Hom model, Lambert and Johnston hypothesized that an important factor in disinfection kinetics was the disinfectant concentration, as available for inactivation of microorganisms [50]. They concluded, that in any model examining disinfection rates, time dependence of the reaction constant must be considered. Based on the findings of Johnston et al. (2000) [49], Lambert and Johnston developed their own model, the intrinsic quenching model (IQ model). The theoretical basis for their model is the Chick-Watson Law. However, Lambert and Johnston assume that the disinfection rate constant decreases over time as shown in the following equation:

$$\frac{dN}{dt} = -k\,(t)\,N \tag{9}$$

According to their model, apart from rate constant k_1, which governs how an inoculum N is inactivated, there is a second rate constant k_2, governing how the concentration C of the disinfectant becomes inactivated during the process, to form inactive product X. They propose the following law for the dependence of C on time t [50]:

$$C = C_0 - e^{k_2 t} \tag{10}$$

where C_0 is the initial concentration of the disinfectant [mg/L], t is the time [min] and k_2 is the rate of disinfectant inactivation.

Substitution of equation (10) into equation (9) results in:

$$\frac{dN}{dt} = -k_1 = C_0^n e^{-k_2 nt} N \tag{11}$$

where C_0 is the initial concentration of the disinfectant [mg/L], t is the time [min], k_1 is the rate constant of bacterial inactivation, k_2 is the rate constant of disinfectant inactivation.

Finally, integration results in the "Intrinsic Quenching Model" [50]:

$$\log\left(\frac{N}{N_0}\right) = -\frac{k_1}{k_2 n}(1 - e^{k_2 nt}) \tag{12}$$

As indicated, the model contains a parameter Q (= k_2n), termed by the authors as the "Intrinsic Quenching Parameter" [50].

Q is a measure of how fast the rate of inactivation slows down with a given biocide inactivation rate k_2. The model assumes that this inactivation rate is independent of the biocide concentration. As their model seems to reflect the tailing effect observed in inactivation experiments, Lambert and Johnston suggest that Q is a physical property of the microbes used.

According to Lambert's and Johnston's study, the parameters obtained for disinfection rate constant and dilution coefficient for their IQ model are similar to those in the Hom model. They deduce that results obtained with their model are compatible with those of older studies which used the Chick-Watson model or the Hom model. Furthermore, they suggest the re-examination of older data with the IQ model. As to achieve log-linear survival curves as predicted by the Chick-Watson model, one of the reactants needs to be present in high excess to the other. Accordingly, Lambert and Johnston interpret the Chick-Watson model as a "special case" based on their own model and comparable models [50].

3.1.6 Biexponential Model

As has been shown, the vitalistic theory of microbial inactivation kinetics assumes that the shape of inactivation curves is determined by varying resistance of the exposed microbes to the disinfection agent. The biexponential model [79], sometimes simply referred to as biphasic model [80], is a quite popular vitalistic model for fitting biphasic survival curves. The model presumes that two subpopulations are inactivated at two individual rates.

It is expressed by the equation [68, 81]:

$$\log\left(\frac{N}{N_0}\right) = \log\left(Pe^{-k_1 t} + (1-P)e^{-k_2 t}\right) \tag{13}$$

where N_0 is the initial concentration of microorganisms [n/ml], t is the time [min], N is the concentration of microorganisms at a time point t [n/ml], and k_1 and k_2 are the individual reaction rate constants of two subpopulations of varying sensitivity to the treatment.

3.1.7 Weibull Model

As the name indicates, the Weibull model assumes that the heterogeneous distribution of stress tolerance in a microbial population can be described by a Weibull equation [79].

Weibull distribution is a continuous probability distribution model. As a statistical tool, it is applied in a variety of fields to predict the occurrence of certain events (e.g. in weather forecasting to describe wind speed distributions and in electrical engineering to describe overvoltage in electrical systems).

With respect to microbial inactivation, the Weibull model has been applied predominantly to interpret thermal inactivation [82, 83]. In the context of disinfection kinetics, the Weibull model basically provides a statistical tool to fit the distribution of inactivation times while taking into account biological variation within a tested population [83]. In other words, the model tells us how often an individual inactivation time will be present in an experiment with a certain mean inactivation time.

The Weibull distribution can be expressed as [82-85]:

$$N = N_0 e^{-kt^n} \tag{14}$$

where N is the number of surviving cells after a treatment time t, N_0 is the initial cell number, k is a scale parameter effecting time t, and n is an dimensionless shape parameter [84, 85].

Peleg and Cole (1998) [85] extracted from this the decimal logarithmic form [84]:

$$\log\left(\frac{N}{N_0}\right) = -kt^n \tag{15}$$

Applying the two parameters k and n, the model accounts for non-linear semi-logarithmic survival curves. The shape parameter n may provide for linearity (n = 1), as well as upward concavity (n < 1) or downward concavity (n > 1). The first-order approach represented by the Chick-Watson model is considered a special case of the Weibull model [85].

3.2 Materials and Methods

3.2.1 Test Species Selection

Three bacterial species have been selected for this study, each one for its unique set of characteristics.

The Gram-negative species *Escherichia coli* was selected for its long-time use in pool maintenance. While this species is normally non-hazardous to human health, there also exist pathogenic strains [86]. *E. coli* is a common part of vertebrates' intestinal flora [87]. Consequently, in water hygiene, this species is used as an indicator for fecal contaminations [2, 19, 88, 89] .

The second Gram-negative species used was *Pseudomonas aeruginosa*, an inhabitant of moist habitats, including man-made environments such as water pipes and swimming pool filters. Integrated into biofilms [90], *P. aeruginosa* may persist under conditions otherwise detrimental for this organism. Since, in addition to its ubiquitous distribution and its persistence, *P. aeruginosa* is also an opportunistic pathogen of significant medical importance [91], this species is considered a potential health hazard for bathers in swimming pools [2]. Accordingly, it is used as a process indicator [19].

Staphylococcus aureus is a non-spore forming, Gram-positive species. Like other members of the *Staphylococcus* genus, *S. aureus* is part of the natural human skin flora [33, 92, 93]. However, unlike most related species, strains of *S. aureus* are often pathogenic [93]. Consequently *S. aureus* is considered a possible health hazard for bathers in swimming pools [2]. For the experiments performed for this study, this germ was selected as a representative of all those species entering swimming pool water through the shedding of skin particles by bathers [94].

3.2.2 Test Strains

The following strains of the bacterial species described above were used in experiments:

- *Escherichia coli*: ATCC® 25922™ (origin: clinical isolate; LGC Standards GmbH, Germany).
- *Pseudomonas aeruginosa*: mucoid wildtype isolated from domestic drinking water installation in Germany
- *Staphylococcus aureus*: ATCC® 6538™ (origin: human lesion; LGC Standards GmbH, Germany).

3.2.3 Bacteria Growth Conditions

Suspensions of the bacterial strains in CASO broth (Merck Life Science, Germany) were produced using stock preparations suspended in skimmed milk (Merck, Germany) and stored at -80 °C. To this end, fractions of frozen medium were removed with sterile inoculation loops (Looplast®, LP Italiana SPA, Italy) and streaked on Columbia agar plates (BD BBL™ stacker™ plates, BD Biosciences, Germany). After incubation at 36 ± 1 °C for 20 h, a single colony was transferred to 30 ml of sterile CASO broth and incubated at 36 ± 1 °C on an incubation shaker (Köttermann Labortechnik, Germany) until the stationary growth phase was reached. Before an experiment, an inoculum with a volume of 3 ml was removed, transferred to a sterile Falcon tube (Cellstar® tubes, Greiner bio-one, Germany) and washed twice by centrifugation at 2,000 rpm, followed by dispersal of the supernatant and resuspending of the pellet in 10 ml of test medium. Bacterial concentrations were determined using dilution series of the original suspension, plated on TSA (BD BBL™ stacker™ plates, BD Biosciences, Germany) and incubated at 36 ± 1 °C for 24 h.

3.2.4 Experimental Setup

Apart from parameters such as temperature and pH, the type of reactor used also has a strong impact on the outcome of experiments on inactivation kinetics [11]. The setup applied for the experiments of this study is illustrated in figure 13. The design was developed in consideration of the OECD guideline on testing disinfecting agents for pools and spas [95]. As recommended [96], a large ratio of test volume to inoculum volume was used. Accordingly, an aquarium with a volume of 50 liters was used as a test vessel. During an experiment, the water body was stirred constantly by a pitch blade turbine impeller (Carl Roth GmbH + Co. KG, Germany) connected to an agitator (RW 20, IKA®-Werke GmbH & Co. KG, Germany).

The medium was composed of sterile deionized water containing standardized concentrations of additives (based on [97]): 26 mg/L calcium chloride, 7.4 mg/L magnesium sulfate heptahydrate, 3.5 mg/L sodium chloride, 0.088 μg/L potassium chloride, 0.623 μg/L di-sodium hydrogen phosphate, 0.118 μg/L potassium dihydrogen phosphate and 153 mg/L sodium bicarbonate (Merck, Germany). Stock solutions of hypochlorous acid were prepared afresh before each experiment by diluting a sodium hypochlorite solution (12% Cl content, Carl Roth GmbH + Co. KG, Germany) in the test medium. Chlorine concentrations in the test vessel were adjusted using portions of the prepared stock solution and verified by the N,N-diethyl-p-phenylenediamine (DPD) colorimetric method [98, 99].

Further parameters were chosen as representative for conditions as commonly found in public swimming pools. Accordingly, water temperature was adjusted to 30 ± 2 °C by adding portions of deionized water preheated to 100 °C. As indicated before, water temperature has a significant effect on inactivation kinetics. Accordingly, during an experiment, temperature was kept by using aquarium heating mats (ThermoLux, Witte + Sutor GmbH, Germany) regulated via a thermostat (Biotherm® professional, Aquatico Ltd., Ireland).

In accordance with European norms on swimming pool maintenance [19, 100, 101], the pH was adjusted using sodium hydroxide (Fluka® Analytical, Germany) or hydrochloric acid (Merck KgaA, Germany) respectively.

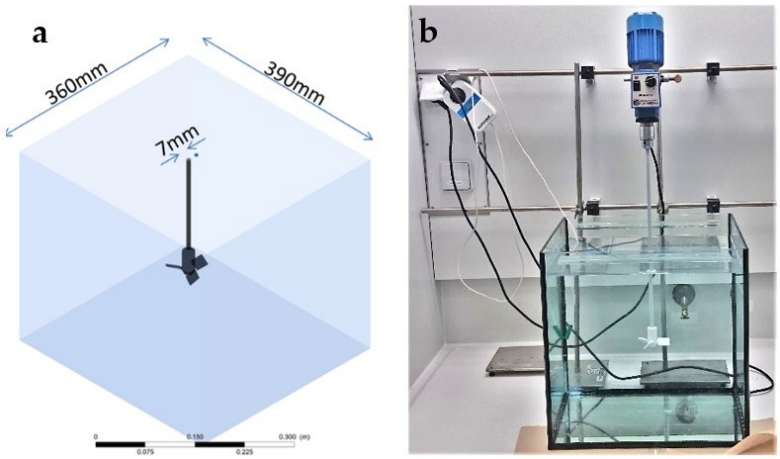

Fig. 13: Setup of the experiments performed on bacterial inactivation kinetics: **(a)** Dimensions of the test vessel and indication of the position of the turbine impeller. The red spot marks where a bacteria inoculum was added when starting an experiment. **(b)** Photo of the setup showing the test vessel, turbo impeller and heating mats for temperature maintenance.

3.2.5 Experimental Procedure

Before the experiments, a pretest was performed to determine the speed by which an inoculum was dispersed in the aquarium under the given conditions. Results indicated a complete mixture within 10 to 20 seconds.

Then, two lines of experiments were performed. In the first line, various concentrations of hypochlorous acid were tested for their efficiency in inactivating inoculated bacteria within a given time interval. In these experiments, the pH was fixed at 7.2. This pH was selected to resemble conditions slightly below the optimum for chlorine disinfection, as may frequently be found in public swimming pools. In the second line of experiments, several pH values were tested for their influence on inactivation success at fixed concentrations of the chlorine disinfectant. Respective chlorine concentrations were defined in consideration of specifications made in current German norms on swimming pool maintenance [19], where a lower limit of 0.3 mg/L and an upper limit of 0.6 mg/L of free available chlorine are determined. The mentioned norms furthermore define, that proper disinfection conditions are reached if a “reduction of *Pseudomonas aeruginosa* by 4 orders of magnitude within 30 seconds” is achieved. Initially, the tests performed were based on this definition, using concentrations of 0.3 mg/L for determination of the effect of pH on inactivation kinetics.

However, based on the results of the first line of experiments, in the second line, also experiments with 0.2 mg/L of free available chlorine were performed. In doing so, inactivation of bacteria was slowed down to an extent which allowed for better interpretation of the results achieved.

Bacteria counts were determined as colony forming units (cfu) at several time intervals after addition of the inoculum to the previously prepared test setup. During the first minute after inoculation, samples were taken at intervals of 10 seconds each. For the next three minutes, one sample was taken every minute. Samples had a volume of 1 ml and were collected in sterile Eppendorf tubes (Safe-Lock Tubes 2.0 ml, Eppendorf AG, Germany). Before sampling, the tubes were filled with 1 ml of a sterile thiosulfate solution (20 mg/L) to remove all remaining active chlorine the moment a sample was taken. To enumerate viable bacteria, per sample, portions of 100 µl were cultivated on TSA plates (BD BBL™ stacker™ plates, BD Biosciences, Germany). Samples taken in the first minute were additionally cultivated in 1:20 dilution on TSA plates. Each sample was streaked in triplicate. Before colony counting, the plates were incubated for 24 h at 37 ± 1 °C in a hot cabinet (Thermo Fisher Scientific, Germany).

3.2.6 Data Analysis

Fitting data plots with the models introduced was performed using Excel (Office 365 Student, Microsoft) and OriginPro (OriginLab, versions 2018b and 2019b respectively). Analysis was performed separately for each experiment. For the respective calculations, the coefficient of dilution of hypochlorous acid was considered to be 1 [56, 78].

3.2.7 Control Approaches

Results from experiments on inactivation kinetics are always dependent on the methods used. With respect to the experimental setup, presumably particularly incubation conditions and selection of growth media had an impact on the outcome.

In order to evaluate the influence of the selected methods on the results, two complementary approaches were included in the present study. Beyond evaluation of the setup, inclusion of these approaches allowed a critical reconsideration in general of the proceeding in determining indicator bacteria counts from swimming pool water using culture dependent methods. The question behind this is, if these culture dependent methods are appropriate for examining swimming pool hygiene, or if it would be more beneficial to replace them (e.g. by molecular techniques).

The first approach involved comparison of the results received using TSA agar (as described above) with those gained using an alternative growth medium. Reasoner's 2A agar (R2a agar) was chosen for this purpose. By comparing the results produced with these two media, a measure of the influence of growth medium composition can be determined.

Originally, R2a was developed to assess the presence of bacteria in potable water [102]. In comparison, R2a agar is poorer in total nutrients than TSA. However, on the other hand, R2a provides a broader range of nutrients than its counterpart. R2a may therefore be used to cultivate slow growing bacteria species. For the experiments performed as control approach to the original experiments, R2a was included to evaluate if the mentioned alternative composition of the growth medium results in a higher rate of recovery of bacteria after exposure

to chlorine. The hypothesis behind this procedure was that stressed and/or injured bacteria cells could lack the competence to grow on a rich medium like TSA but may recover and grow on a less rich medium.

The second alternative approach was based on the use of a non-culture dependent method allowing examination of the presence of intact bacteria cells lacking the ability to grow. To this end, the molecular technique called fluorescence *in situ* hybridization (FISH) was used.

Since completely independent of growth conditions, FISH allowed assessment of the general influence of cultivability on the results. Comparing the results achieved with FISH and those from the original inactivation experiments provided insights into the survival of bacteria including the occurrence of viable but non-cultivable cells (VBNC). In the context of swimming pool water hygiene, such VBNC stated bacteria are of potential importance, as their occurrence would indicate that the standard methods for assessment of hygiene have a blind spot regarding non-cultivable bacteria. If chlorine disinfection induces a switch to survival states which are temporarily non-cultivable but may regain cultivability after removal of this stressor, using culture-dependent methods could lead to misinterpretations on disinfection success. Since the same applies to the experiments performed, FISH was included as a control approach.

Alternative Growth Medium R2a

At random, some of the samples taken in inactivation experiments were additionally plated on Reasoner's 2a agar (R2a). cfu counts achieved on R2a were compared to those recovered on TSA. The composition of R2a agar is indicated in table 4. The composition of TSA agar is presented in table 15 in chapter 6.

Table 4: Composition of R2a agar [103].

Component	Grams per Liter purified water
Yeast Extract	0.5
Proteose Peptone Nr. 3	0.5
Casamino Acids	0.5
Dextrose	0.5
Soluble Starch	0.5
Sodium Pyrovate	0.3
Dipotassium Phosphate	0.3
Magnesium Sulfate	0.05
Agar	15.0

Fluorescence *in situ* Hybridization (FISH)

Fluorescence *in situ* hybridization (FISH) is a molecular technique used to detect the presence of specific DNA sequences. The technique uses fluorescent probes binding to parts of a given nucleic acid sequence only if this sequence possesses a high degree of complementarity. By using fluorescence microscopy, it can be determined where a fluorescent probe is bound. The FISH technique was developed in 1982 [104].

In the present study, FISH was used to examine if there were cells remaining intact after exposure to chlorine. In a second step, it was determined if there were significant differences

in the results achieved using FISH and by using culture-dependent methods. Furthermore, a possible influence of aggregate formation on the survival of individual bacteria cells was examined.

Samples of the bacteria suspensions were used for fluorescence *in situ* hybridization and subsequently examined under a laser scanning microscope (Leica TCS SP5, Leica, Germany). Bacteria cells were marked using the EUB 338 probe [105] with an Alexa Fluor 488 tag (Invitrogen™, Thermo Fisher Scientific, Germany). To this end, 2 ml of a sample were transferred to an object slide (Paul Marienfeld GmbH & Co. KG, Germany) and air dried overnight. The next day, samples were dehydrated by incubation in 50%, 70% and 96% ethanol solution for 3 minutes each. After a second air drying step, 1 ml of hybridization buffer (0.9 M NaCl, 20 mM Tris [pH 8.0], 0.01% SDS, 40% (v/v) formamide und 10 ng/µL oligonucleotide probe EUB338 labelled with Alexa Fluor 488) were added to each object slide. The object slides were then incubated at 46 °C in a heat cabinet (Heraeus Group, Germany) for 90 minutes. Afterwards, they were incubated in washing buffer (56 mM NaCl, 20 mM Tris [pH 8.0], 0.01% SDS and 5 mM EDTA) for 15 minutes at 46 °C. The last steps were to rinse the object slides with sterile deionized water and to seal them with CitiFluor™ medium (Science Services, Germany), cover glasses (VWR International, Germany) and nail polish (Cosmetica Fanatica). Additionally, samples taken at the 1-minute and 4-minute time points respectively were taken and examined for surviving bacteria by the methods described.

3.3 Results

Inactivation kinetics were examined by plotting the log 10 ratio of survivors against exposure time [min]. Figures 14 and 18 show the raw data for experiments on the effect of chlorine concentration and pH respectively. The data points of individual time points in the graphs represent the mean values for the results of three independent runs. Raw data was fitted with several mathematical models. These models are listed in table 5.

Raw data was fitted with the Chick-Watson model and the resulting information was used to calculate Ct values. The respective results are presented in figures 15 and 19, as well as figures 23 and 24. Additionally, assuming multiple log-linear phases occurred, corresponding drawings were created based on the raw data obtained (compare figures 15 and 19). Two additional mechanistic models were used to fit the raw data, the Hom model and the Intrinsic Quenching Model. Figures 16 and 20 show the results achieved using these two models. Furthermore, two vitalistic models were used, the biphasic model and a model based on Weibull distribution. The results achieved using these two models are presented in figures 17 and 21. In all cases, the coefficient of dilution n was assumed to be 1, based on information given in existing literature on chlorine disinfection [11].

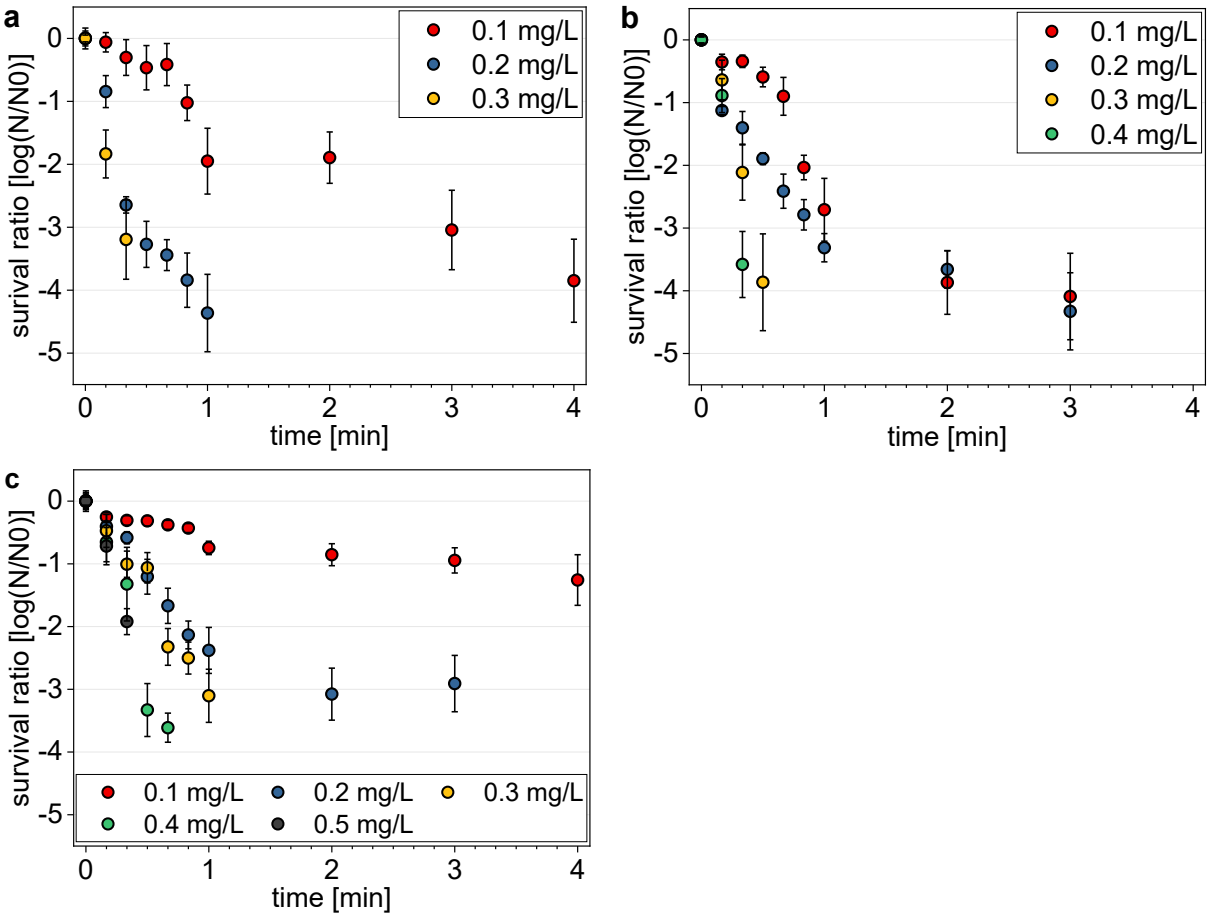

Fig. 14: The effect of chlorine concentration on inactivation kinetics - raw data from the performed experiments. With increasing concentrations of free chlorine, the pace of bacterial inactivation also increased. Inactivation of **(a)** *E. coli*, **(b)** *P. aeruginosa*, and **(c)** *S. aureus*.

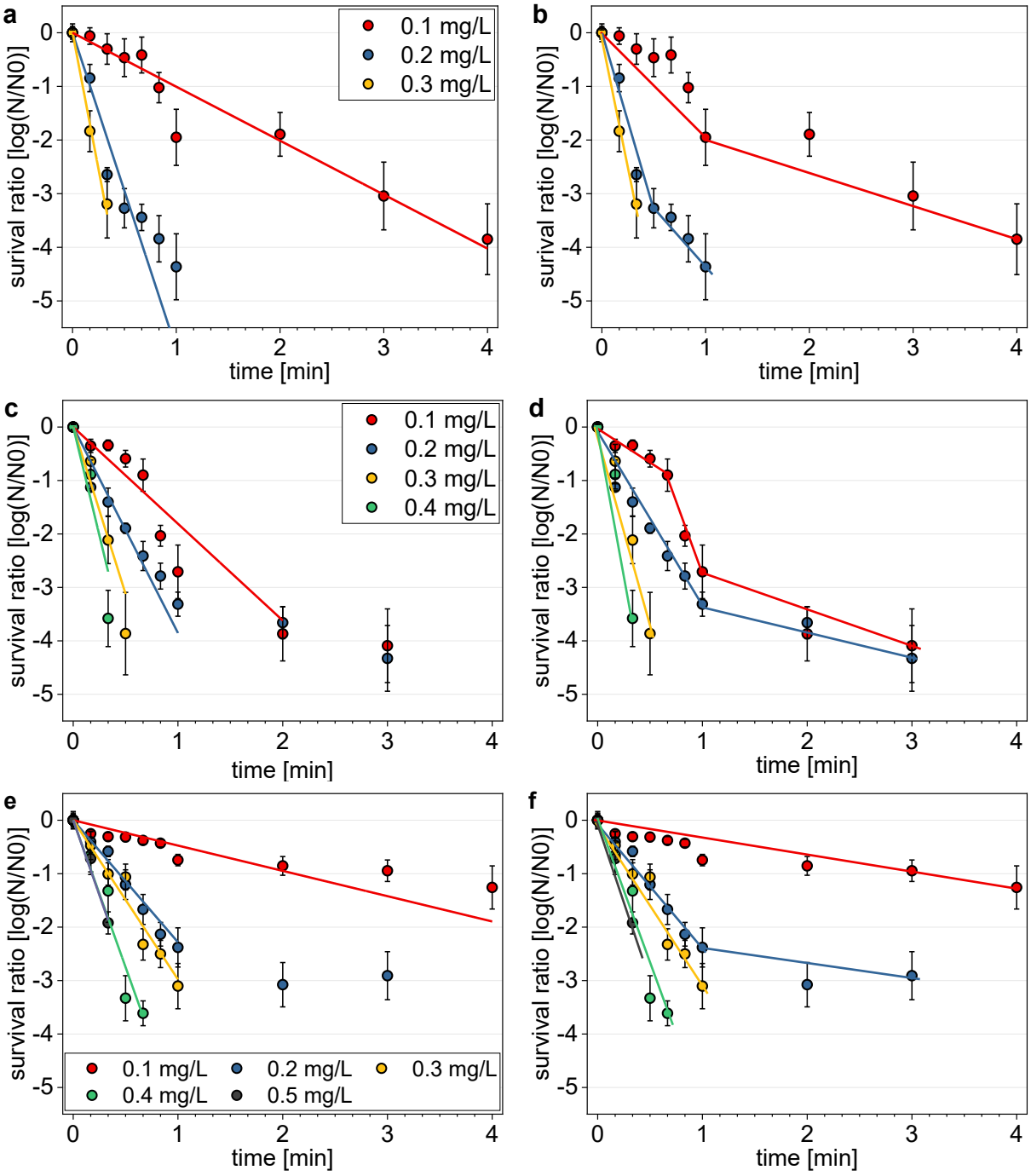

Fig. 15: The effect of chlorine concentration on inactivation kinetics - Fitting with the Chick Watson model. As can be seen, in most cases, it was not possible to fit all data points with this model: **(Left column)** Survival curves of **(a)** *E. coli*, **(c)** *P. aeruginosa*, and **(e)** *S. aureus* at varying concentrations of free chlorine [mg/L] and a pH of 7.2. **(Right column)** Hypothetical division into several log linear phases of the survival curves of **(b)** *E. coli*, **(d)** *P. aeruginosa* and **(f)** *S. aureus*.

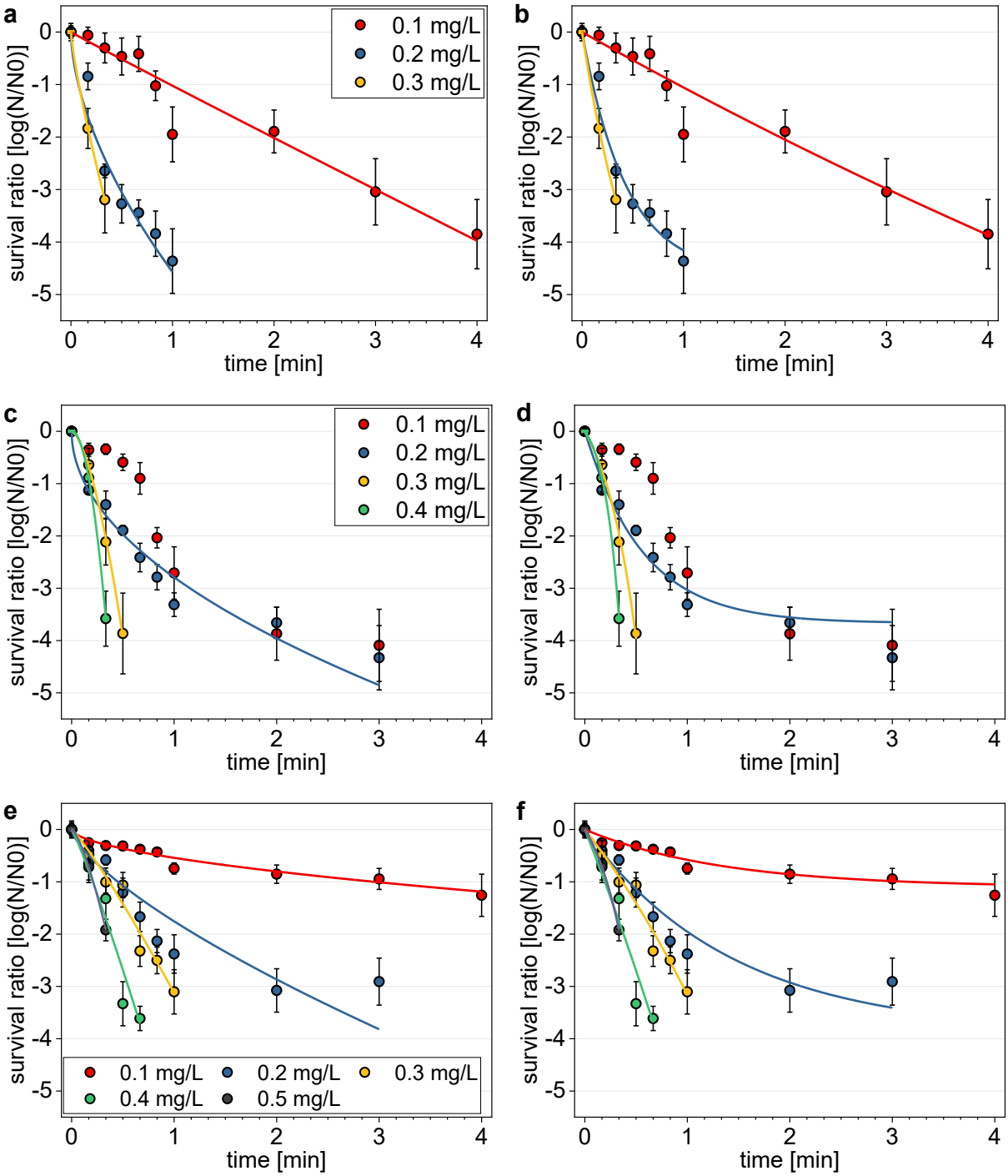

Fig. 16: The effect of chlorine concentration on inactivation kinetics - Fitting with the Hom model **(left column)** and the Intrinsic Quenching model **(right column)** respectively. In most cases, both models allowed fitting of curves with tailing. Survival curves of **(a, b)** *E. coli*, **(c, d)** *P. aeruginosa*, and **(e, f)** *S. aureus* at varying concentrations of free chlorine [mg/L] and a pH of 7.2.

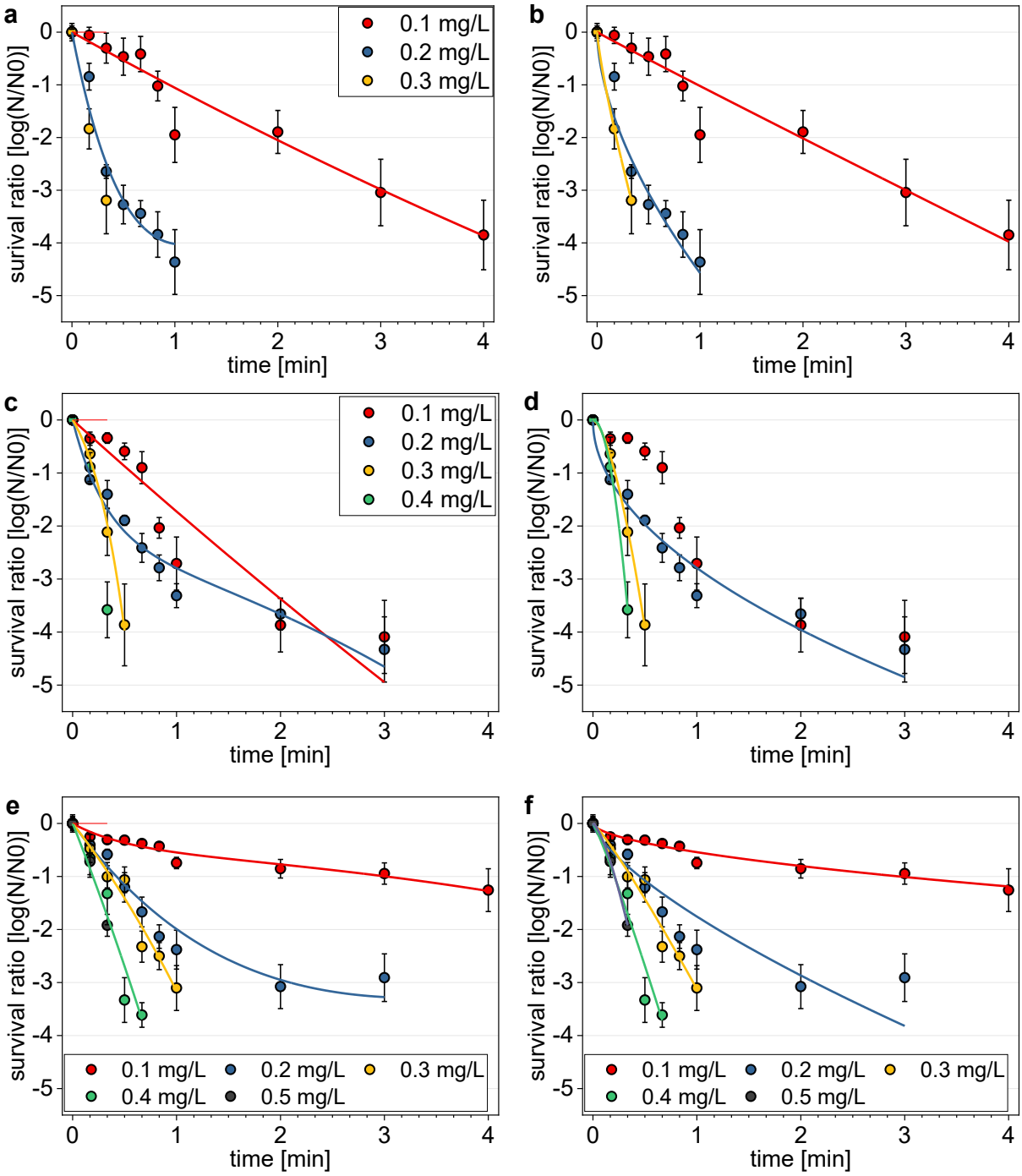

Fig. 17: The effect of chlorine concentration on inactivation kinetics - Fitting with the biphasic model **(left column)** and Weibull model **(right column)** respectively. In most cases, both models allowed fitting of curves with tailing. Survival curves of **(a, b)** *E. coli*, **(c, d)** *P. aeruginosa*, and **(e, f)** *S. aureus* at varying concentrations of free chlorine [mg/L] and a pH of 7.2.

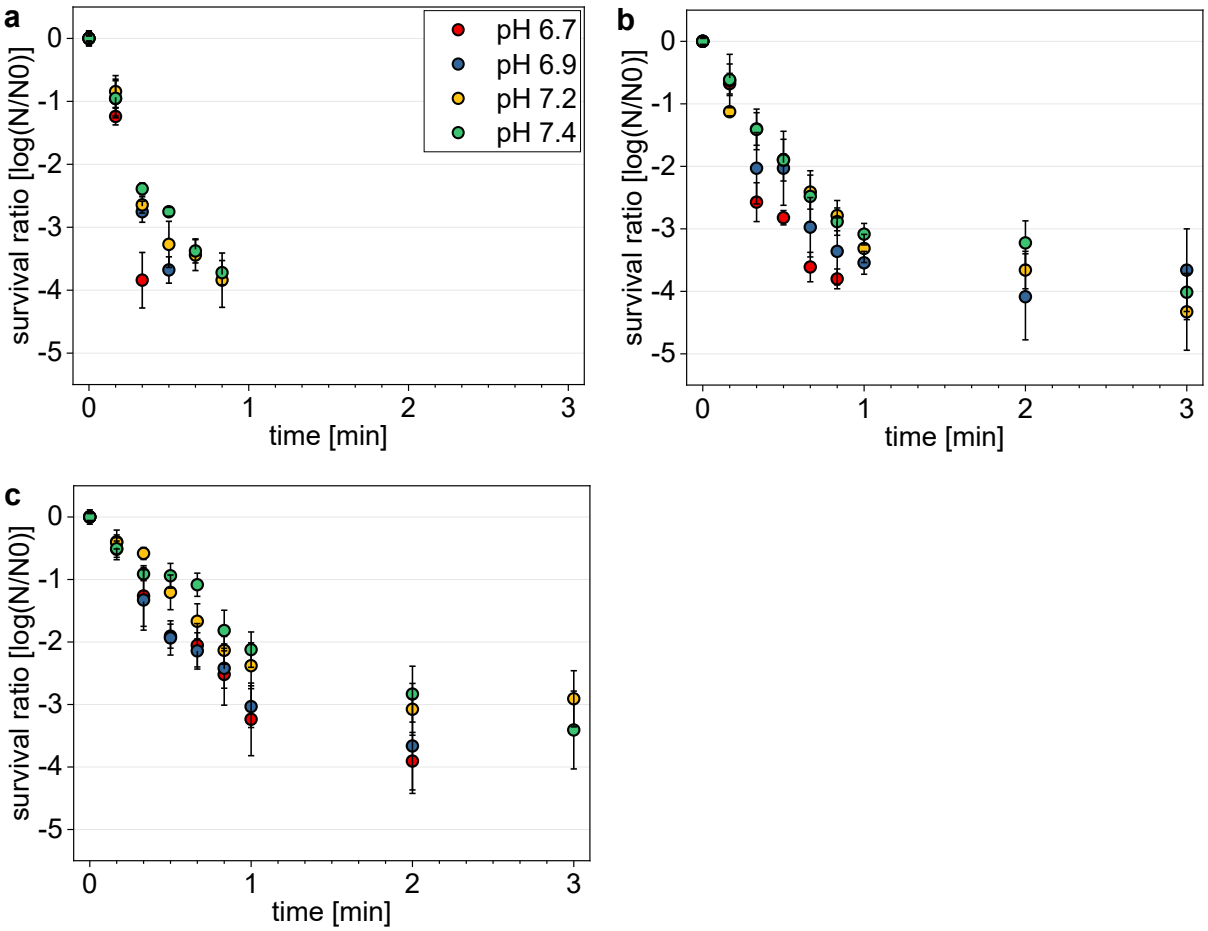

Fig. 18: The effect of pH on inactivation kinetics - raw data from the experiments performed. With decreasing pH, the pace of bacterial inactivation increased. Survival curves of **(a)** *E. coli*, **(b)** *P. aeruginosa*, and **(c)** *S. aureus*.

Table 5: Models used in this study for the interpretation of data on inactivation kinetics.

Model/Approach	Formula	References
Chick-Watson	$y = -k * C^n * x$	[74]
Multiple Log-Linear Phases	$y = -k * C^n * x$	[106]
Hom	$y = -k * C^n * x^m$	[11]
Intrinsic Quenching	$y = -(k/(l*n)) * (1-10^{(l*n*x)})$	[49, 50]
Biphasic	$y = P * 10^{-kt} + (1-P) * 10^{-lt}$	[68, 80, 81]
Weibull	$y = -k * x^n$	[82-85]

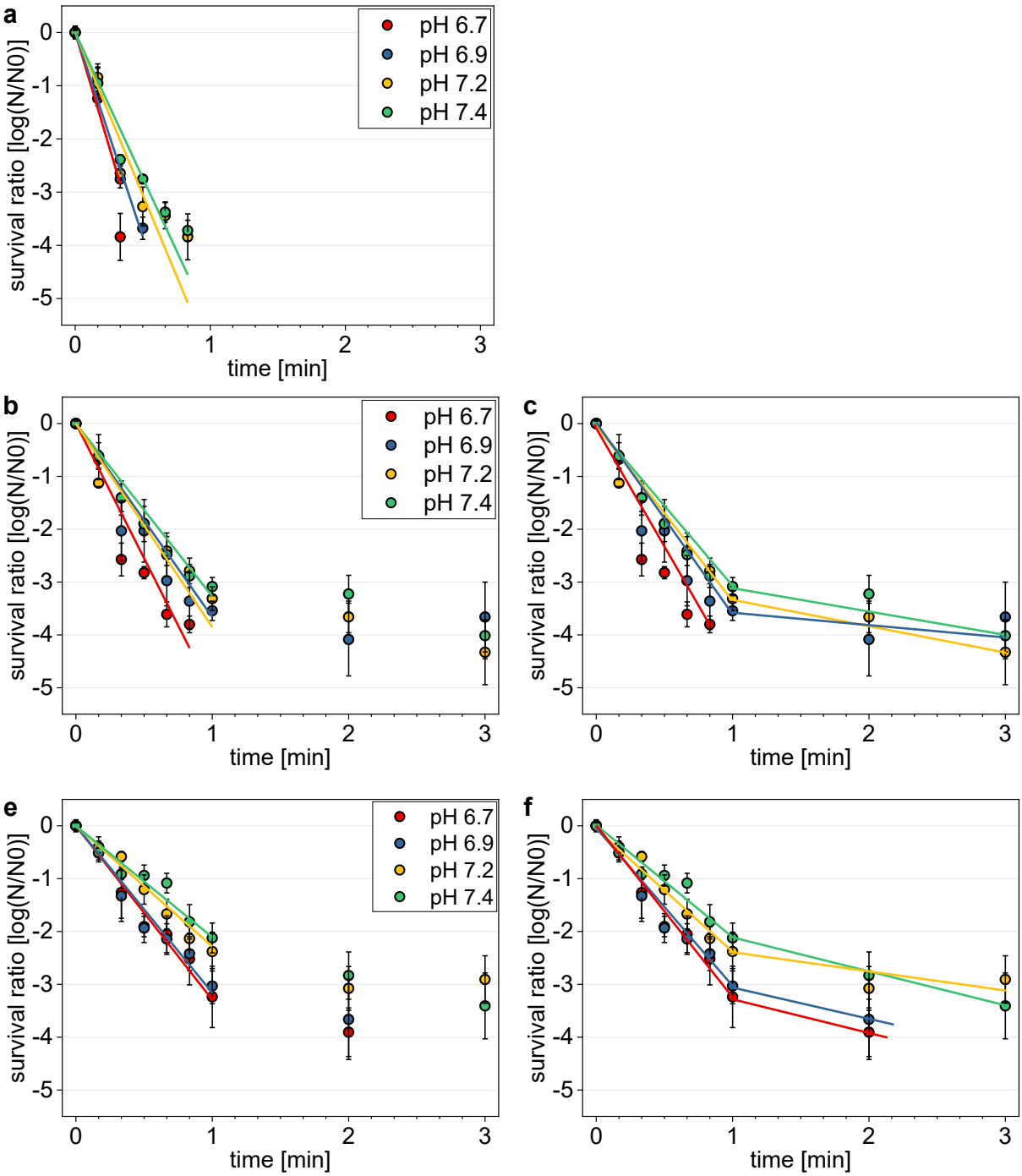

Fig. 19: The effect of pH on inactivation kinetics - Fitting with the Chick Watson model. As can be seen, in most cases, it was not possible to fit all data points with this model: **(Left column)** Survival curves of **(a)** *E. coli*, **(c)** *P. aeruginosa*, and **(e)** *S. aureus* at varying pH chlorine concentration of 0.2 mg/L. **(Right column)** Hypothetical division into several log linear phases of the survival of **(b)** *E. coli*, **(d)** *P. aeruginosa* and **(f)** *S. aureus*.

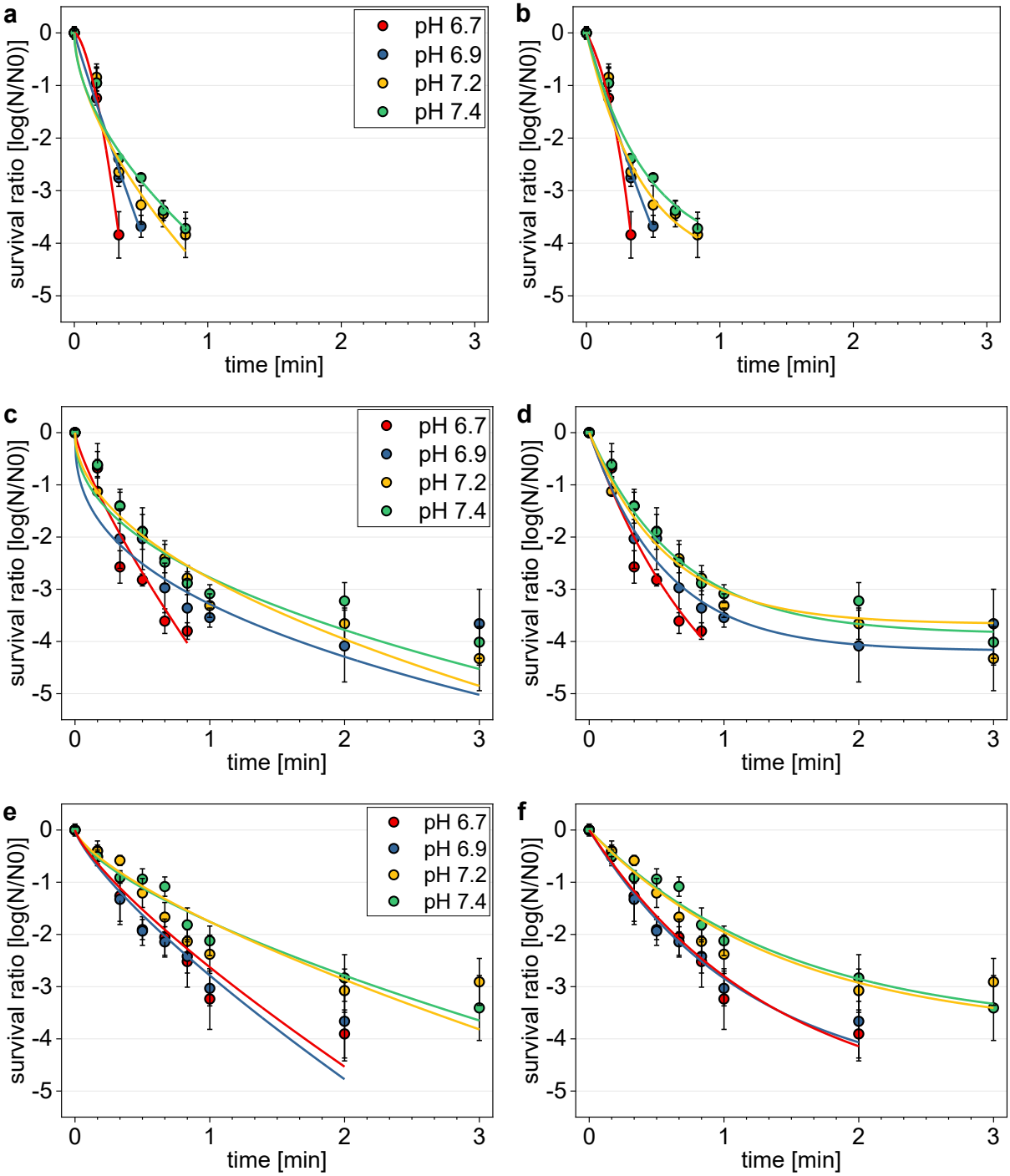

Fig. 20: The effect of chlorine concentration on inactivation kinetics - Fitting with the Hom model **(left column)** and the Intrinsic Quenching model **(right column)** respectively. In most cases, both models allowed fitting of curves with tailing. Survival curves of **(a, b)** *E. coli*, **(c, d)** *P. aeruginosa*, and **(e, f)** *S. aureus* at varying pH and a chlorine concentration of 0.2 mg/l.

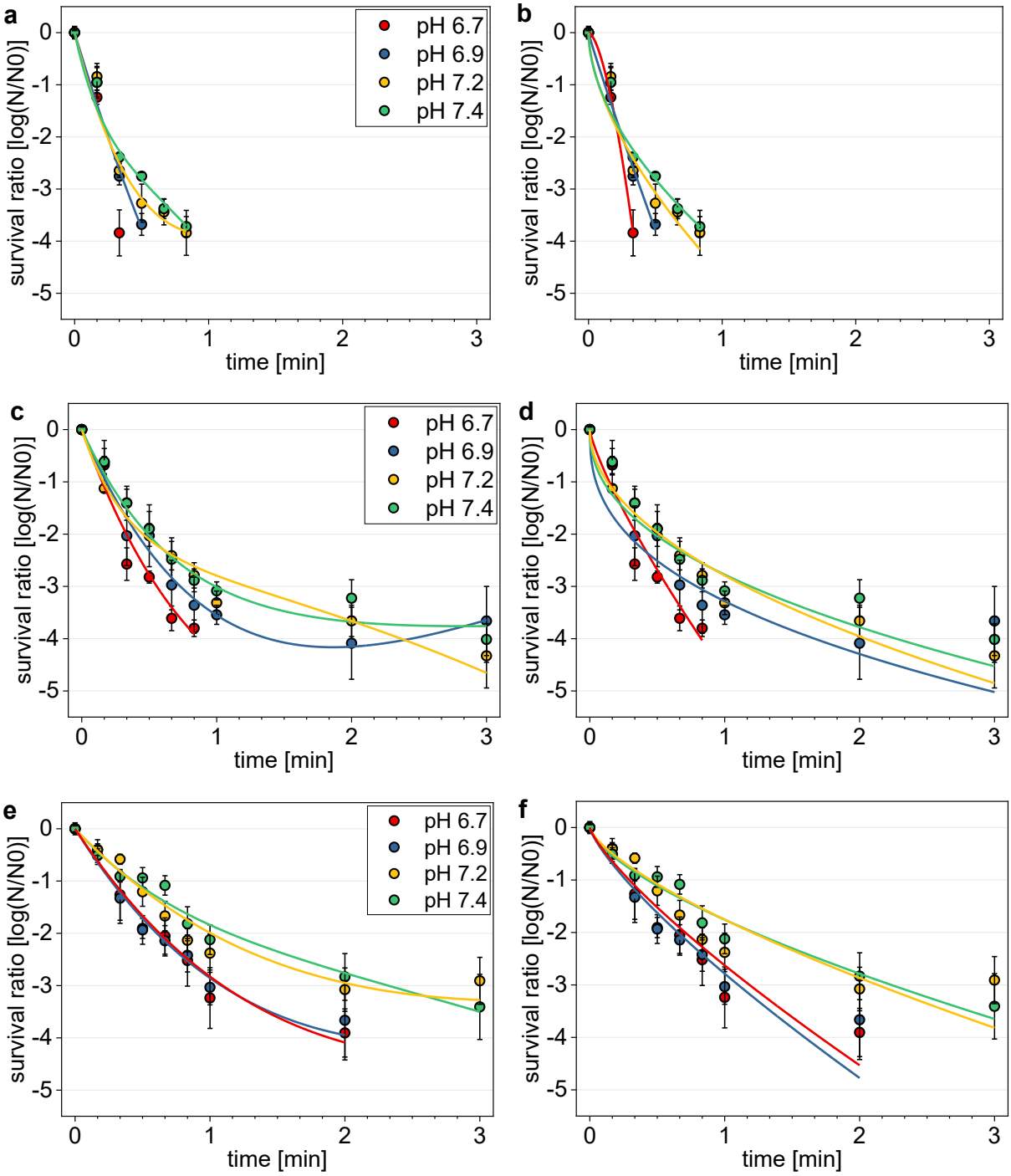

Fig. 21: The effect of pH on inactivation kinetics - Fitting with the Biphasic model (**left column**) and Weibull model **(right column)** respectively. In most cases, both models allowed fitting of curves with tailing. Survival curves of **(a, b)** *E. coli*, **(c, d)** *P. aeruginosa*, and **(e, f)** *S. aureus* at varying pH and a chlorine concentration of 0.2 mg/l.

3.3.1 Control Approaches

Alternative Growth Medium R2a

As explained, R2a agar is a medium designed to support the growth of slow-growing bacteria and such species adapted to low nutrition. In table 6, colony counts as determined on R2a and TSA are presented in comparison to the cell counts determined using the FISH technique. In most cases, higher colony counts were detected on R2a agar plates than on their TSA counterparts. Yet, for an interpretation of these findings, it is important to know that R2a was used only in some runs of the respective inactivation experiments. Therefore, the mean colony count determined using TSA is calculated using more individual values (9 each) than the mean colony count determined using R2a (3 each). Nonetheless, a tendency was determined towards slightly higher colony counts on R2a in comparison to TSA. This applies both to bacteria exposed to hypochlorous acid for 10 seconds and 1 minute. Interestingly, after an exposure to the disinfectant for 4 minutes, colonies still frequently grew on R2a, but only in very few cases also on TSA.

Table 6: Comparative presentation of the results obtained using TSA, R2a or microscopy for determining bacteria counts in inactivation experiments.

Experiment Run	Time Point	Mean Colony Count on TSA [1)] [cfu/ml]	Mean Colony Count on R2a [2)] [cfu/ml]	Cell Count under the Microscope [3)] [cells/2 ml]
E. coli, pH 6.9, 0.2 mg/L HOCl	10 sec.	61	93	---
	60 sec.	0	4	8
	240 sec.	0	0	0
P. aeruginosa, pH 6.9, 0.2 mg/L HOCl	10 sec.	102	135	---
	60 sec.	2	14	24
	240 sec.	0	1	2
S. aureus, pH 6.9, 0.2 mg/L HOCl	10 sec.	118	188	---
	60 sec.	7	26	76
	240 sec.	0	3	0
E. coli, pH 7.4, 0.2 mg/L HOCl	10 sec.	80	101	---
	60 sec.	0	1	3
	240 sec.	0	0	1
P. aeruginosa, pH 7.4, 0.2 mg/L HOCl	10 sec.	272	184	---
	60 sec.	6	14	31
	240 sec.	0	2	2
S. aureus, pH 7.4, 0.2 mg/L HOCl	10 sec.	146	148	---
	60 sec.	60	31	55
	240 sec.	0	2	0

[1)] mean value calculated from 9 values (3 runs); [2)] mean value calculated from 3 values (1 run); [3)] cell count of 1 run (2 ml of the test medium); `---´ not measured

Fluorescence *in situ* Hybridization (FISH)

The cell counts determined using microscopy are presented in table 6 next to those of the approaches using TSA and R2a respectively. In figure 22 some exemplary pictures of cells of the used strains of *E. coli*, *P. aeruginosa* and *S. aureus* are presented.

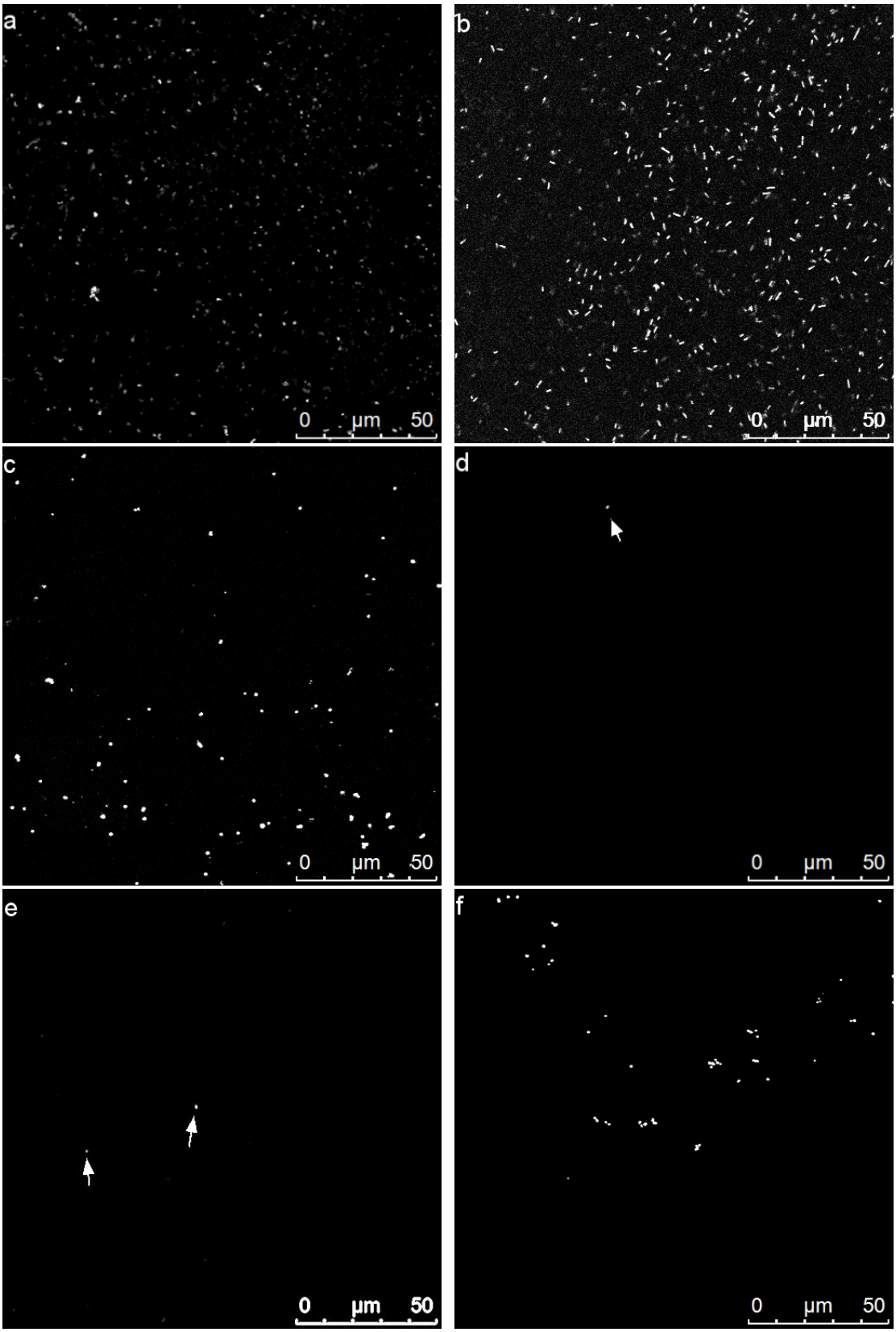

Fig. 22: False color photography images of bacteria cells labelled with an EUB 338 probe connected to an Alexa Fluor 488 tag: **(a - c)** before an experiment, **(e - f)** after exposure to 0.2 mg/L of free chlorine. **(a)** *E. coli*; **(b)** *P. aeruginosa*, **(c)** *S. aureus*, **(d)** *E. coli* (pH 6.9, 240 sec.), **(e)** *P. aeruginosa* (pH 6.9, 240 sec.), and **(f)** *S. aureus* (pH 7.4, 60 sec.). As can be seen, some cells remained intact over prolonged times exposed to hypochlorous acid.

The false color settings (grayscale) used in the presented images were either employed already in the microscope settings, or, in some cases, adjusted afterwards using ImageJ (public domain, developed by Wayne Rasband). Subsequent adjustment was performed in order to have a consistent presentation of the results. Furthermore, ImageJ was used to stamp identifying alphabetic characters on the pictures and to brighten up the images (as some signals were comparably weak, compare discussion of this chapter).

Examination under the microscope was performed only for some of the experiments on inactivation kinetics. Nonetheless, the few performed examinations lead to an interesting observation. Frequently, single cells were detected even after 4 minutes of exposure to chlorine. In the approaches using TSA, cfu counts reached 0 at this time point. This means, that some cells were still intact at this time of an experiment, although they were no longer detectable using culturing methods. The implications of this finding are part of the discussion of this chapter.

3.4 Discussion

3.4.1 Evaluation of the Selected Methods

To this day, in order to pass legislation, disinfectants are assessed using traditional methods [56] which rely on the cultivation of bacteria. Disinfectants are tested by single time exposure of an inoculum. Inactivation of microbes is assessed at defined contact times. However, as these methods originally were not designed to provide concise scientific information, they are associated with difficulties regarding their reproducibility [56, 107]. The results of the present study were produced using culture-dependent methods. Hence, they may have been influenced by these weaknesses. The intention was to achieve results relatable to those from routine control, where plate counting is still the gold standard. Yet, consequently, all those bacteria not detectable with culturing methods, e.g. individuals in the dormant-like “viable but non-culturable state” [108] or generally non-cultivable environmental bacteria, have to be excluded from any considerations made.

Furthermore, the experimental setup designed for the performed experiments will have influenced the results in some way. For example, due to the large test volume, the setup provided a comparably strong dilution of the initial inoculum, which potentially lead to an error in determining colony counts. Although steady mixture of the medium was guaranteed, it is still not completely certain, that the inoculum was always completely dispensed when samples were taken. Other potential sources for errors are the washing steps before using an inoculum or the sampling at the defined time points. However, in order to minimize the occurrence of effects deriving from the test procedure, every step of a respective experimental runs was performed with the high diligence demanded. The influence of the test setup and performance are therefore considered negligible by the author.

Another point to be considered is that - in experiments with higher concentrations of free chlorine or low pH values respectively - less data points were available for interpretation, than in those experiments using lower concentrations of chlorine or higher pH values respectively. As a result, coefficients of determination (as calculated by fitting the plotted data with models for inactivation kinetics) sometimes had better values in those runs with low concentrations of free chlorine or high pH values. This created a bias, which, if interpreted inattentively, may falsely indicate a better correlation for some runs than for others. Nonetheless, based on the

coefficient of determination, the results achieved may be compared with each other to understand which of the used models best to fit the data. If, for instance, one model resulted in a better coefficient of determination than another and, at the same time, more data points were included in the calculation or the first compared to the latter, this proves that the respective model had a better application for the given data.

More importantly, inactivation curves of some of the experiments performed lacked significance with respect to the differences between individual runs. Having a look at the standard deviations of data points in the semi-logarithmic plots, one will notice that there was a large overlap between data from different runs of an individual approach. This is especially the case for those experiments on the effect of the pH, but to a lesser extent it also applies to the experiments on chlorine concentrations.

Fluorescence microscopy revealed that some cells remained intact for longer periods of time than cultivable bacteria were detected on TSA. The question is, how these cells survived exposure to hypochlorous acid. In general, in the examined samples, clumping of cells was not detected. Yet, in some cases, cells were located relatively close to each other. This could indicate that adhesion of cells to other cells had occurred, which, in turn, would provide an explanation for their prolonged survival. However, the observed phenomenon could also go back to the preparation procedure. As the samples were left for drying on object slides overnight, a gradual shrinking of the area covered by fluid occurred, which, in turn, may have led to converging of the cells contained.

Additionally, in comparison to samples taken at the start of an experiment, cells frequently appeared smaller and the fluorescent signal was weaker in samples taken after exposure of the respective bacteria to hypochlorous acid. On the one hand, this could mean that bacteria cells were damaged to some extent, although they did not lose their integrity completely. On some slides, cloudy spheres presumably composed of the fluorescent marker could be seen. Perhaps, these indicated the former location of cells which disintegrated due to exposure to chlorine and/or the treatment before microscopy. In this case, both mentioned phenomena would be explainable as different stages of cellular decay. However, this could also mean, that respective cells were in a state allowing their survival, accompanied by a smaller cell volume and/or downregulation of DNA replication or transcription. Also, the fluorophore may have been damaged to some extent by the chlorine disinfectant.

3.4.2 Recapitulation of the Results

In figure 14, results on the effect of chlorine concentration on the pace of inactivation are presented. A predictable trend was determined independent of the test species: the higher the initial concentration of chlorine, the faster the inactivation of bacteria proceeded. Results on the effect of pH value on inactivation kinetics are presented in figure 18. Here, results are less distinct. However, as expected, with increasing pH, the speed of inactivation decreased.

As indicated, the experimental design was developed considering the current German DIN standard on swimming pool maintenance [19]. Here, for chlorine, a lower limit of 0.3 mg/L is defined. A reduction of *Pseudomonas aeruginosa* by 4 orders of magnitude within 30 seconds is regarded as sufficiently effective disinfection. Within this framework, the results achieved may be interpreted as follows: At a pH of 7.2, 0.3 mg/L of free chlorine were suitable only for the inactivation of *Escherichia coli*. However, under given premises, inactivation kinetics were

borderline to insufficient for *Pseudomonas aeruginosa* and clearly insufficient for *Staphylococcus aureus*.

The lowest used concentration of chlorine (0.1 mg/L) was inadequate for the determination of inactivation kinetics. Results were significantly different from those achieved in runs with higher concentrations of free chlorine. On the other hand, using comparably high concentrations of free chlorine (>0.4 mg/L) resulted in very fast proceeding inactivation of bacteria, consequently leading to results not allowing an interpretation based on kinetic models. Already after 10 seconds, there were no cultivable bacteria remaining. Concentrations of 0.2 and 0.3 mg/L of free chlorine brought results allowing an interpretation based on kinetic models.

3.5 Conclusions

3.5.1 Fitting Models

Frequently, inactivation kinetics did not follow strictly log-linear courses. As shown in figures 15 and 19, many inactivation curves apparently were composed of at least two separate phases. Each of these phases can be interpreted as an individual phase of log-linear decline with its own inactivation rate constant. As indicated, a period of faster decay (or a labile regime) was followed by a period of comparably slower decay (or a resistant regime) [79]. However, while such a tailing was the most frequent phenomenon, also initial lag-phases were observed.

Although equal deviations from monophasic decay have often been described in studies investigating inactivation kinetics, assumptions of first-order kinetics are still common to this day [11, 79]. Actually, there are even studies concluding that there was no improvement in using alternative models and not exclusively the Chick-Watson model [11, 74]. However, regarding the results of the present study, log-linear curves were only then achieved, if either chlorine concentrations or pH value were within an ideal range. If, however, any factor varied towards non-ideal conditions (e.g. by raising the pH), the shape of respective inactivation curves exhibited curvatures.

Except for the Chick-Watson model, all used fitting models were suitable for the fitting of most of the inactivation curves. However, in all cases, some inactivation curves could not be fitted with the selected models. Both the classical approach by Hom [54] and the more recently developed Intrinsic Quenching model (IQ model) [50] were used to interpret the data in a mechanistic way. The IQ model was originally developed for examination of inactivation kinetics as induced by non-oxidizing disinfectants. This model was used in the present study, although chlorine disinfectants are obviously oxidizing agents, in order to examine the effect of disinfectant decay on inactivation kinetics. On the other hand, vitalistic models assume that multiphasic bacterial decay is the result of the composition of an inoculated population of subpopulations differing in their individual resistance towards a given treatment [80]. Subpopulations may, for instance, go back to the occurrence of different strains of a given species, mutations within individuals, differences in the phase of growth etc. [79]. In the present study, the popular bi-exponential model and a model based on Weibull distribution were used.

Table 7 lists how many data points were used for the calculation of fitting curves in the respective runs. Furthermore, the coefficients of determination (r^2) are listed for each approach. With respect to the results, both mechanistic and vitalistic models succeeded or failed fitting the given data in comparable scopes. As shown in table 7, all selected models lead to quite good correlations. The Hom model, which does not provide any explanation for

what was observed in the experiments performed, was equal to the Weibull based model. Assuming the Weibull model was right, the inactivation curves achieved would indicate a distribution of resistances, with most of the exposed bacteria being comparably sensitive, while a minority was less sensitive, which lead to the observed tailing. The most obvious reason, according to Cerf, is genetic heterogeneity of the exposed population of bacteria [68].

If two subpopulations of diverging resistance are mixed, this would results in a biphasic survival curve [68, 109, 110]. For the present study, results have been interpreted as two independent log-linear phases. Following Cerf's line of thought, the occurrence of two inactivation curves would indicate that two subpopulations of diverging resistance towards chlorine were present. Cerf [68] furthermore states that some studies have succeeded in selecting individuals with increased resistance (e.g. [111]). Selecting survivors of a treatment resulted in following generations of increased resistance to this treatment.

In 1918, Lee and Gilbert presented their concept to explain upward-concave or sigmoidal survival curves [11, 66]. They assumed the shapes of these curves to be determined by a distribution of resistances within exposed populations, suggesting that such individuals of average resistance would be the majority. Hence, they concluded that survival times could be expected to be normally distributed. Later, Withell found evidence that the logarithm of survival times of some microbes was actually normally distributed [69]. However, a distribution opposite to the one proposed by Lee and Gilbert was found, with microbes with low resistances being the majority, while such individuals more resistant being the minority [69].

At any rate, as also the Intrinsic Quenching model succeeded in fitting most of the data, next to the vitalistic approach mentioned above, also mechanistic reasons should be considered. Another potential reason for the occurrence of tailing is a heterogeneity of the treatment, e.g. variants in temperature, pH or disinfectant concentration [68]. Although the experimental setup was designed with the intention of minimizing the effect of these factors, changing conditions during disinfection may still have played a role. For instance, dilution of the inoculum during an experiment could have had an influence on the outcome. Since a large ratio of inoculum size to test volume was selected, the inoculum was diluted during an experiment, meaning that over time the respective bacteria became more assailable by chlorine. Consequently, also inactivation frequencies would increase over time, resulting in a convex curvature in semi-logarithmic representation. Regarding the results achieved in the present study, this applies to data on both high concentrations of free chlorine and low pH values respectively. Here, inactivation frequencies apparently increased over time.

Another factor which needs to be considered is the consumption of chlorine during the disinfection process. Although a large ratio of inoculum volume to test volume was chosen, as mentioned above, declining concentrations of the disinfectant still may have impacted inactivation kinetics. A removal of chlorine would have slowed down inactivation frequencies, expressed in a flattening of respective curves in the rear section. Presumably, this applies especially for those experiments conducted with low concentrations of free chlorine. Curves expressing a concave shape (or tailing) were observed in several experiments with comparably low concentrations of free chlorine or high pH values. As has been explained by Johnston et al. (2000) [49], the concept of using concentration/time data to assess disinfection efficacy does not work if the used test concentration of the disinfectant is assumed to be dependent on time. As indicated by its creators [50], the IQ model becomes reduced to the simpler model by Chick-Watson, when the constant Q (k_2n) is appropriately small.

Table 7: Data points used and r^2 of the performed inactivation experiments.

Chlorine Concentration	*E. coli* Data Points	*E. coli* r^2	*P. aeruginosa* Data Points	*P. aeruginosa* r^2	*S. aureus* Data Points	*S. aureus* r^2	pH Value	*E. coli* Data Points	*E. coli* r^2	*P. aeruginosa* Data Points	*P. aeruginosa* r^2	*S. aureus* Data Points	*S. aureus* r^2
Chick-Watson model													
0.1	10	0.940	8	0.862	10	0.499	6.7	3	0.935	6	0.971	7	0.974
0.2	7	0.931	7	0.903	7	0.958	6.9	4	0.99	7	0.990	7	0.988
0.3	3	0.995	4	0.948	7	0.963	7.2	6	0.939	7	0.903	7	0.958
0.4	1	-	3	0.897	5	0.988	7.4	6	0.969	7	0.991	7	0.947
0.5	1	-	1	-	3	0.990							
Hom model / Weibull model													
0.1	10	0.933	9	-	10	0.869	6.7	3	1	6	0.975	8	0.935
0.2	7	0.975	9	0.986	9	0.858	6.9	4	0.986	9	0.966	8	0.970
0.3	3	1	4	0.999	7	0.960	7.2	6	0.972	9	0.988	9	0.858
0.4	1	-	3	1	5	0.985	7.4	6	0.995	9	0.964	9	0.969
0.5	1	-	1	-	3	1							
Intrinsic Quenching model													
0.1	10	0.935	9	-	10	0.823	6.7	3	1	6	0.982	8	0.965
0.2	7	0.984	9	0.971	9	0.919	6.9	4	0.988	9	0.994	8	0.919
0.3	3	1	4	0.993	7	0.961	7.2	6	0.983	9	0.975	9	0.987
0.4	1	-	3	1	5	0.984	7.4	6	0.995	9	0.992	9	0.965
0.5	1	-	1	-	3	1							
Biphasic model													
0.1	10	0.925	9	0.824	10	0.813	6.7	3	1	6	0.977	8	0.960
0.2	7	0.981	9	0.976	9	0.915	6.9	4	0.986	9	0.996	8	0.915
0.3	3	-	4	0.987	7	0.951	7.2	6	0.973	9	0.976	9	0.986
0.4	1	-	3	-	5	0.976	7.4	6	0.995	9	0.991	9	0.960
0.5	1	-	1	-	1	-							

In other words, if the biocide is subjected to quenching only weakly, the plot of the respective experiment will be a log-linear survival curve. *Vice versa*, with increasing Q, the degree of the curvature of the respective curve will also increase. With Q > 1, the rate of inactivation will rapidly fall to zero [50].

With respect to the results achieved in the present study, this means, that chlorine consumption could have led to the concave shape of some survival curves as has been determined. However, the IQ model was developed for the fitting of data from experiments using such disinfectants which are only removed, if the individual target is present. Quenching agents used in disinfection experiments often include emulsifying agents (e.g. Tween or lecithin), as do microbial membranes (e.g. phosphatidyl ethanolamine and triacylglycerols). Johnston et al. hence assume that, if a biocide ruptures a microbe, the cell membrane may quench out the biocide. However, they do also argue, that this self-quenching phenomenon works well for surfactant-type biocides, such as quaternary ammonium compounds, but does not work with hypochlorite [49]. As the authors indicate, this intrinsic quenching reaction will be dependent on the biomass present per milliliter of solution. The quenching reaction would then be related to cell numbers. They also state, that “a truly predictive disinfection model” should also include a term for the inoculum size [49]. However, because of the oxidative character of chlorine disinfectants, they are consumed also by reactions with material other than living bacteria (e.g. the remains of dead bacteria). Chemical reactions will thus result in a reduction of hypochlorite concentration, which in turn, to some extent is independent of disinfection. This needs to be kept in mind, when interpreting the results, as it will have a strong influence on the observed kinetics. Interestingly, the scientists who developed the IQ model also state [49] that the idea has become dogma, that the reagent in excess in disinfection processes always is the disinfectant [112]. They criticize this dogma, intending to proof that it is wrong [50].

3.5.2 Role of the Test Species

According to the results, the species most susceptible to chlorine was *Escherichia coli.* This is a coherent finding, as, in general, *Escherichia coli* is considered quite sensitive to chlorine disinfection [42, 43, 113]. If *E. coli* was used in experiments, inactivation kinetics followed approximately log-linear courses. However, if either *P. aeruginosa* or *S. aureus* were used in experiments, inactivation curves often displayed a gradual slowing of inactivation kinetics over time.

In line with the findings of the present study, Seyfried and Fraser [114] discovered, that *Pseudomonas aeruginosa* was present more frequently in swimming pool water, if the levels of free chlorine dropped below 0.4 mg/L. They also correlated increasing pH values with decreasing disinfection success. This correlation is as well indicated by the results of the experiments performed in the present study.

Species-specific sensitivity to a treatment may be attributed to diverse factors including aggregation of cells, secretion of unique proteins and attachment of bacteria to suspended particulate matter [115-117]. Using *P. aeruginosa* in the experiments performed, secretion of protective substances may have played a role [57]. The strain used was a mucoid wild type. Hence, substances like mucopolysaccharides could have protected cells from chlorine exposure [114, 116, 118, 119]. For *S. aureus*, if anything, the achieved results suggest an influence of the comparably thicker cell wall and/or aggregate formation. Being protected by a coat of inactivated cells, some individuals may have survived chlorine exposure for comparably

longer periods than their sister cells [68]. However, laser scanning microscopy did not reveal the occurrence of clustering, although for *S. aureus*, cells were located comparably close to each other (compare 3.2.7). In line with this, Wickramanayake et al. in their study on inactivation of clump-free protozoan cysts achieved survival curves with a lagging-phase [120]. Weavers and Wickramanayake [11] seduced from these results, that lag phases in microbial inactivation may not be entirely attributed to aggregation.

3.5.3 On the Use of Ct Values

Ct values were determined using the data generated using the Chick-Watson model [75]. As shown in figures 23 and 24, Ct values remained at approximately the same level independent of chlorine concentration. Yet, apparently, they were dependent on the pH.

As has been explained, Ct values may be used to compare the relative resistance of several microorganisms to a given disinfectant [11]. Such information is needed, if a given matrix commonly contains several microbial species. Since this is the case in swimming pool water, in the present study the Ct value was used for comparative assessment of disinfection success. Of the experiments on the effect of chlorine concentration on inactivation kinetics, the approaches with 0.1 mg/L of free chlorine did not provide reliable results. Presumably, here, chlorine concentrations dropped too fast for a steady inactivation of the bacteria exposed.

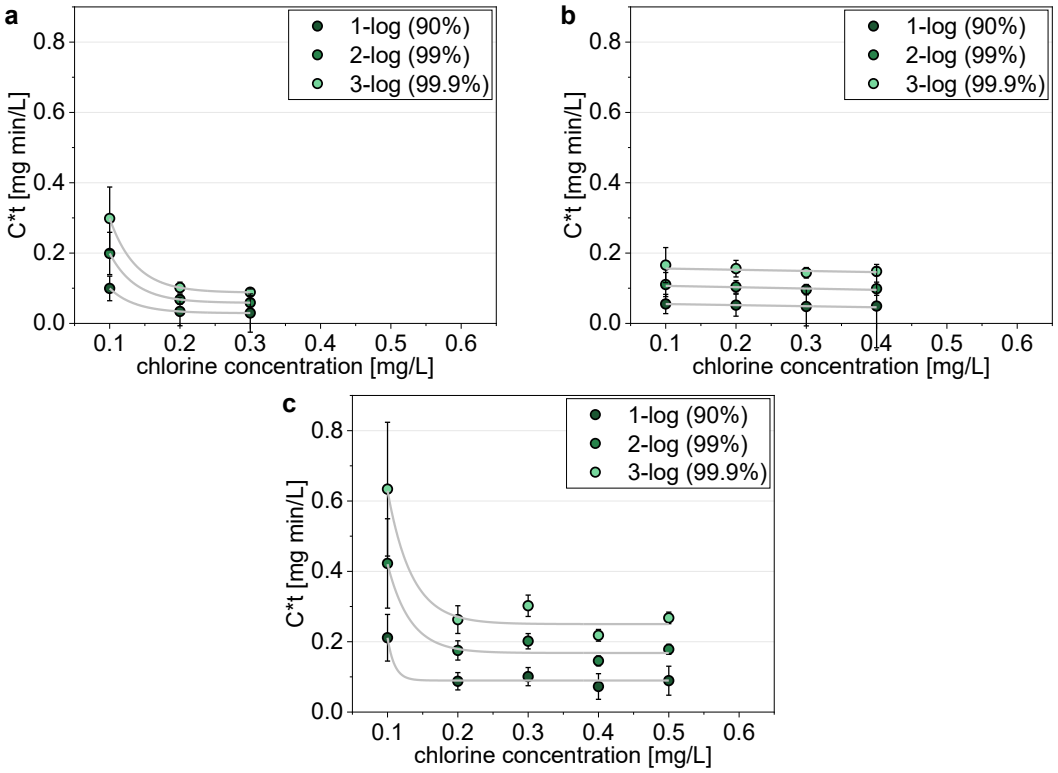

Fig. 23: Ct values (1-log, 2-log, and 3-log) dependent on the initial concentration of free chlorine [mg/L] using **(a)** *E. coli*, **(b)** *P. aeruginosa*, and **(c)** *S. aureus*. As can be seen, the Ct value does not vary much between the individual approaches using varying concentrations of chlorine (with exception of 0.1 mg/L). The highest Ct values were reached using *Staphylococcus aureus*, the lowest Ct values were achieved using *Escherichia coli*.

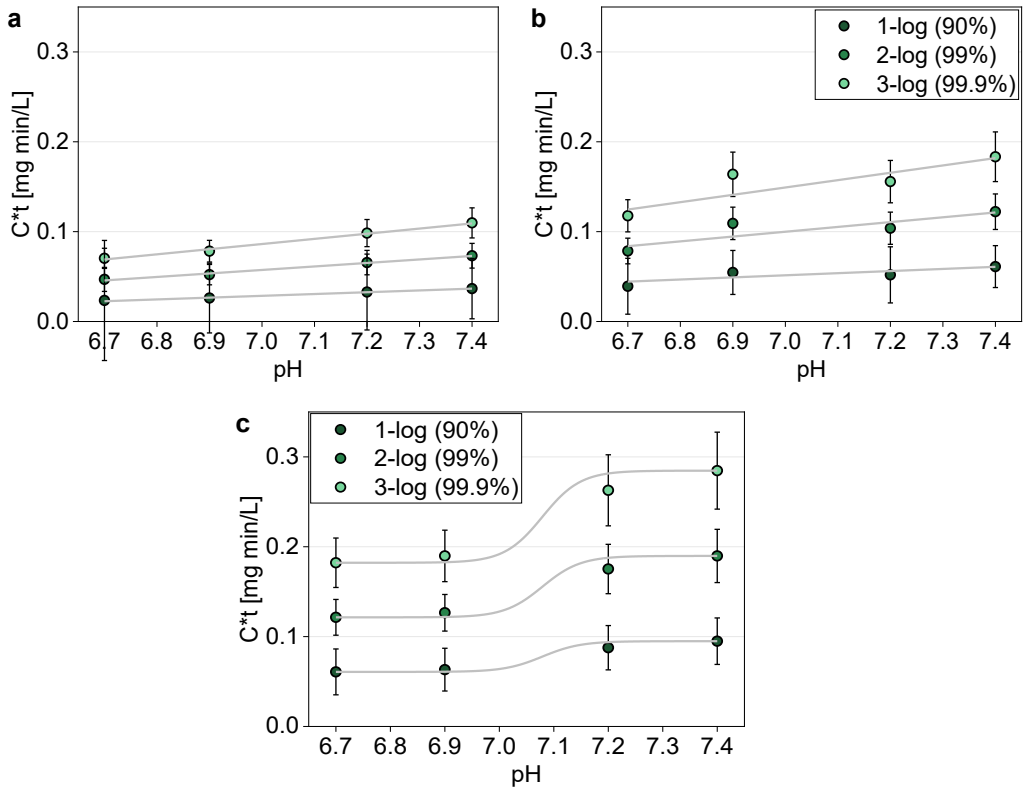

Fig. 24: Ct values (1-log, 2-log, and 3-log) dependent on the pH value of the medium using **(a)** *E. coli*, **(b)** *P. aeruginosa*, **(c)** *S. aureus* at 0.2 mg/L of free chlorine. As can be seen, the Ct value increases with increasing pH values. The highest Ct values were reached using *Staphylococcus aureus*, the lowest Ct values were achieved using *Escherichia coli*.

As explained already in 1986, the use of Ct values is, at any rate, associated with some difficulties [75]. The respective study lists the following: variances in the susceptibility of different isolates of the same test species, failure to fit data achieved with the Ct equation, variances between species of the same group (e.g. bacteria), effects of the state of the organisms used (e.g. prior growth conditions, aggregation), effects by the experimental conditions (e.g. variations in disinfectant concentration, intensity of mixing), and transferability of laboratory data to field conditions. Furthermore, the study concludes that Ct values at some occasions express only minor variations between different concentrations of the same disinfecting agent, but sometimes express a large variation.

In a nutshell, identification of underlying mechanisms is difficult, because many factors, some of them unknown, contribute to the outcome. According to the study cited, therefore, the use of Ct values should be done only “with considerable caution and incorporation of appropriate safety factors”. The Ct concept was nonetheless included in the present study, as the concept is considered beneficial for data analysis in pilot scale tests covering only a few points of a broader range of environmental conditions [11]. As this applies to the present study, the author considered inclusion of the concept beneficial for this study.

3.5.4 Potential Role of Non-Culturable State Bacteria

Comparing the results of the approach using TSA as growth medium with those using R2a, a tendency towards higher colony counts on the latter medium became apparent. With increasing time of exposure to chlorine, the difference between growth on these two mediums was also increasing. At short times of exposure, colony counts differed only slightly. However, after an exposure to chlorine for 4 minutes, growth nearly exclusively could be detected on R2a agar plates. Although these findings are based only on a low number of experiments and cannot be verified statistically, the findings still appear coherent. Presumably, R2a allowed growth of cells in an injured or near-dormant state, which did not form colonies on TSA.

As illustrated in figure 22, single bacteria cells were detected under the microscope even at time points at which no cfu were counted. This means, that some cells remained intact over the whole examination period but, apparently, were no longer able to form colonies. Thus, these cells did not contribute to the dataset used for determination of inactivation kinetics. However, if these cells were able to restore their cultivability remains uncertain and was not part of the examinations performed. If these cells were able to regain their ability to grow on media, this would indicate that they switched into a state allowing their survival only temporarily. A comparable behavior could then also occur in swimming pools and would provide an explanation for recontaminations after removal of initially detected bacterial contaminations.

In this connection, it appears interesting, that, based on older publications, Pernitsky et al. (1995) [55] concluded that the main sources of contamination in drinking water are the shedding of biofilms and the release of colonized particles from filters. The same authors also state, that bacteria residing on “granular activated carbon” (GAC) may survive for increased periods of time [55]. A paper written by Cerf [68] mentions that the enumeration of survivors could be a source for artefacts. As techniques which are based on counting low numbers have a very high variability, according to Cerf, this could lead to tailing due to a selective counting procedure [51]. Accordingly, growth conditions and the selection of test strains may influence the outcome of inactivation experiments [57]. With respect to the results achieved with R2a agar in the present study, that the inactivation kinetics determined were influenced by the selected growth medium. The results achieved using the FISH technique indicate that single cells may have survived during inactivation experiments. The question remains, whether these cells, which presumably switched to a non-culturable state, were able to regain their ability for growth and multiplication.

Chapter 4

Small Colony Variants of *Pseudomonas aeruginosa* [3)]

Chapter Abstract

The present chapter provides additional information on a phenomenon observed in some of the experiments described in chapter 3. After chlorination, colonies of the used wild type strain of *P. aeruginosa* frequently showed an atypical morphology. Such colonies were characterized by their reduced size and the lack of a normally occurring prominent coloration. This chapter is dedicated to the interpretation of this phenomenon. Both the smaller size and the lack of coloration of colonies have also been described by clinical studies on infections with *P. aeruginosa*. Apparently, both go back to adaptions related to persistence and virulence. Furthermore, the lack of coloration indicates reactions of *Pseudomonas aeruginosa* towards oxidative stress as induced by hypochlorous acid. Potentially, the observed changes are symptoms of the gradual passage of bacteria cells from a cultivable and susceptible state towards a temporarily non-cultivable but also less susceptible state.

4.1 Introduction and Materials and Methods

The present chapter addresses results produced by experiments on the resistance of *Pseudomonas aeruginosa* to chlorine, as performed for chapter 3. Here, colonies of atypical morphology and size were detected frequently after treatment of the test germ with hypochlorous acid. Corresponding colonies were smaller in size and lacked the typical coloration of normal colonies of the strain on the selected growth media.

After recognizing that colonies of atypical morphology occurred, additional steps were taken to characterize the observed. Atypical colonies were compared to typical colonies as performed in similar studies [121]. In addition to the experiments described in chapter 2, smaller scale experiments on chlorine disinfection were performed*;* 500 ml beakers were used as test vessels. A magnetic stirrer (IKAmag RET-GS, IKA Janke & Kunkel) was used to provide mixing. Except for these modifications, the experimental procedure was adopted from the experiments described in chapter 3. The same media were used, and sampling procedure and time points were the same as in these experiments.

[3)] Parts of this chapter were presented in talks and posters at several occasions:

Kreuter, L., Skarke T. and L. Erdinger, *Persistence of planktonic Pseudomonas aeruginosa under swimming pool conditions*, How Dead is Dead? V conference, 6^{h} to 8th of September 2017, Vienna (Austria)

Kreuter, L., *"Minimum necessary chlorine concentration" - recent additions*, IntelliPool Project Meeting, 02.11.2016, Southampton (England)

Kreuter, L., Schlosser T. and L. Erdinger, *Evaluation of disinfection kinetics in swimming pools, taking into account the Occurrence of bacterial subpopulations*, ÖGHMP Annual Conference 2016, Zell am See (Austria).

Kreuter, L., *Determination of minimal necessary chlorine concentration for disinfection*, IntelliPool Project Meeting, 17.09.2015 Barcelona (Spain)

However, for the purposes of the present chapter, inactivation kinetics were ignored as the occurrence of atypical colonies after treatment with chlorine was the only parameter examined.

The used concentration of free chlorine was 0.2 mg/L. The pH of the medium was adjusted to 7.2 by the same means described in chapter 3. Atypical colonies were verified to be formed by *P. aeruginosa* by subjecting them to MALDI-TOF identification as described in chapter 6.

4.2 Results

Exposure to chlorine resulted in the occurrence of atypical colonies in most of the cases examined. Atypical colonies did not occur without exposure to chlorine. However, exposure to chlorine did not always result in the switch of all colonies to the atypical morphology. At exposure times of 10 seconds and 20 seconds respectively, approximately one third to half of the colonies exhibited the changed morphology. At later time points, either 0 or 100 percent of the colonies were of reduced size. However, the lack of pigmentation in many cases affected both such colonies of normal size and such colonies of reduced size. Examples of both types of colonies are presented in figure 25.

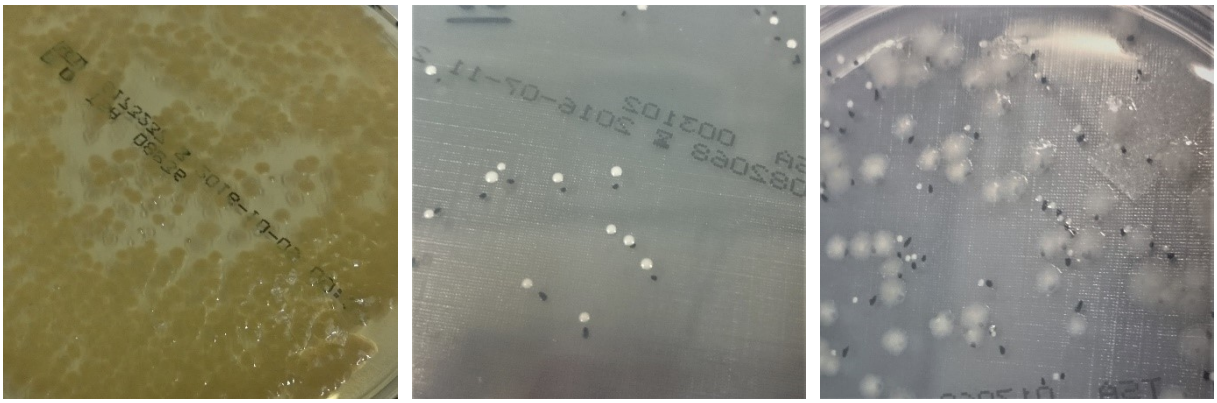

Fig. 25: Changes in the morphology of colonies of the used wildtype strains of *P. aeruginosa* on TSA agar induced by exposure to hypochlorous acid: **(a)** Without exposure to hypochlorous acid. Colonies are approximately 10 mm in diameter and express a strong green coloration; **(b)** Atypical morphology after exposure to hypochlorous acid (0.3 mg/l of free chlorine, pH 7.2. Colonies are marked by nearby black dots. Colonies are approximately 1 mm in size and express no green coloration; **(c)** Co-occurrence of such colonies of normal size and such colonies of reduced size after exposure to hypochlorous acid (0.3 mg/L of free chlorine, pH 7.2). Colonies of the smaller type are marked by nearby black dots. In this case, both types of colonies lack the typical green coloration.

4.3 Discussion

4.3.1 Altered Colony Morphology

The occurrence of colonies of atypical morphology, especially formed by such bacteria exposed to chlorine, could be an indication of persisting states of the respective bacteria.

In literature, phenomena such as those observed, are often referred to as small colony variants. As indicated by the name, small colony variants are characterized by comparably low growth rates and unusual biochemical features, resulting in an overall smaller size and abnormal appearance of colonies [122]. SCVs were first identified in 1910 for *Salmonella enterica serovar* Thyphi [122, 123]. Since then, SCVs have been described for a broad range of bacterial species, some of which are particularly interesting for the present study (e.g. *Escherichia coli* [124], *Pseudomonas aeruginosa* [125], and *Staphylococcus aureus* [126]).

Due to their difference to wild type colonies, SCVs are often difficult to identify with classical methods. Nonetheless, descriptions of SCVs of *Pseudomonas aeruginosa* match the observations made in the present study. Atypical colonies were described as being smaller than their wildtype counterparts, exhibiting a reduced pigmentation, and needing comparably longer periods to grow [121, 122, 125]. Comparable results were also achieved in studies with *S. aureus* [122, 127].

Bayer et al. [121, 128] suggest that, due to the characteristics of SCVs mentioned and their association with further defects and the retention of *in vivo* virulence, SCVs of *P. aeruginosa* presumably are related to the occurrence of persister cells. Persisters are defined as bacteria which are temporarily more tolerant to exposure to antibiotics than the rest of a given population [129]. Commonly slow-growing (or even non-growing), persister cells are able to restore growth after removal of a stress event (even if the event would normally be lethal for bacteria of the respective species and strain) [129]. The occurrence of persister cells (or in other words, the heterogenicity of a bacterial population) presumably plays an important role in the adaption of bacterial populations to environmental changes [129].

As SCVs presumably are more resistant to both host immune reactions and antibiotics than their wildtype counterparts [122], their occurrence indicates the presence of persister cells. With respect to the results of the present study, the described has the following implications: Atypical colonies, as observed after the treatment of inoculi of *P. aeruginosa* with hypochlorous acid, could go back to some cells momentarily being adapted better to the treatment and the accompanied oxidative stress than most of the cells. These cells would therefore survive the exposure to the disinfecting agent for longer periods of time than other cells. However, their growth on agar plates presumably is affected by their current state.

4.3.2 Lack of Pigmentation

Apart from the changed overall appearance of the colonies mentioned above, most of these atypical colonies also lacked pigmentation. While commonly, colonies of *Pseudomonas aeruginosa* showed a blue-green color going back to distinct sets of pigments excreted by this bacterium, atypical colonies were characterized by the complete lack of this coloration.

The coloration of colonies of *P. aeruginosa* mainly goes back to two metabolites - pyocyanin (blue) and pyoverdin (green). Apart from these, some strains furthermore excrete pigments such as pyorubin and pyomelanin [130], changing the coloration of colonies of the respective strains towards a more reddish or yellowish spectrum.

Presumably, Pyocyanin (PCN) is a metabolite produced exclusively by the species *Pseudomonas aeruginosa* [131]. The chemical structure of this pigment is presented in figure 26. As a zwitterion, the molecule may assume one of three states - oxidized, monovalently reduced, and divalently reduced [131]. Therefore, at blood pH, PCN molecules can easily cross

the membranes of host cells (buffered at ~7.4). Furthermore, due to these characteristics, PCN is also a redox-active compound [132], oxidizing and reducing other molecules in its path [133]. Presumably, the resulting generating of reactive oxygen species is used by *P. aeruginosa* as a biocidal weapon against other bacteria [133, 134]. Furthermore, PCN enhances the oxidative metabolism of neutrophils in the human body [135], thereby increasing oxidative stress for other cells.

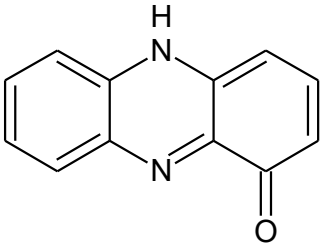

Fig. 26: Chemical structure of the pigment Pyocyanin (ACD/ChemSketch 2017.2.1).

Together, the traits mentioned make pyocyanin an effective tool to kill other cells and, hence, a significant virulence factor (for instance being associated with CF lung infections) [131]. Although the actual impact on clinical infection is still the subject of debate, recent studies on *in vivo* virulence of PCN suggest a considerable importance [131, 136, 137]. This hypothesis is further supported by the finding that PCN is present in the sputum of colonized patients at significant concentrations [138].

Pyoverdines (PVDs) are a group of diffusible fluorescent siderophores secreted by diverse species of *Pseudomonas* [139, 140]. Since their discovery in 1982, these compounds have been handled under several alternative names such as fluorescein and pseudobactin [139]. PVDs provide the primary iron uptake system of fluorescent pseudomonads [141, 142]. However, some species also excrete additional siderophores like pyochelin, or even recruit exogenous compounds such as heterologous siderophores [143]. Next to iron acquisition, PVDs presumably are also involved in the ecology of *Pseudomonas* species [139] in other ways, including *quorum* sensing [144] and the setup of virulence [145]. Today, the structures of over 50 Pyoverdines are known [139, 146]. Generally, the structure is composed of three main units: a conserved fluorescent chromophore (dihydroxyquinoline), an acyl side chain (either dicarboxylic acid or an amide), and a variable peptide chain bound to the C1 (or in some rare cases C3) carboxyl group of the chromophore [139]. Individual pyoverdines, however, differ in their structure not only between species, but also between strains of one species [139]. For *Pseudomonas aeruginosa*, for instance, three groups of PVDs can be distinguished. Their chemical structures are presented elsewhere [139, 147, 148].

As described above, the switch of some colonies to a SCV morphology could be an expression of an adaption to the oxidative stress deriving from chlorination. The same applies for the lack of pigmentation. Regarding Pyocyanin, a throw-off and subsequent downregulation can be explained as the removal of a source potentially generating additional free radicals [133, 134].

Following this line of thought, Pyoverdines would have been removed because of their function to bind iron. Based on a chemical principle called the Fenton reaction [149], the presence of labile iron in a cell may strongly contribute to damage deriving from oxidative stress [150-154]. The Fenton reaction describes the oxidation of organic substrates catalyzed by iron salts at

acidic pH values. As presented in figure 27, it proceeds in two steps. Both these steps are still subject to ongoing discussion. Nonetheless, the given summary by the author is considered adequate for the purposes of this study.

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+}HO \cdot + OH^-$$
$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + HOO \cdot + H^+$$

Fig. 27: First and second step of the Fenton Reaction

In a first step, iron(II) is oxidized by hydrogen peroxide to iron(III). Consequently, a hydroxyl radical and a hydroxide ion are generated [155]. Iron (III) is then reduced back to iron (II) by a second molecule of hydrogen peroxide. This time, a hydroperoxyl radical and a proton are released. In total, the disproportionation of hydrogen peroxide creates two oxygen-radical species and one molecule of water (H^+ and OH^-). The generated free radicals may subsequently engage diverse secondary reactions. For instance, hydroxyl radicals react with all types of biomolecules [153]. By removing the source of these radicals, a cell may reduce the level of detrimental effects induced by exposure to oxidative conditions to an extent allowing the respective cell to survive. Yet, these interpretations are only hypotheses taken from what was observed and would need further examination in order to confirm or abandon the theory.

4.4 Conclusions

Most lifeforms on earth are highly dependent on controlling oxidative stress. Diverse ways have been developed to overcome the permanent threat posed by radical oxygen species. These include specific defenses as well as global responses [155-158]. For that matter, defense mechanisms either keep concentrations of radicals at acceptable levels or are involved in repairing the damage introduced by radicals [159].

Generally, oxidative stress will arise, when concentrations of reactive oxygen species reach levels exceeding a given cell's defense capacity [159]. Both switching to a mode of reduced colony growth and the throw-off and downregulation of pyocyanin and pyoverdines respectively could be mechanisms used by *Pseudomonas aeruginosa* to reduce the damage induced by chlorine. In literature on this topic, evidence can be found supporting this hypothesis.

For instance, one study highlighted the relationship between the administering of divalent metals other than iron and the observation of a depigmentation of colonies of *Pseudomonas aeruginosa* [160]. Based on the Fenton reaction (compare previous section), divalent metal ions (e.g. Cu(II), Cr(II), and Co(II)) may induce oxidative stress. Apart from other effects, a depigmentation of colonies of *P. aeruginosa* was observed. When exposed to cadmium, pyocyanin, but not pyoverdine was excreted by the exposed bacteria. Copper and chromium, on the other hand, had effects on pyoverdin, but not on pyocyanin excretion. Finally, mercury and cobalt blocked both the secretion of pyocyanin and pyoverdin.

The findings on the occurrence of potential persisting states of *P. aeruginosa* could be relevant with respect to the contracting of diseases associated with exposure of bathers in swimming pools to contaminations with *P. aeruginosa*. As explained above, small colony variants are believed to play a role in the development of infections by *P. aeruginosa* in immunocompromised people. Apart from the explained role in persistence, the occurrence of SCVs also influences the virulence of *P. aeruginosa*. For instance, SCVs have been reported to have impact on the development of infections in patients suffering from cystic fibrosis [122, 161, 162]. Fittingly, SCVs of *P. aeruginosa* were isolated in 38% of the patients taking part in a study [161]. Also, the lack of pigmentation has been linked to SCVs of *P. aeruginosa* [121, 122, 125]. In the clinical context, SCVs are characterized by an increased tendency to form biofilms, an improved antibiotic resistance and fitness under stationary growth conditions, as well as a greater adhesion to host cells [122, 161-163]. The presence of persisters presumably influences recalcitrance and relapse of bacterial infections, as well as the risk of antibiotic resistance [129].

According to a review [163], although several phenotypic variants of *P. aeruginosa* may be isolated from CF patients [164], commonly only a few genotypes can be distinguished [165]. As explained in the review, for a long time, research interest was limited to “the most common mucoid […] phenotype”. However, several other phenotypes, including SCV, presumably are particularly adapted to biofilm formation e.g. in the human lung [166, 167].

Yet, the findings of the present study are relativized by their context. Normally, exposure to *Pseudomonas aeruginosa* in a swimming pool will occur at low to moderate levels. The likelihood of ingesting bacteria in infective doses is considerably low. Accordingly, diseases associated with the use of swimming pools commonly proceed having only comparably mild consequences for the people concerned, while severe infections (e.g. of the lung tract) have, to date, not been linked to swimming activities. Therefore, the author considers the observed phenomenon an interesting side aspect of the overall results of the present study, which highlights the complexity of the topic of swimming pool disinfection rather than providing information from a medical perspective.

Chapter 5

Chlorine Consumption during the Inactivation of Bacteria

Chapter Abstract

In addition to the inactivation experiments described in chapter 3, chlorine consumption during disinfection of the selected bacterial strains was examined. Chlorine decay was measured by means of an instrument used in routine control in public swimming pools. The experimental setup described in chapter 3 was adapted accordant to the new issue addressed. While the used bacterial strains were identical to those used in the experiments on inactivation kinetics, higher concentrations were used in the experiments on chlorine consumption. Subsequently to determination of chlorine decay, the data obtained was analyzed for the underlying kinetics. It was examined which concentrations of free chlorine were consumed under the given conditions. The development of chlorine concentrations over time was interpreted in comparison with literature on this topic. Apparently, chlorine consumption during inactivation followed a trend composed of two main phases: an initial short phase of fast decay followed by comparably slower decay. Both these phases followed approximately linear courses in semi-logarithmic representation. Presumably, the first phase indicated the inactivation of the exposed bacteria, while second phase decay went back to non-specific reactions with cellular residuals and other material. These results indicate that although a large ratio of inoculum volume to test volume was selected, chlorine decay may still have influenced the outcome of the inactivation experiments performed. Additionally, the achieved results provide information on the kinetics of chlorine consumption during disinfection.

5.1 Introduction

Being strongly oxidative compounds, chlorine disinfectants are consumed both during inactivation of microorganisms and during reactions with other inorganic and organic materials (e.g. nitrogen and ammonia in the water, biofilms, corrosion processes) [11, 168-172]. As the consumption of chlorine has an impact on disinfection and maintenance of hygiene in swimming pools, this aspect was included in the considerations made in the present thesis.

To date, research on chlorine consumption has mainly focused on the chlorine demand in drinking water [168]. To a lesser extent, grey water has also been examined [173]. This research has implications for chlorine disinfection in swimming pools and other recreational areas. It was determined that the amount of chlorine consumption is highly dependent on the microbial species exposed [113, 174]. Furthermore, several studies found out that chlorine demand was dependent on initial chlorine concentrations [113].

In differentiation from chlorine consumption during disinfection, the term chlorine demand describes the difference between the concentration originally used in a disinfection approach and the concentration still available after a given time. For chlorine, the remaining quantity is named chlorine residual. If the ratio of Cl_2 to given reaction partners is below a certain breakpoint, chlorine residuals will be present mostly as combined chlorine (i.e. chloramines). If, on the other hand, the ratio is beyond this breakpoint, free chlorine species predominate (i.e. Cl_2, HOCl and OCl^-) [11].

In public swimming pools, the chlorine demand may be enhanced by the presence of organic matter in the water (e.g. bather skin particles, lotion residuals, fecal residuals). One group of chemicals of interest in chlorine decay are organic-nitrogen compounds [175], as they consume free chlorine [176, 177] and act as precursors for volatile substances transferred to the gas phase [178]. Such organic-nitrogen compounds may derive from diverse human sources, such as urine, saliva, hair and skin, the respective precursors for volatile products including urea, creatinine, and amino acids [175, 179, 180].

The question remains, whether chlorine consumption during microbial inactivation is dependent on cell survival. Of particular interest in this context is, if chlorine disinfectants are consumed only during the process of microbial inactivation [113, 174].

5.1.1 Modelling Chlorine Consumption

Chlorine readily reacts with all kinds of organic and inorganic compounds (e.g. ammonia, sulfides, ferrous iron, or humic acids). Therefore, chlorine disinfectants will always be subjected to gradual dissipation [168]. Referring to older publications [181-184], a review states that the consumption of chlorine by reaction with organic or inorganic chemicals is “reasonably well defined” in the bulk aqueous phase of drinking water [168]. The review describes that reaction rates can be characterized by either first-order decay or second-order decay processes.

Regarding chlorine consumption during inactivation of microbes, biphasic kinetics have been proposed by diverse studies [113, 174, 185, 186]. Chlorine demand, for instance, was modeled with the following biphasic exponential decay model [113]:

$$C = (1 - Q)e^{-k_1 t} + Qe^{-k_2 t} \quad (16)$$

where C is the free chlorine fraction at a point in time t and defined as C_t/C_0, where C_t is the free chlorine concentration at point in time t and C_0 is the initial chlorine concentration. 1-Q is the free chlorine fraction demanded in the first phase; k_1 is the chlorine decay kinetic coefficient for this initial phase; k_2 is the chlorine decay kinetic coefficient for the second phase.

According to the study proposing this model [113], parameters Q, k_1, and k_2 are dependent on the initial free chlorine concentration (C_0) applied, as well as on initial cell concentration (X_0) and the microbial species used.

5.2 Materials and Methods

5.2.1 Preparation of Test Bacteria

The selected test strains and the respective growth conditions were like those explained in chapter 3. However, unlike in the approach on inactivation kinetics, inoculi with volumes of 10 ml (instead of 3 ml) were used in the experiments on chlorine consumption. By increasing the inoculum volume, more distinct results were achieved, thus allowing for a more precise and valid analysis.

5.2.2 Experimental Conditions

The experimental setup for determination of chlorine consumption was developed based on the concept for determination of inactivation kinetics (compare chapter 3). An on-line measurement instrument (AMI Trides, SWAN analytical instruments, Swiss) was connected to the water body in the aquarium by silicone tubing plunged into the water in the test vessel. Through an external aquarium pump, water was transferred into the inlet of the measurement device at a constant rate. Backflow was provided by connecting the outlet with the aquarium. The setup is presented in figure 28.

Like in the experiments on inactivation kinetics, an aquarium having a volume of 50 liter was used as a test vessel. The water body was stirred constantly by a pitch blade turbine impeller (Carl Roth GmbH + Co. KG, Germany) connected to an agitator (RW 20, IKA®-Werke GmbH & Co. KG, Germany). The test medium was composed and prepared exactly as described in chapter 3. Furthermore, parameters such as temperature and pH were adjusted in the same way as described in the chapter.

Fig. 28: Setup of the performed experiments on chlorine consumption during inactivation of bacteria inoculi. A measurement instrument (AMI Trides; top left) was connected to the test vessel via silicone tubing. The setup was adapted from the setup used for determination of inactivation kinetics.

5.2.3 Measurement of Free Chlorine and the Hypochlorous Acid Fraction

An AMI Trides (SWAN analytical instruments, Switzerland) was used in this study for the continuous measurement of free chlorine and hypochlorous acid respectively. According to the manual on the device, the AMI Trides is “used to measure and control” several oxidative disinfectants (e.g. hypochlorous acid, ozone, and bromine) in swimming pool water [187]. The manual describes the control of a set-point as one main application. The instrument is used to maintain a defined disinfectant value. The disinfecting agent is added with a linked dosing unit.

In the respective manual [187], the measuring principle is described as 3-electrode amperometry.

In general, the method of amperometry is based on the measurement of an electric current on a work electrode, while having applied a constant electrochemical potential. The potential is applied between two electrodes positioned in the effluent. The measured current changes if an electroactive analyte is oxidized at the anode or reduced at the cathode. Conveniently, when exposing a sensor to a soluble compound, the measured current is directly proportional to the concentration of the used compound. This allows determination of unknown concentrations of this compound with the help of a calibration curve [188].

The sensor of the AMI Trides is composed of two platinum electrodes and a reference electrode [187]. An electric potential is established between measuring electrode (platinum) and counter electrode (platinum) of the sensor. Proportional to the agent's concentration, the disinfectant generates a current between the electrodes. Meanwhile, the reference electrode controls this potential, thereby guaranteeing optimal measuring conditions. As the signal of amperometric systems depends on flow, to guarantee optimal sensitivity, a rotor provides a continuous flow. A connected sensor measures these rotations to guarantee flow. Furthermore, temperature composition is carried out automatically by the device.

5.2.4 Data Analysis

Data was fitted using this biphasic exponential decay model by nonlinear regression (OriginPro 2018b, OriginLab). Data was, furthermore, interpreted as two linear phases.

5.3 Results

Total chlorine and the fraction of hypochlorous acid [mg/L] were measured every 5 seconds. Accordingly, over a time of 60 minutes (or 3,600 seconds respectively), 721 data points were generated. The results achieved with *E. coli*, *P. aeruginosa* and *S. aureus* are presented in figures 29 and 30. Mean values of three independent measurements are indicated as solid lines in blue (for total chlorine) and red (for hypochlorous acid). The respective standard deviations are indicated in light blue and light red. Black lines indicate curves generated with fitting models. Since the test system needed an initial phase for evening out, the first 5 reading points were excluded from analysis.

Over the course of one hour, chlorine consumption followed a trend which can be characterized as exponential decay (Fig. 29). This exponential decay may be subdivided into two linear phases with distinct decline rates, connected by a transitional phase of concave shape (Fig. 30). The initial phase of fast proceeding linear decline was calculated including data points between reading points 5 and 20. The second linear phase of comparably slower proceeding decline included reading points 240 to 721. Accordingly, the transitional phases between first and second phase comprised reading points 21 to 239.

Rice et al. (2005) [189] calculated CT values based on a "first-order exponential relationship for chlorine decay". They calculated chlorine decay from the slope of a curve plotting chlorine concentrations against exposure time. The same experiment was performed with the results from the experiments of this study. The two formulae used are presented in table 8.

In a first step, exponential decay was assumed. The respective results are presented in figure 29. In a second step, the assumption that two linear phases could be distinguished was tested. The results on this are presented in figure 30.

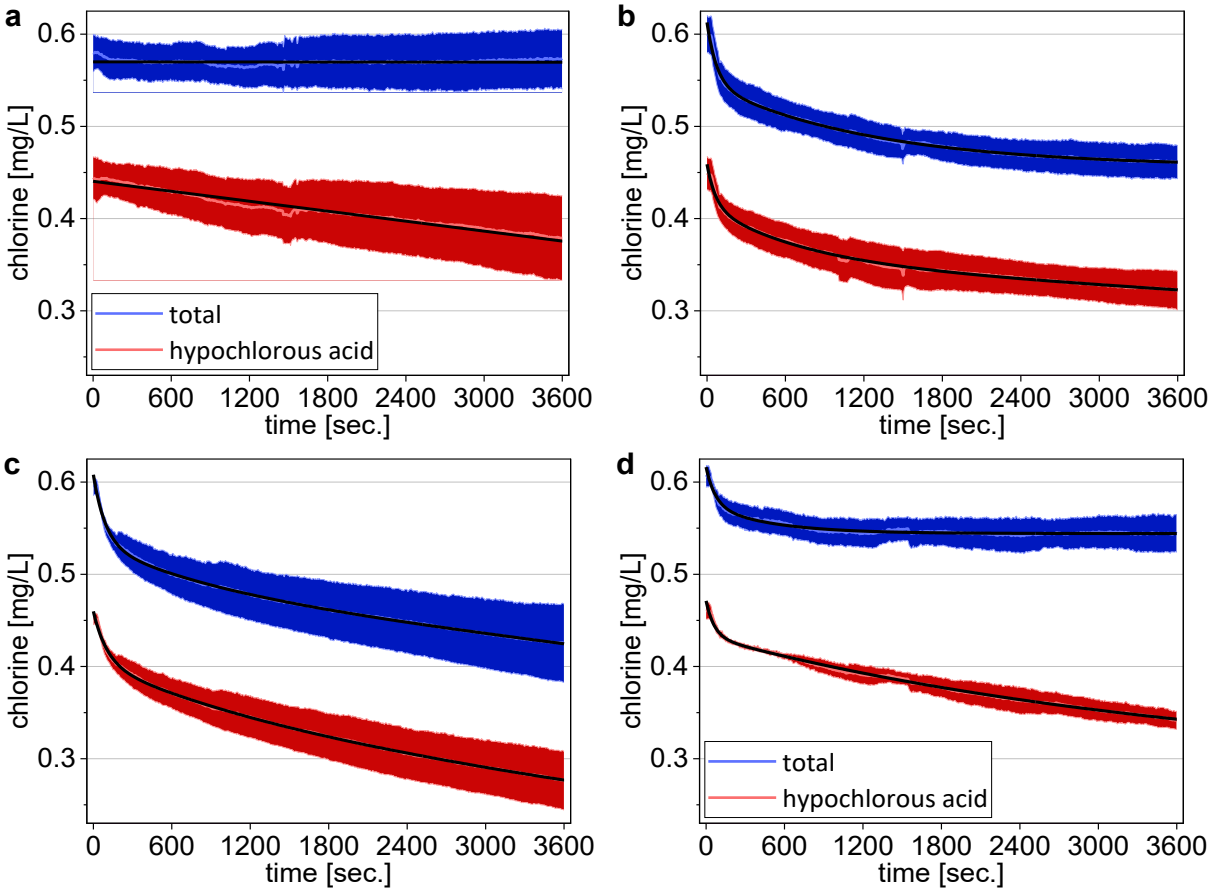

Fig. 29: Development of chlorine concentrations during a period of 60 minutes after addition of an inoculum of bacteria. Addition of bacteria inoculi resulted in a changing shape of the decline curve from linear to concave upwards. The graphs express mean values and standard deviations from 3 independent measurements per approach. Development of chlorine concentrations using **(a)** no bacteria inoculum (blank run), **(b)** an inoculum of *E. coli*, **(c)** an inoculum of *P. aeruginosa, and* **(d)** an inoculum of *S. aureus*. The black lines indicate curves as calculated using **(a)** the linear fitting model, and **(b - d)** the exponential fitting model.

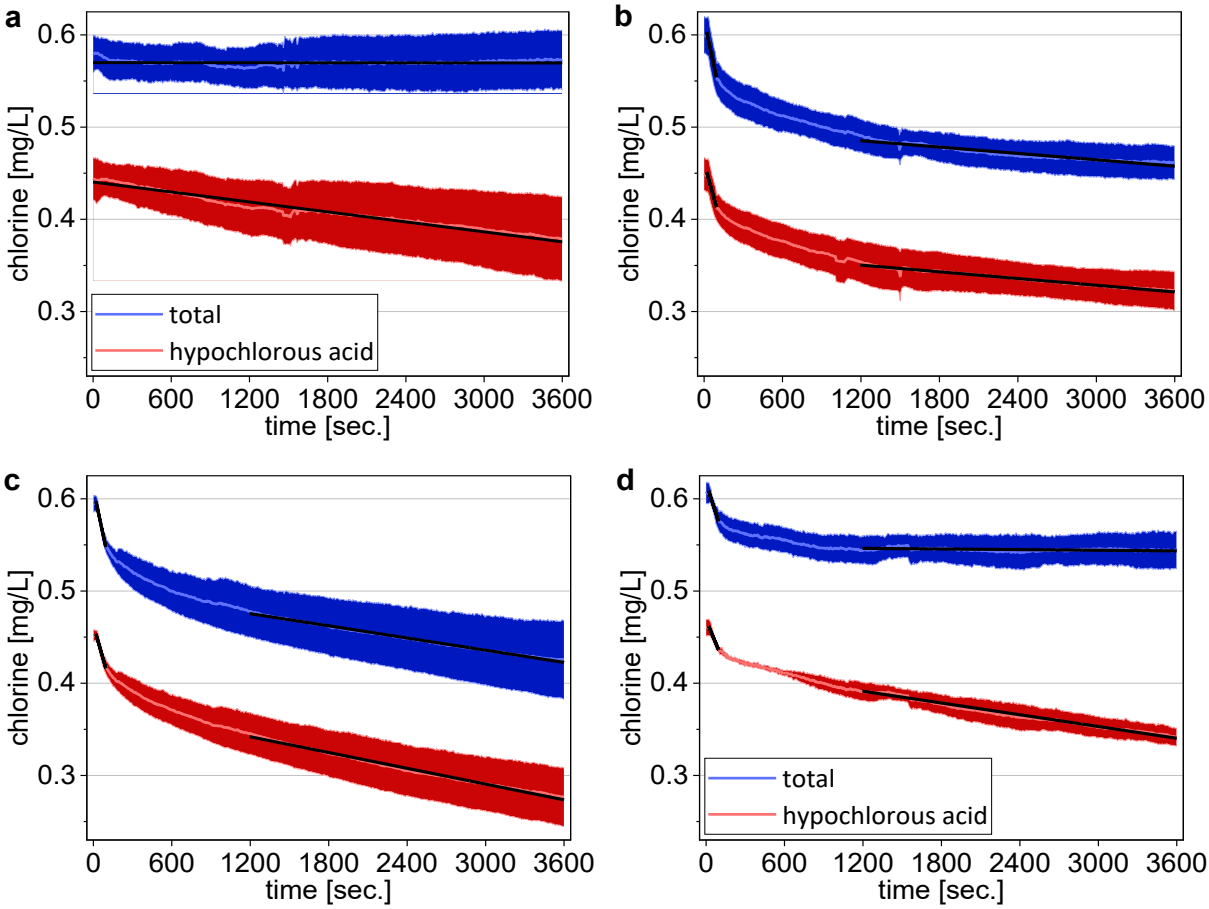

Fig. 30: Development of chlorine concentrations during a period of 60 minutes after addition of an inoculum of bacteria. The black lines indicate the position of two assumed phases of individual linear decay. The graphs express mean values and standard deviations from 3 independent measurements per approach. Development of chlorine concentrations using **(a)** no bacteria inoculum (blank run), **(b)** an inoculum of *E. coli*, **(c)** an inoculum of *P. aeruginosa, and* **(d)** an inoculum of *S. aureus*.

Table 8: Models used for fitting data on chlorine consumption during inactivation of bacteria.

Model/Approach	Formula
Exponential Decay	$y = A1*\exp(-x/t_1) + A2*\exp(-x/t_2) + A3*\exp(-x/t_3) + y0$
Two Phases of Linear Decay	$y = a + b * x$

5.4 Discussion

5.4.1 Evaluation of the Selected Methods

Chlorine disinfectants have strong oxidizing features. Accordingly, they will react with a broad variety of potential partners. For the experiments performed, this could mean, that chlorine consumption did not exclusively go back to the inactivation of bacteria, but in part also to reactions with components of the experimental setup such as hoses and measurement instrument. Yet, the author considers the effect to have had minimal impact. All components used were made of inert materials (e.g. glass, silicone). Hence, presumably, reactions did not occur at high frequencies. Correspondingly, the blank run indicates, that reactions of the disinfecting agent with components of the setup did not interfere with the production of consistent results. By subtracting the consumption during the blank runs from the respective rates in experimental runs, both consumption due to setup components and due to transition of chlorine from water to gas phase should be excluded from the results.

Interpreting the plots of chlorine consumption as curves composed of distinct phases becomes complicated as the issue arises whether these phases are comparable to each other in terms of the coefficient of determination. Since the respective fittings rely on different counts of reading points, also the coefficients of determination are based on varying numbers of cases. While the first, linear phase included only approximately 2% of all reading points, the transitional phase comprised approx. 30% and the second linear phase even about 67% of the reading points. Therefore, the coefficient of determination of the second linear phase is, comparably, more reliable than the coefficient of determination for the respective first linear phase of a given data plot.

5.4.2 Recapitulation and Interpretation of the Results

Like the data plots on inactivation kinetics (compare chapter 3), the data plots on chlorine consumption indicate that disinfection was completed within the first 75 seconds of an experiment (15 reading points of the AMI Trides instrument). Over this short period, concentrations of hypochlorous acid dropped significantly and in a linear fashion. Thereafter followed a phase of modulation and then a second, less steep phase of approximately linear decline.

The results were interpreted in a way described in the following:

Chlorine consumption was determined as milligrams per liter per second after addition of the bacteria inoculum. In a first step, the background decline, as determined in the blank run, was compared to second phase decline in the approaches with inoculi of one test species each. Consumption per minute was calculated from these data. The respective results are presented in table 9.

As can be seen, in all three examined cases, decline during the second linear phase had slopes comparable to the blank run. Therefore, the second phase decline in the approaches using bacteria inoculi was interpreted as not dependent on the addition and inactivation of bacteria cells. Presumably, this background decline went back to other mechanisms such as chlorine consumption by reactions with the components of the setup and volatilization. At any rate, this decline was not to be considered in determining chlorine consumption during inactivation of bacteria.

Table 9: Decline of hypochlorous acid [mg/L] per time unit (per second, per minute) - Slope of second phase linear decline.

Test Bacterium	Consumption [mg/L * sec.]	Difference to Blank Run	Consumption [mg/L * min]	Difference to Blank Run
Blank Run	-1.8×10^{-5}	---	-1.1×10^{-3}	---
E. coli	-1.2×10^{-5}	0.6×10^{-5}	-7.2×10^{-4}	3.8×10^{-4}
P. aeruginosa	-2.9×10^{-5}	-1.1×10^{-5}	-1.7×10^{-3}	-6.0×10^{-4}
S. aureus	-2.1×10^{-5}	-0.3×10^{-5}	$-1{,}3 \times 10^{-3}$	-2.0×10^{-4}

'---' not applicable

In a second step, the slope of the decline in the first linear phase was calculated. The decline not related to the inactivation of bacteria was excluded from the result by subtracting the slope of the blank run curve from the results achieved in the individual approaches. Again, chlorine consumption was both calculated per second and per minute. The results are presented in table 10.

Table 10: Decline of hypochlorous acid [mg/L] per time unit (per second, per minute) - Slope of first phase linear decline.

Test Bacterium	Consumption [mg/L * sec.]	Difference to Blank Run	Consumption [mg/L * min]	Difference to Blank Run
Blank Run	-1.8×10^{-5}	---	-1.1×10^{-3}	---
E. coli	-5.0×10^{-4}	-4.8×10^{-4}	-3.0×10^{-2}	-2.9×10^{-2}
P. aeruginosa	-4.9×10^{-4}	-4.7×10^{-4}	-2.9×10^{-2}	-2.8×10^{-2}
S. aureus	-3.4×10^{-4}	-3.2×10^{-4}	-2.0×10^{-2}	-1.9×10^{-2}

'---' not applicable

As shown in the table, the slopes of the linear decline during the first phase did not vary that much between the experiments using different test species. In all three experiments, approximately 3-5 $\times 10^{-4}$ mg/L per second were consumed during the process.

The concentration of bacteria in the used inoculi was approximately 1×10^{9} cfu/ml. The volume of the inoculum used in one experiment was 10 ml. This means that in every run, approximately 1×10^{10} bacteria cells were exposed to the disinfecting agent. Since the test volume was ca 50 L, in theory, approx. 2×10^{5} bacteria were present per milliliter.

Based on this hypothetical count of bacteria, the concentrations of hypochlorous acid consumed per bacteria cell were calculated. First, chlorine consumption was calculated by subtracting the decline in the blank run from the first phase decline determined for a respective bacteria strain (compare table 10). The resulting value was then multiplied with 75 seconds (the assumed duration of first phase linear decline in the graphs). Finally, this value was divided by 1×10^{5} bacteria per ml. The results are presented in table 11.

Table 11: Consumption of hypochlorous acid [mg/L] per bacterium cell.

Test Bacterium	Consumption per Bacterium [mg/L*Bacteria Cell]
E. coli	1.8×10^{-7}
P. aeruginosa	1.8×10^{-7}
S. aureus	1.1×10^{-7}

According to a review [190], typical cell volumes of cells of *Bacillus subtilis*, *Staphylococcus aureus* and *Escherichia coli* range between ~0.4 to 3 μm^3 (0.4 to 3 femtoliters or ~10^{-15} liters). In general, free-living cells tend to be small, having a large surface area in relation to their volume. For *E. coli*, the review mentions a surface- to volume ratio of ~3.7 μm^2 to 1 μm^3 [190].

In order to simplify calculations, the mentioned volumes were assumed to be correct for all three test species. Assuming a volume 1 μm^3 for cells of any bacteria strain used in the experiments, also a surface area of approximately 3.7 μm^2 was assumed for cells of all three strains. Really, cells of *S. aureus* presumably had a lower surface area compared to the rod-shaped Gram-negative species used. However, this was not considered for the calculations.

For all three species, approximately 10^{-7} mg/L of hypochlorous acid were consumed during the inactivation of one bacteria cell. This equals 100 pg/L (picogram per liter) per bacteria cell.

Using the equation n [mol] = m [g] / M [g/mol], we can furthermore calculate the number of molecules of hypochlorous acid coming on 1 μm^2 of bacteria surface:

10^{-7} mg/L = 10^{-10} g/L

The test vessel had a volume of 50 L

50 x 10^{-10} g = 5 x 10^{-9} g

The molar mass of hypochlorous acid is 52,46 g/mol

5 x 10^{-9} g / 52.46 g/mol = 9.531 x 10^{-11} mol

95 pikomol hypochlorous acid were used up per bacterium

~6 x 10^{23} molecules x 9.5 x 10^{-11} = ~57,000,000,000,000

~5.7 x 10^{13} molecules hypochlorous acid were used up per bacterium

~5.7 x 10^{13} molecules / 3.7 μm^2 = ~1.5 x 10^{13} molecules per μm^2

~1.5 x 10^{13} molecules hypochlorous acid were used up per μm^2 bacterium surface

So, theoretically, about 5.7 x 10^{13} molecules of hypochlorous acid were consumed during inactivation of one bacteria cell, with 1.5 x 10^{13} molecules taking one μm^2 of the surface of a given bacteria cell. According to Neidhardt et al. (1990), an average *E. coli* cell contains around 2.2 x 10^7 lipid molecules and around 1.2 x 10^6 lipopolysaccharide molecules [50, 191]. Even if more than one hypochlorous acid molecules reacted with any given lipid or polysaccharide molecule, hypochlorous acid molecules would still be in large excess. This means, that after reaction with every molecule of the cell's membrane, most of the molecules of the disinfecting agent remained available for further reactions. Presumably, these molecules reacted with other molecules of bacteria cells such as intracellular components.

5.5 Conclusions

As may have been assumed, the consumption of chlorine during disinfection of bacteria was, at least to some extent, dependent on the respective bacterial test species. This result is well in line with the findings of other studies on this topic [113, 174]. In general, chlorine consumption apparently was reciprocal to chlorine resistance of the respective test species. Accordingly, inactivation of the species most resistant to chlorine treatment, *Staphylococcus aureus*, was accompanied by the lowest observed chlorine usage. On the other hand, the inactivation of the most sensitive species, *Escherichia coli*, was accompanied by the highest chlorine consumption.

In their study, Shang and Blatchley indicated a role of cell wall structures [174]. In line with this, in the present study, a slightly higher consumption of chlorine was determined for experiments using Gram-negative species compared to the Gram-positive species *Staphylococcus aureus*.

Furthermore, second phase decline was considerably steeper in experiments using *Pseudomonas aeruginosa* than in experiments using the other two test species. Also, standard deviations of the kinetics examined were considerably larger in experiments using *Pseudomonas aeruginosa* in comparison to the other species. This indicates that, apart from differences in cell wall structure, secreted material could have had an influence on the chlorine consumption during inactivation of bacteria. The test strain of *P. aeruginosa* used was a mucoid wildtype. Hence, it appears realistic, that secretion of a protective slime could have increased chlorine consumption. As has been demonstrated by several studies [192-194], reaction rates of chlorine with natural matter may vary strongly, depending on which organic species are present. It can be assumed, that, in the determination of the kinetics of chlorine kinetics, the overall error was comparably larger in the experiments using *P. aeruginosa*, as this species secreted extracellular material influencing chlorine consumption. However, with respect to the results in inactivation kinetics, the secretion of molecules did not protect cells of *Pseudomonas aeruginosa* effectively against the oxidative capacities of hypochlorous acid.

Independent of the test species, chlorine consumption followed a bi-linear trend. The mentioned study by Shang and Blatchley achieved comparable results. Interpreting their results, they hypothesized that there were “two general types of chlorine demand expression by bacterial cells” [174]. Following this line of thought, the first, steep phase of chlorine decline would be linked to the inactivation of living bacteria. The second, less steep phase would then be defined by `non-targeted´ reactions with the remaining debris.

Several studies dealing with chlorine demand during the inactivation of bacteria [113, 174] came to the conclusion that higher initial chlorine concentrations would lead to higher chlorine demands compared to lower initial chlorine concentrations. Helbling and VanBriesen (2007) [113] interpreted this observation as the result of “solutions with higher oxidation potential (higher free chlorine concentration) either reacting longer with the microbial material or continuing to react with intermediates formed by the initial oxidation reaction”. This would mean that increasing chlorine concentrations in the water would always lead to increased consumption of chlorine, but not necessarily also to increased levels of inactivation of microbial cells.

Apart from this, Helbling and VanBriesen compared the chlorine demand using varying bacterial test species [113]. They found out that *Staphylococcus epidermidis*, a comparably close relative of *Staphylococcus aureus*, consumed more chlorine during inactivation that *Escherichia coli*. The results of the present study point in another direction, as they indicate a

greater decline in free chlorine concentrations using *Escherichia coli*, than using *Staphylococcus aureus*. This difference in the results achieved by the two compared studies could derive from the use of different strains of the used bacteria or factors given by the selected experimental setups. However, with respect to the similarity of the results achieved in the present study using any of the test bacteria, the differences may also have occurred by chance.

In a nutshell, the results provide information on the general measure of chlorine consumption during the inactivation of bacteria under conditions like those given in the basins of swimming pools. The results achieved are comparable to those described by studies from other fields of research. However, the results need critical interpretation, as, based on the experimental setup, they may be accompanied by an error. Nonetheless, they provide an insight into the usage of chlorine during disinfection, which, when related to the results of the other chapters of this study, may help to improve swimming pool maintenance procedures.

Chapter 6

Bacteria Recovered from Public Swimming Pools [4)]

Chapter Abstract

Complementing chapter 2, the present chapter provides additional information on which bacteria can be recovered from public swimming pools in Germany. However, where chapter 2 deals with indicator bacteria, the present chapter is concerned with cultivable bacteria in general. Next to indicator bacteria, a broad range of other bacteria species contribute to the overall contamination in swimming pool water. Culture-dependent methods were used to recover some of these bacteria. The intention behind using these methods was to recover the fraction of bacterial communities also detectable by methods used for routine monitoring. In doing so, results were achieved which can be related to data provided by standard control protocols. By selecting appropriate media, the collected data also indicates the pathogenicity of the detected species. Two categories of samples were examined for bacteria - swimming pool water and sand filter material samples. Swimming pool water samples were taken at several German public swimming pools. Filter material samples were taken at one of these venues when the relevant filter was restored. Bacteria were recovered by cultivating them on growth media. Subsequently, colonies were identified using the MALDI-TOF technique. The respective results indicate a strong presence in swimming pool water of bacteria associated with human skin microflora. Furthermore, the community detected in sand filter material apparently was composed of species adapted to the conditions given in the filter. In both cases, primary pathogens were detected only at comparably low frequencies. However, many of the bacteria detected are considered opportunistic pathogens. Indicator bacteria did not contribute to the detected communities at considerable rates.

6.1 Introduction

As has been described, routine evaluation of microbial water quality is based on the detection of indicator bacteria rather than on assessment of the presence of specific pathogens. The indicators used have in common that they are relatively susceptible to disinfection, while many pathogenic species are significantly more resistant. On the one hand, the presence of indicator bacteria will hence indicate conditions which presumably also allow survival or even multiplication of more resistant species. Yet, on the other hand, the goal behind using indicators is to provide safety of human health. The question is how useful extrapolation of information achieved by the assessment of indicator bacteria is.

This chapter summarizes which steps were taken to produce information on which (cultivable) bacteria could be isolated from swimming pool water. Collected data was related to the incidence of several common indicators in order to assess if occurrence of the latter allowed

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[4)] Parts of this chapter were presented in talks and posters on several occasions:

Kreuter, L., Schlosser T. and L. Erdinger, *Disinfection kinetics in swimming pool water*, Symposium on Improving Pool Water Quality, 30.05.2016, Zell am See (Austria).

Kreuter, L., *Determination of minimal necessary chlorine concentration for disinfection*, IntelliPool Project Meetings, 17.09.2015 Barcelona (Spain)

correlation to the first. To this end, fractions of water samples taken at public swimming pools were transferred to growth media. Subsequently, occurring microbial colonies were counted and identified using MALDI-TOF (matrix-assisted laser desorption/ionization - time-of-flight).

6.2 Materials and Methods

6.2.1 Measurement Procedure

MALDI-TOF was used for the identification of microbes from water and sand samples.

As the name implies, the ionization technique termed as “matrix-assisted laser desorption/ionization” (MALDI) [195], uses a matrix to absorb the energy of a laser. This energy is then used to create ions from large molecules [196]. Commonly, one run is separated into three steps. Firstly, a sample is mixed with the matrix material and put on a metal plate (the target). Secondly, a laser is used to irradiate the sample, thus causing ablation and desorption of sample and matrix. Thirdly, in the ablated gases, analytes are ionized and may be accelerated into a mass spectrometer [197], as, in case of this study, a time-of-flight spectrometer (TOF).

Time-of-flight mass spectrometry relies on the determination of an ion’s mass-to-charge ratio by measuring the time of flight of a given ion over a defined range. To this end, ions are accelerated by an electric field [198]. Any ion of a given charge will have the same kinetic energy as any other ion of the same charge. Consequently, the velocity of an ion will be dependent on the mass-to-charge ratio. While heavier ions with a given charge will reach lower speeds, lighter ions of the same charge will reach higher speeds. Utilizing this, the time an ion needs to reach a detector at a known distance is measured. From the measured time and other factors, the ion can be identified.

MALDI/TOF spectra are a common tool for the identification of microorganisms. A portion of a colony of an unknown microbe is placed onto a metal target and mass spectra are generated. The achieved mass spectra are then analyzed, using dedicated software and stored comparative profiles. Since species diagnosis by MALDI/TOF is much faster, more accurate, and cheaper than immunological and biochemical test methods, this technique has become a standard for the identification of microbes in medical research [199, 200]. According to one study on this topic [199], MALDI-TOF did not only improve and accelerate routine identification of bacteria, but also proofed very useful for the identification of such species only rarely described as pathogenic agents. This feature makes MALDI-TOF a very promising tool for the identification of bacteria from environmental samples (as conducted in the present study), since it can be assumed, that at least some germs isolated from such sources belong to genera and species which are uncommon agents of disease in humans.

For the measurements performed, a Microflex MALDI-TOF mass spectrometer was used (Bruker Daltonik GmbH, Germany). The respective device produces fragments of a size of 2,000 to 20,000 Dalton/charge. The device was equipped with software for the identification of microbes (flexControl, BioTyper, database). Before placing the respective target metal plates into the device, the samples were treated with formic acid (Merck KGaA, Germany) and covered with a matrix (α-cyano-4-hydroxy cinnamic acid, Merck KGaA, Germany).

6.2.2 Sample Matrices

Two different matrices associated with swimming pool hygiene were assessed for the cultivable bacteria contained. Water samples were taken at several public swimming pools (table 12). Additionally, samples of sand filter material were isolated from one public swimming pool. Bathing water samples were plated directly; sand samples were rinsed with sterile water of standardized hardness [97].

Swimming Pool Water

Table 12 lists the sites at which samples of bathing water have been taken. Samples were taken in accordance with the specifications given in DIN 19643 [19]. Sterile sampling vessels equipped with 2,5 mg thiosulfate were used (aqua Laborservice e. K., Wertheim, Germany).

Table 12: Sampling venues included in the examinations described in this chapter.

Site Number	Swimming Pool Type	Number of Samplings
2	Public Indoor	12
3	Public Outdoor	1
5	Public Indoor	1
15	Public Indoor	1
19	Sports Club	2
24	Public Outdoor	1
26	Public Indoor	4
33	Public Outdoor	1
50	Public Outdoor	1
51	Public Indoor	1
52	Hotel	1

Sand Filter Material

Sample material was taken on August the 17th 2016, when the material was completely removed from the filter tank. After cleaning and disinfection of the filter walls, new material composed of glass pearls was introduced to the filter on August the 24th 2016, restoring the function of the device. The sampled filter had a height of 2.5 m and a diameter of 2 m. 256 nozzles at the bottom provided pervasion. Table 13 specifies the arrangement of layers of the given filter.

In total, 16 individual samples were taken. Samples 1 to 3 were taken at the bottom porthole. Samples 4 to 6 as well as 10 to 14 were taken at the bottom porthole after removal of the layers beneath. Sample 7 was taken from the baked cake on the porthole. Samples 8 and 9 were taken from the top porthole before removal of any material through the bottom porthole. Samples 15 and 16 were taken from two nozzles at the bottom of the filter. All samples were taken using sterile sampling vessels (aqua Laborservice e. K., Wertheim, Germany).

Figure 31 shows two exemplary samples of varying grain size.

Table 13: Arrangement in layers of the sampled filter from the top to the bottom.

Layer	Thickness [mm]	Material [Grain Size mm]	Samples Taken [Nr.]
Flood Freeboard	825	-	-
1	-	Activated Carbon	8, 9
2	1,300	Sand [0.71 – 1.25]	4, 5, 6, 10, 11, 12, 13, 14
3	100	Gravel [1.0 – 2.0]	1, 2, 3
4	100	Gravel [2.0 – 3.15]	
5	100	Gravel [3.15 – 5.6]	
6	100	Gravel [5.6 – 8.0]	

Fig. 31: Examples of samples taken. Left - sample 2 (gravel); Right - sample 12 (sand mixed with activated carbon).

6.2.3 Growth Media

One ml of each sample was cultivated on Columbia agar with 5% sheep blood (Becton Dickinson GmbH, Germany) and TSA (Becton Dickinson GmbH, Germany) for 48 h at 36 ± 2 °C. Columbia agar was selected for its common application in MALDI-TOF operations and because this medium allows growth of many pathogenic bacteria species. TSA was selected for its common application in the assessment for bacterial (as well as mold and yeast) contaminations.

Columbia agar is a nutrient-rich medium commonly used in many European countries for the isolation and cultivation of non-fastidious and fastidious microorganisms from clinical samples [201]. The medium was developed by Ellner et al. [202]. Growth of microorganisms is supported by the combination of two peptones and yeast extract as a source of vitamin B [201]. Columbia agar is recommended as medium for isolation by German microbiological and infectiological quality standards [201, 203, 204].

Trypticase Soy Agar is a medium supporting the growth of non-fastidious and moderately fastidious microorganisms [205]. A combination of casein and soy peptones supplies organic nitrogen, sodium chloride maintains the osmotic equilibrium. This medium is used for many purposes like plate counting and isolation of microorganisms from various materials including water, foods and clinical specimens [205-207]. However, according to the supplier [205], TSA is not recommended for the primary isolation of microorganisms from clinical specimens, as it supports growth of many fastidious bacteria. It is hence not used as medium for primary isolation but for further differentiation and identification.

Tables 14 and 15 indicate the composition of the two media as specified by the supplier.

Table 14: Composition of Columbia agar [201].

Component	Grams per Liter Purified Water
Pancreatic Digest of Casein	12.0
Peptic Digest of Animal Tissue	5.0
Yeast Extract	3.0
Beef Extract	3.0
Corn Starch	1.0
Sodium Chloride	5.0
Agar	13.5
Defibrinated Sheep Blood	5 %

pH 7.3 ± 0.2

Table 15: Composition of tryptic soy agar (TSA) [205].

Component	Grams per Liter Purified Water
Pancreatic Digest of Casein	15.0
Papaic Digest of Soybean	5.0
Sodium Chloride	5.0
Agar	15.0

pH 7.3 ± 0.2

6.3 Results

6.3.1 Identification of Colonies Using MALDI/TOF

Commonly, microbial communities in swimming pools and similar environments include viruses, bacteria, protozoans and fungi [1]. As explained above, the present study focused exclusively on the detection and identification of bacteria. The main reason for this was which methods were applied, as MALDI-TOF requires accumulated material as provided by bacterial colonies. Furthermore, proceeding in this way had the benefit of producing results which could be embedded in the context of the other chapters.

As may be expected from an approach using growth media, apart from bacteria, also some yeasts and molds were detected and identified. Identified species of bacteria were classified, based on their Gram-type, the assumed origin and their pathogenic potential.

In this connection, the term skin flora applies to species commonly found on human skin as well as for such species residing on mucous membranes (e.g. in the mouth, nose, and genitals). Intestinal flora indicates any species commonly deriving from human intestines. Environmental refers to species inhabiting soil, water or any other media from the natural or man-made environment. Pathogenicity was classified as, negligible, if literature research indicated none or only very rare cases of disease caused by the given agent, opportunistic for all those species exhibiting pathogenicity under certain circumstances (e.g. mutants of commensals, nosocomial infections etc.) and primary for primary agents of diseases. This classification is, of course, debatable and, thus, was factored into the discussion.

Swimming Pool Water

Figures 32 to 34 present illustrations of the composition of the identified community with respect to Gram-type, assumed origin and pathogenicity. However, it needs to be borne in mind that the community examined is artificial, as the graphs presented reflect the aggregated information of all samples taken, regardless of the sampling venue. In the figures, the classes examined are shown related to both the detected species and total cases of detection respectively.

Tables 16 and 17 summarize the results on bacterial species isolated from water samples. The detection frequency indicates the number of independent samples the species was detected in, independent of the individual concentrations of bacteria in each sample.

Table 16: Species (bacteria, molds, yeasts) detected in 1 sample each.

Species Name	Frequency [n]	Gram-Type	Assumed Origin	Rating Pathogenicity	Literature Cited
Achromobacter denitrificans	1	Negative	Environmental	Opportunistic	[208, 209]
Acidovorax emperans	1	Negative	Environmental	Negligible	[210]
Acinetobacter ohnsonii	1	Negative	Shed	Opportunistic	[211]
Acinetobacter pittii	1	Negative	Shed	Opportunistic	[212, 213]
Aeromonas bestiarum	1	Negative	Environmental	Other	[214]
Aeromonas hydrophila	1	Negative	Fecal	Opportunistic	[215, 216]
Bacillus licheniformis	1	Positive	Environmental	Negligible	[217]
Bacillus megaterium	1	Positive	Environmental	Negligible	[218]
Bacillus thuringiensis	1	Positive	Environmental	Negligible	[219, 220]
Brevibacterium casei	1	Positive	Environmental	Opportunistic	[221-223]
Brevibacterium paucivorans	1	Positive	Environmental	Negligible	[224]
Corynebacterium minutissimum	1	Positive	Shed	Primary	[225]
Escherichia coli	1	Negative	Fecal	Opportunistic	[86, 226]
Herbaspirillum huttiense	1	Negative	Environmental	Opportunistic	[227]
Kocuria marina	1	Positive	Environmental	Negligible	[228]
Methylobacterium radiotolerans	1	Negative	Environmental	Opportunistic	[229]
Microbacterium testaceum	1	Positive	Environmental	Negligible	[230]
Moraxella osloensis	1	Negative	Shed	Opportunistic	[231, 232]
Pantoea agglomerans	1	Negative	Fecal	Opportunistic	[233, 234]
Paenibacillus timonensis	1	Positive	Environmental	Opportunistic	[235]
Paenibacillus urinalis	1	Positive	Environmental	Opportunistic	[236]
Pseudomonas fulva	1	Negative	Environmental	Negligible	[237]
Pseudomonas koreensis	1	Negative	Environmental	Negligible	[238]
Pseudomonas monteilii	1	Negative	Environmental	Opportunistic	[239]
Pseudomonas putida	1	Negative	Environmental	Negligible	[240]
Rothia amarae	1	Positive	Shed	Opportunistic	[241, 242]
Sphingomonas paucimobilis	1	Negative	Environmental	Opportunistic	[243, 244]
Staphylococcus lugdunensis	1	Positive	Shed	Primary	[245]
Staphylococcus pasteuri	1	Positive	Shed	Negligible	[93]
Streptococcus dysgalactiae	1	Positive	Shed	Opportunistic	[246]
Streptococcus sanguinis	1	Positive	Shed	Opportunistic	[246]
Streptomyces violaceoruber	1	Positive	Environmental	Negligible	[247]
Aspergillus fumigatus	1	Mold	Environmental	Opportunistic	[248]
Candida glabrata	1	Yeast	Environmental	Opportunistic	[249]

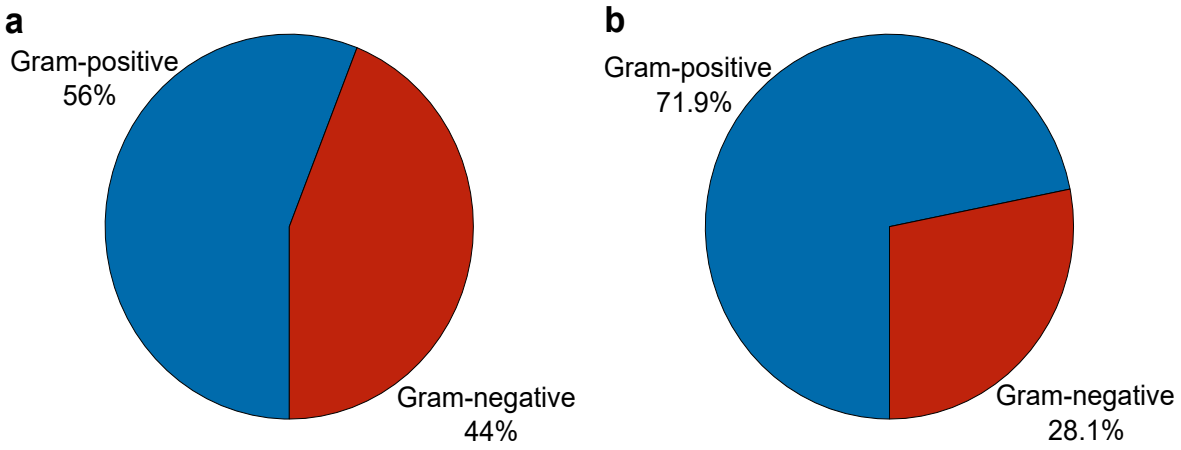

Fig. 32: Composition (percentage) of the sampled community of bacteria with respect to Gram type, relating to **(a)** species (n = 50) and **(b)** total cases of detection (n = 128).

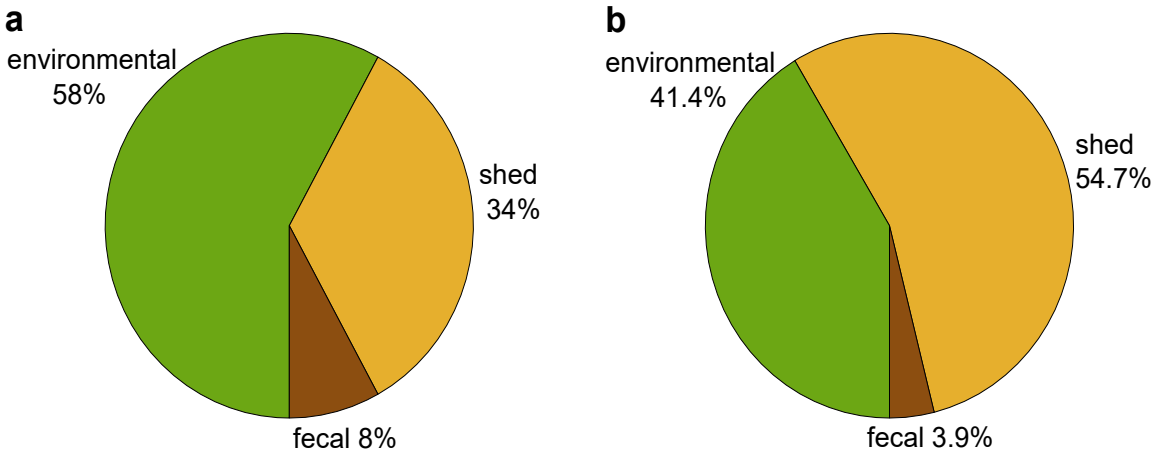

Fig. 33: Composition (percentage) of the sampled community of bacteria with respect to their origin, relating to **(a)** species (n = 50) and **(b)** total cases of detection (n = 128).

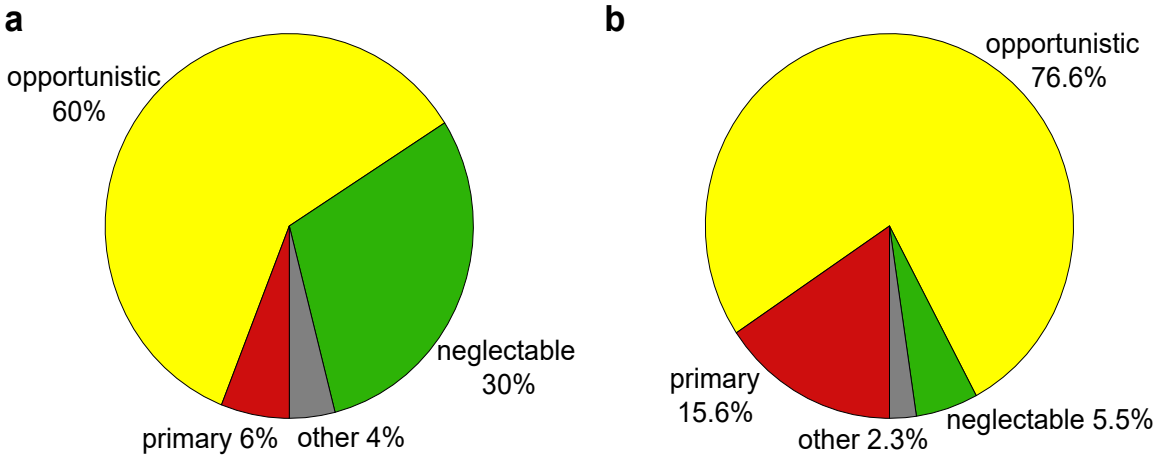

Fig. 34: Composition (percentage) of the sampled community of bacteria with respect to their pathogenicity, relating to **(a)** species (n = 50) and **(b)** total cases of detection (n = 128).

Table 17: List of bacterial species isolated from water samples more frequently than once.

Species Name	Frequency [n]	Gram-Type	Assumed Origin	Rating Pathogenicity	Literature Cited
Staphylococcus epidermidis	14	Positive	Shed	Opportunistic	[2, 250-252]
Staphylococcus warneri	12	Positive	Shed	Opportunistic	[253]
Micrococcus luteus	10	Positive	Shed	Opportunistic	[93, 254-256]
Pseudomonas alcaligenes	7	Negative	Environmental	Opportunistic	[257, 258]
Bacillus cereus	6	Positive	Environmental	Primary	[259, 260]
Staphylococcus capitis	6	Positive	Shed	Opportunistic	[93, 253]
Staphylococcus hominis	6	Positive	Shed	Opportunistic	[93, 261]
Ralstonia insidiosa	5	Negative	Environmental	Opportunistic	[262]
Staphylococcus haemolyticus	5	Positive	Shed	Opportunistic	[93, 252, 253]
Corynebacterium amycolatum	4	Positive	Shed	Opportunistic	[263-265]
Kocuria palustris	4	Positive	Environmental	Opportunistic	[266, 267]
Staphylococcus aureus	4	Positive	Shed	Opportunistic	[268, 269]
Kocuria rhizophila	3	Positive	Environmental	Negligible	[266]
Bacillus simplex	2	Positive	Environmental	Negligible	[260]
Citrobacter freundii	2	Negative	Fecal	Opportunistic	[270]
Paracoccus yeei	2	Negative	Environmental	Negligible	[271, 272]
Pseudomonas aeruginosa	2	Negative	Environmental	Opportunistic	[273, 274]
Sphingomonas melonis	2	Negative	Environmental	Other	[275]

Filter Material Samples

Table 18 lists microbes detected in filter samples related to the detection frequency.

In contrast to the results presented in tables 16 and 17, results are related to one sampling rather than multiple samplings. The detection frequency indicates in which quantities the given species was detected in all filter samples. In figure 35, the composition of the communities of each of the layers in the sand filter is illustrated in the form of a pie chart.

Additionally, sand samples were examined for the parameters heterotrophic plate count at 22 and 36 °C, as well as *E. coli* and coliform bacteria. The methods used were taken from the German standard on swimming pool hygiene [19]. The results are presented in figure 36.

Table 18: Community of bacteria, molds and yeasts isolated from sand filter samples.

Species Name	Frequency [n]	Gram-Type	Assumed Origin	Rating Pathogenicity	Literature Cited
Cupriavidus necator	930	Negative	Environmental	Negligible	[276, 277]
Pseudomonas putida	300	Negative	Environmental	Negligible	[240]
Hydrogenophaga flava	75	Negative	Environmental	Negligible	[278]
Pseudomonas alcaligenes	69	Negative	Environmental	Opportunistic	[257, 258]
Staphylococcus haemolyticus	38	Positive	Shed	Opportunistic	[93, 252, 253]
Staphylococcus capitis	16	Positive	Shed	Opportunistic	[93, 253]
Pseudoarthrobacter sulfonivorans	12	Positive	Environmental	Negligible	[279, 280]
Clostridium cochlearium	9	Positive	Environmental	Opportunistic	
Pseudomonas rhodesiae	5	Negative	Environmental	Negligible	[281]
Bacillus firmus	3	Positive	Environmental	Negligible	[282]
Staphylococcus epidermidis	3	Positive	Shed	Opportunistic	[250-252]
Novosphingobium subterraneum	3	Negative	Environmental	Negligible	[283, 284]
Bacillus cereus	2	Positive	Environmental	Primary	[259, 260]
Bacillus licheniformis	2	Positive	Environmental	Negligible	[217]
Bacillus simplex	2	Positive	Environmental	Negligible	[260]
Corynebacterium striatum	2	Positive	Shed	Opportunistic	[285]
Staphylococcus hominis	2	Positive	Shed	Opportunistic	[93, 261]
Staphylococcus pettenkoferi	2	Positive	Shed	Opportunistic	[286-288]
Micrococcus luteus	2	Positive	Shed	Opportunistic	[289, 290]
Neisseria meningitidis	2	Negative	Shed	Primary	[291, 292]
Aeromonas caviae	1	Negative	Fecal	Opportunistic	[293]
Bacillus arsenicus	1	Positive	Environmental	Negligible	[294, 295]
Bacillus megaterium	1	Positive	Environmental	Negligible	[218]
Comamonas aquatica	1	Negative	Environmental	Negligible	[296]
Corynebacterium amycolatum	1	Positive	Shed	Opportunistic	[263-265]
Escherichia hermannii	1	Negative	Fecal	Negligible	[297-299]
Pseudomonas nitroreducens	1	Negative	Environmental	Negligible	[300, 301]
Candida tropicalis	100	Yeast	Environmental	Primary	[302, 303]
Pichia occidentalis	1	Yeast	Environmental	Negligible	[304, 305]

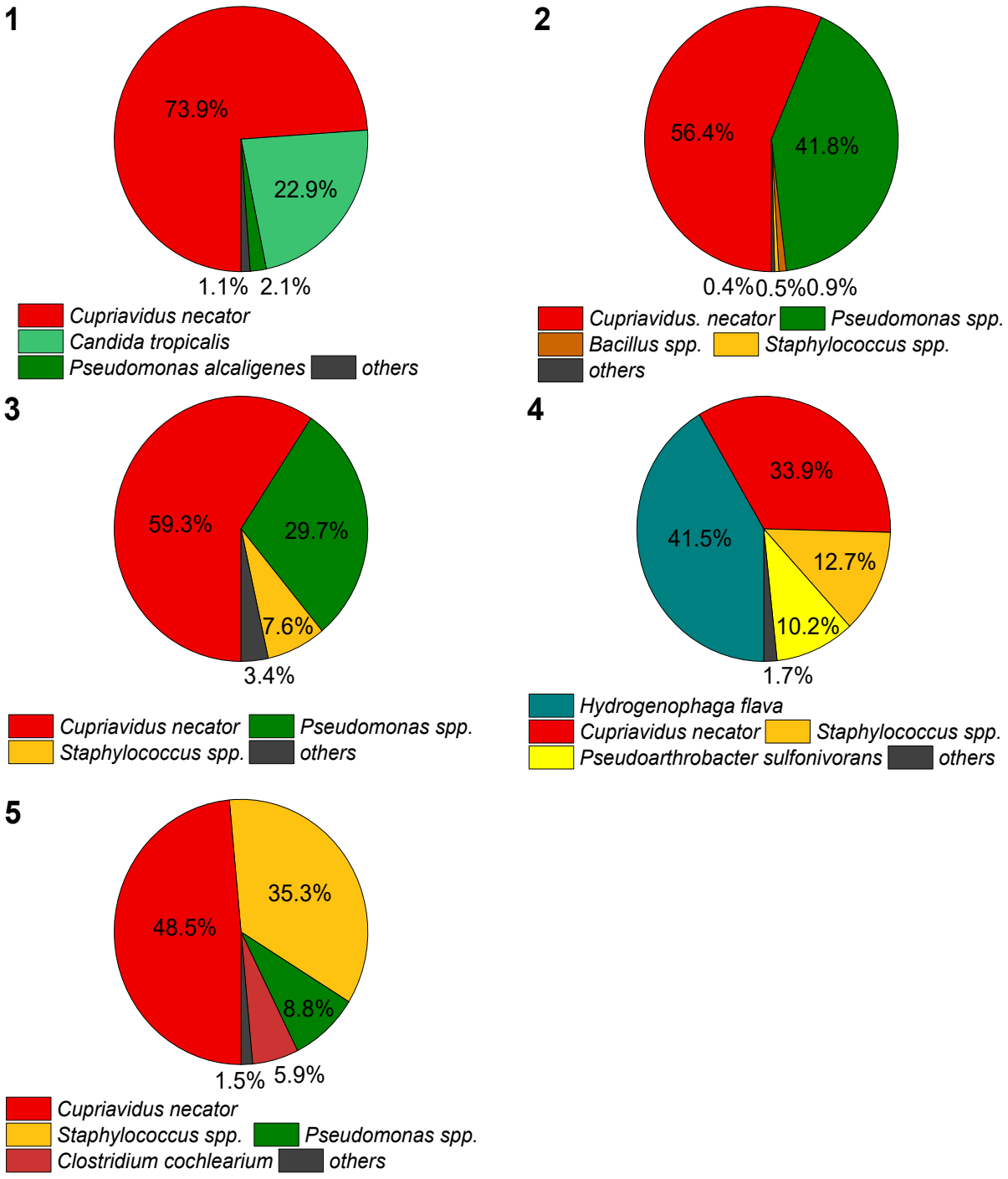

Fig. 35: Composition of the microbial communities detected in five layers of the sampled sand filter. A dominance of the predatory species *Cubriavidus necator* was determined: **(1)** activated carbon, samples 8 and 9; **(2)** activated carbon mixed with sand, samples 10 and 11; **(3)** sand, samples 12, 13, and 14; **(4)** sand mixed with gravel, samples 4, 5, 6; **(5)** gravel, samples 1, 2 and 3.

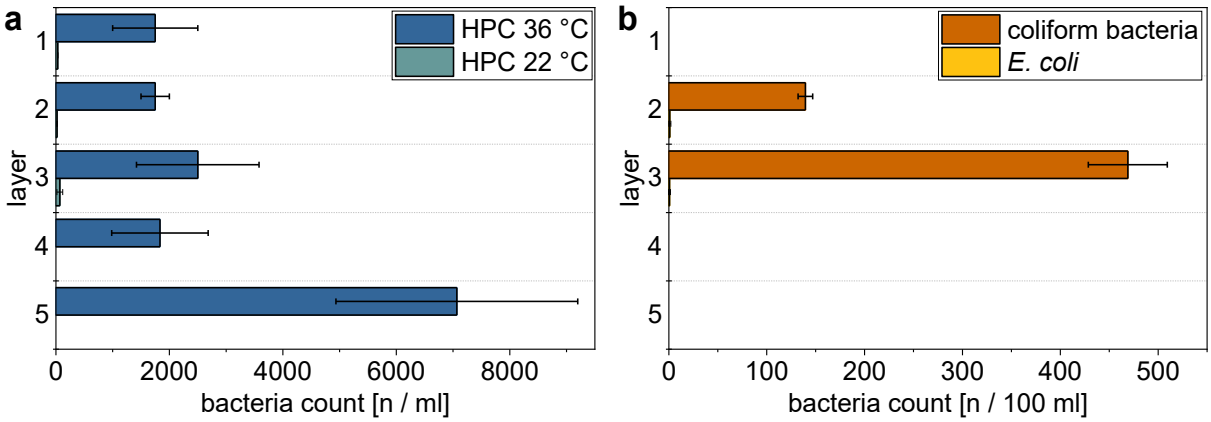

Fig. 36: Distribution of several microbial indicators over the height of the sampled sand filter: **(a)** Heterotrophic plate count at 22 °C and 36 °C respectively; **(b)** coliforms and *E. coli*. Layers: **(1)** activated carbon, samples 8 and 9; **(2)** activated carbon mixed with sand [0.71 - 1.25 mm], samples 10 and 11; **(3)** sand [0.71 - 1.25], samples 12, 13, and 14; **(4)** sand [0.71 - 1.25 mm] mixed with gravel [1.0 - 3.15 mm], samples 4, 5, 6; **(5)** gravel [3.15 - 8.0], samples 1, 2 and 3.

6.4 Discussion

6.4.1 Evaluation of the Selected Methods

The presented results of this chapter rely exclusively on culturing methods. For this study, data was generated and interpreted from a hygienist perspective. Both detection and identification of microbes focused on bacteria and particularly such species potentially harmful for bathers. Accordingly, examination procedures and methods were selected not solely out of necessity (e.g. as culture media fit the requirements for MALDI-TOF identification), but also with the intention to particularly detect such species possessing pathogenic features. However, due to this proceeding, results do not reflect the entire spectrum of microbial life potentially present, but rather the fraction which can be cultured under the given conditions. Presumably, this excludes a broad range of environmental bacteria, which, in many cases, cannot be cultured at all [306]. Furthermore, any individuals in the “viable but non-culturable state” (VBNC) [307] are not reflected. Yet, while these organisms would be detectable by methods non-dependent on cultivation, e.g. deep-sequencing [308, 309], results gathered with these methods lack indication of the impact of generated information for hygienics. Some scientists even recommend the parallel use of culture-dependent methods and modern molecular techniques as any method will always introduce a bias [310].

As mentioned above, identified species were classified by their Gram-type, assumed origin and pathogenic features. In doing so, distinct patterns in the relative abundances of individual species became apparent. However, while the Gram-type is an established tool, the other two categories are both subject to debate. For instance, the question is still being discussed to date what distinguishes a primary pathogen from an opportunistic pathogen [311-313]. Hence, the results presented here may not be used to draw easy conclusions regarding swimming pool hygiene.

Furthermore, during examination of the generated data, an overall tendency for a stronger database was identified for such species detected more frequently than for species detected less frequently. Occurrence of such a trend raises the question if the literature used here introduced a bias to the results. This bias could, for instance, derive from the higher importance

of some species in the clinical context. The methods and equipment used for the present study are commonly used for the assessment of clinical samples. The prevalence of such species known for their importance in the clinical context could, hence, go back to the selected methods being more suitable for their identification than for the identification of less important species. Identification of such bacteria with a large database from clinical sampling is presumably performed more easily and reliably, compared to such species involved only in a few cases per decade.

Culturing Methods

Comparably small numbers of colonies were detected on most of the agar plates examined (in average 1-10 colonies both on Columbia agar and TSA respectively). In the past, predicating the identification of health risks using culturing methods has frequently been criticized. For example, a study by Roszak and Colwell mentioned several common points of this criticism [314]. These include the fact that there are frequently significant differences between plate counts and cell numbers examined under the microscope, that there is no single medium adequate for the culturing of all bacteria species potentially present in a sample, and that incubation temperatures are potentially lethal for diverse environmental bacteria.

The first point indicates that, although present, a considerable fraction of microbial cells possibly does not grow into colonies. For the present study, this could be of importance, as not the entire present community of bacteria could have been captured with the methods used. However, since the goal was to gather information on overall distribution patterns of pathogenic bacteria in swimming pools, the introduced bias should be negligible.

Regarding the second fact, Roszak and Colwell conclude that growth media were generally inherently selective and, consequently, deficient for the evaluation of health risks [314, 315]. There is no doubt that growth media will always provide varying combinations of nutrients which allow the growth of some species, while others are hindered from growing under the given conditions. However, there is also a lack of suitable alternatives for routine identification of bacteria. Accordingly, culture-dependent methods are still the gold standard in many routine applications including maintenance of swimming pools and, thus, were also applied for this study.

The fact that, presumably, temperatures higher than 20 °C are lethal for a broad range of aquatic bacteria (even including some species of “possible public health importance”) [314, 316] is obviously of importance for the outcome of the used approach. Yet, this factor presumably would also have the strongest effect on results on such species of minor impact regarding public health.

6.4.2 Classification of Bacteria from Water Samples

In total, 26 samples from 11 venues were analyzed. Of the 52 species identified, 50 were bacterial species. Eighteen of these species were detected repeatedly, while the majority were detected only once. Additionally, 2 fungal species were detected. Both belong to genera (*Candida* spp. and *Aspergillus* spp.) which have also been detected in larger-scale studies on microbial swimming pool water quality [1, 317].

Gram-Type

A predominance of Gram-positive species compared to Gram-negative species was determined. Of the species detected at higher frequencies than 1 per 26 samples, 12 were Gram-positive, including the three most abundant species. This finding is substantiated by the results achieved in experiments on disinfection kinetics. Here, Gram-positive bacteria were comparably more resistant to chlorine disinfection, than their Gram-negative counterparts. A higher resistance of Gram-positive bacteria in comparison to Gram-negative species could derive from a thicker cell wall and, in some cases, the ability to form protective endospores. With respect to those species detected only once, no comparable pattern was identified. Both Gram-types were detected at the same frequency (16 species each).

Assumed Origin

Classification of detected bacteria by the assumed origin provided a possible explanation for the observed dominance of Gram-positive bacteria. Fittingly, results indicate a predominance of such genera commonly inhabiting human skin. For instance, the genera *Staphylococcus*, *Micrococcus*, *Corynebacterium*, and *Brevibacterium* were detected frequently. All of them are also very abundant in the natural skin microbiome [93]. Interestingly, the relative abundances of individual *Staphylococcus* species (*S. epidermidis* > *S. warneri* > *S. hominis*) also correlated well with their relative abundances on human skin [318]. Furthermore, several less frequently detected genera contribute to the natural human skin flora, including *Acinetobacter*, *Enterobacter*, *Streptomyces*, *Neisseria* and *Moraxella* [253].

Apart from skin bacteria, also another fraction of considerable dominance was detected. This fraction was composed of such bacteria known for their role in environmental hygiene and included, for example *Acinetobacter*, *Aeromonas*, *Bacillus*, *Enterobacter*, and *Pseudomonas* [117]. Presumably, bacteria of these genera were introduced, for instance, by the addition of raw water to the pool or by showers at the respective venues [319].

Pathogenicity

Based on literature research, most species were categorized as opportunistic pathogens, with only a few exceptions being ranked as primary pathogens. One prominent primary pathogen detected was *Bacillus cereus*. This is a foodborne pathogen causing diarrheal and emetic types of food poisoning [320]. Furthermore, the group of primary pathogens included several species associated with skin diseases [93, 246], like *Corynebacterium minutissimum*, *Staphylococcus lugdunensis* and two species of *Streptococci*. However, those *Streptococcus* species identified, namely *S. dysgalactiae* and *S. sanguinis*, are both rather uncommon agents of disease. On the other hand, the *Streptococcus* species predominantly responsible for infections, *S. pyogenes* [321], was not detected in any sample.

Interestingly, both fungal species detected are potentially pathogenic for human bathers. *Aspergillus fumigatus* is a mold producing several mycotoxins, including fumagillin and gliotoxin [322]. Furthermore, *Aspergillus fumigatus* may cause diverse forms of disease including allergic reactions, aspergilloma and, in immunocompromised people, even aspergillosis [323]. *Candida glabrata* is a yeast and regarded a highly opportunistic pathogen

of the genitourinary system and the blood system [249]. Regarding the affinity for infections of the genitourinary tract, both the origin and consequences of this finding become apparent.

Taxonomy

The composition of bacterial communities in chlorinated waters has frequently been assessed using taxonomic classification. However, comparison of the results achieved in this study with literature on this topic lead only to little congruence. For instance, a study on the bacterial populations in chlorinated drinking water [324] concluded that most residing bacteria were members of the class alphaproteobacteria. A dominance of this class was not determined in the present study for bacteria in chlorinated bathing water. Presumably, this disparity goes back to the occurrence of skin flora bacteria in bathing water, resulting in a shift in ratios in the overall community. Another study on drinking water disinfection [116] discovered that with increasing concentrations of free chlorine, also the fraction of *Actinomycetales* on the microbiome was increasing. Interestingly, this order was also strongly represented in the present study. Detected genera belonging to the *Actinomycetales* were for example *Corynebacterium*, *Micrococcus*, *Mycobacterium*, *Norcardia* and *Streptomyces*. A study from 2017 [325] put emphasis on what effect chlorination had on the bacterial community introduced to swimming pools by the bathers. Before chlorination, the water used for showering by test persons contained seven bacterial families with abundances higher than 5%. Of these, four were still present in higher abundances after chlorination. These were *Flavobacteriaceae*, *Xanthomonadaceae*, *Moraxellaceae*, and *Pseudomonadaceae*. Of the family most abundant in their work, the *Flavobacteriaceae*, only one genus was detected (*Elizabethkingia spec*) in the present study. The second most abundant family, the *Xanthomonadaceae,* was not represented at all. Since both these families are composed mainly of environmental bacteria, this lack of abundance presumably derived from a lack of cultivability. Of the third most abundant family, *Moraxellaceae*, only two members were detected in the present study (*Acinetobacter johnsonii* and *Moraxella osloensis)*. However, the *Pseudomonadaceae* were represented by several species of *Pseudomonas* (e.g. *P. aeruginosa* and *P. alcaligenes*).

6.4.3 Classification of Bacteria from Sand Filter Material

Gram-Type

In contrast to the results of water samples, the bacterial community isolated from sand samples was strongly dominated by Gram-negative species. Contrary to expectation, *Pseudomonas aeruginosa* was not detected in any of the samples taken. Hence, it is assumed that there was no biofilm formed by this species present in the examined filter. However, other members of the genus *Pseudomonas* were detected.

Assumed Origin

Three genera of bacteria were predominant in sand samples: *Pseudomonas* spp., *Staphylococcus* spp. and *Cupriavidus necator*. In four out of five layers, *C. necator* made the majority of the detected microbes (48.5 to 72.8%). In most of these layers, *Pseudomonas* species and *Staphylococcus* species were the second and third most frequently identified genera.

Cupriavidus necator is a Gram-negative species, which is believed to be a non-obligate predator in soil [277, 326]. Fittingly, several other bacterial species detected in swimming pool water and filter samples are part of its presumed range of pray. These include *Bacillus* spp., *Escherichia coli*, *Micrococcus luteus*, *Staphylococcus aureus* and *Streptomyces* spp. [277, 326]. Hypothetically, the population of *C. necator* in the sand filter was specialized in preying on other bacteria held back by the filter material. Hence, since *C. necator* apparently does not exhibit pathogenic traits, the presence of this species may have been beneficial for the water quality of the swimming pool.

The strong presence of *Pseudomonas* species indicates the formation of biofilms on the sand material. The wet environment would have provided ideal conditions for growth. *Staphylococcus* species presumably were detected at high frequencies because they were washed off by bathers. Protected from chlorination by their attachment to skin particles, these bacteria may have reached the sand filter in a state allowing for temporal survival.

The composition of the communities of layers 1 and 4 of the sand filter are particularly interesting. In layer 1, the yeast *Candida tropicalis* was detected at high counts. Presumably, cells were washed there only recently before sampling, as they were only detected at the top of the filter. Furthermore, their larger cell size in comparison to bacterial cells may have played a role. Presumably, they derived from infected bathers. Layer 4 was populated by high counts of *Hydrogenophaga flava* and *Pseudoarthrobacter sulfonivorans*. *H. flava* are facultatively autotrophic hydrogen bacteria [278]. *Pseudoarthrobacter sulfonivorans* is able to reduce dimethylsulfone and dimethylsulfoxide [279, 280]. Furthermore, *Bacillus arsenicus* presumably was detected, an arsenic resistant species [294]. The fact that all these species have in common, that they grow under atypical conditions, layer 4 presumably was characterized by other conditions than the rest of the filter e.g. low oxygen concentrations and mixture of the material with washed of metal from the tank.

Fittingly, several of the other species detected were associated with wet environments, such as the feathers of aquatic birds (populated e.g. by *Bacillus licheniformis* [217]).

Pathogenicity

According to literature, some of the detected species are regarded as primary pathogens. The high frequencies of *Candida tropicalis* detection is particularly concerning. This yeast has been associated with “dramatically” increasing numbers of infections worldwide, thus being classified as an “emerging pathogen” [19]. However, since *Candida tropicalis* was not isolated in water samples at the same time, it can be assumed that no acute health hazard for bathers existed in the concerned swimming pool. Furthermore, as simultaneously with sampling, the sand filter probed was reconstructed, a probable future hazard deriving from the presence of *Candida tropicalis* should have also been removed.

Furthermore, *Pichia occidentalis* was detected. However, to the author’s knowledge, this yeast is not of any clinical importance.

Regarding pathogenic bacteria, two species of interest were detected. The first was *Bacillus cereus*, which has also been detected in water samples of this study. The second, *Neisseria meningitidis*, is part of the microbiome of the nasopharynx in up to 40% of the adult human population. However, as the name suggests, *Neisseria meningitidis* is also a major agent for disease, causing meningitis and sepsis with considerable rates of mortality [291, 292].

Indicator Bacteria

Parallelly to isolation and identification of bacteria from bathing water samples, the same samples were also used for an examination on indicator bacteria. There was no correlation observed between concentrations of HPC 36 °C bacteria and the occurrence of specific indicator species such as *E. coli* or *P. aeruginosa*.

This is in line with the findings of Petti et al. (2004) [327]. Investigating water samples from several sources (e.g. tap, tank, boiler) for the occurrence of *Pseudomonas aeruginosa*, *Legionella pneumophila* and “total cultivable flora”, Petti and colleagues could not find any correlation between frequencies of occurrence of indicator species and total numbers of bacteria.

The same applies for the assessment of bacteria communities as performed for the present chapter. Here, indicator bacteria were detected at very low frequencies, not allowing for correlations to the occurrence of other bacteria. However, one needs to bear in mind that the various indicator bacteria used in routine control are all determined using different test methods with individual culturing media. An impact of the given proceedings on the results cannot be excluded. In rare cases, *E. coli*, other coliforms and *P. aeruginosa* respectively were detected in water samples using the MALDI-TOF technique but have not been detected by the routine methods applied in parallel. These differences could indicate inadequacy of the routine methods to detect the respective indicator bacteria. However, at the encountered frequencies, results are relativized by statistics of uncertainty. Furthermore, the question arises if a single cell of any of the specific indicator bacteria does really pose a health risk. Normally, the infectious dose should be considerably higher.

Staphylococcus Species

Such species of the genus *Staphylococcus,* which commonly inhabit human skin, may also frequently be isolated from chlorinated swimming pool water [328]. It has been suggested that pool water containing high concentrations of *Staphylococcus* presents a risk of infection, comparable to the risk to contract a gastrointestinal illness by bathing in water contaminated with fecal material [329]. According to the WHO, currently there are three species of *Staphylococcus* having clinical importance: *S. aureus*, *S. epidermidis* and *S. saprophyticus*. Of these three, *S. aureus* is considered the most important pathogen [2, 330].

While, on the one hand, *S. aureus* has not been isolated from any pool water samples in this study, on the other hand, *S. epidermidis* was the most prevalent of all species detected. The mentioned document by the WHO [2] also cites the statement of some older publications [331, 332], that *S. aureus* in general would represent 50% or more of total staphylococci isolated from swimming pool water. However, the same document [2] also reviews a more recently published study [333], stating that at chlorine concentrations between 0.8 and 1.2 mg/L, *Staphylococcus aureus* could not be isolated from water samples at all.

6.5 Conclusions

The results from MALDI-TOF identification on colonies of bacteria recovered from samples of swimming pool water and sand taken from a filter have some implications for swimming pool maintenance as currently routinely performed.

For instance, bacteria associated with natural human skin flora were recovered from swimming pool water at high frequencies. Presumably, the predominance of these species goes back to an increased introduction of these species in comparison to other bacteria. In this connection, bacteria cells may both be washed off the skin of bathers and be shed together with skin particles during swimming. According to literature [33, 253, 334, 335], the genera *Staphylococcus* and *Corynebacterium* respectively are the most abundant bacterial genera colonizing moist body areas. Therefore, the frequent occurrence of respective species indicates an inadequate showering behavior of some bathers [35]. However, apart from this, originating from skin could also by other means have influenced the outcome. For instance, bacteria attached to skin particles could have simply been protected from inactivation by their continuing attachment to skin particles. Moreover, skin bacteria are more likely to grow on the selected media, while many environmental bacteria may not be cultivable under the given conditions. Hence, this may also explain a shift of the results towards these species.

Furthermore, in water samples, only very few primary pathogens were detected. In addition, with respect to many of the opportunistic pathogens detected, infections normally are connected to hospitalization. Examples are *Acinetobacter johnsonii* [211], *Methylobacterium radiotolerans* [229], and *Brevibacterium casei* [221-223]. The question arises, if respective species can infect bathers under the given conditions. Regarding the findings of the present study, infections associated with bathing activity in swimming pools appear rather unlikely. However, it needs to be factored in, that bacterial communities in swimming pools have been linked to the occurrence of multi-resistent lines [1, 336]. For example, multi-resistant strains of *Aeromonas hydrophila*, *Staphylococcus aureus*, and several species of *Pseudomonas* (for example *P. aeruginosa*, *P. alkaligenes*, and *P. fluorescens*) have been isolated from swimming pool water. It appears plausible that some of the species detected in swimming pool water could also have spawned multi-resistant descendants or were members of multi-resistant strains.

As described in a review on epidemiological studies [337], the general health risk of bathers is positively correlated to the number of indicator bacteria in the respective recreational waters. According to a study mentioned afore, of all indicator bacteria examined, *Escherichia coli* and enterococci correlated best with health risks for bathers. On the other hand, the absence of indicator bacteria cannot be taken as a guarantee for safety regarding health risks of bathing [2]. In the present study, however, indicator bacteria have been recovered from samples only in very few cases, presumably playing only a minor role.

From the sand filter material examined, a distinct community of bacteria and yeasts was recovered. As described, presumably, *Cupriavidus necator* was preying on other bacteria held back by the filter material. As *C. necator* does not exhibit pathogenic traits, the presence of this species may have been beneficial for the water quality of the swimming pool. Next to this germ, also species commonly found in aquatic environments (e.g. *Pseudomonas* spp.) and members of the skin microbiome on humans (e.g. *Staphylococcus* spp.) were detected. It can be expected, that these were filtered from the bathing water. Moreover, some quite exotic species were detected, which may have been specialized to colonization of areas not reached

properly by hydraulics. However, it may be assumed that these highly specialized species did not play a major role in the development of health risks for bathers.

Summing up, the results indicate that swimming pool water is not a major vector for the distribution of pathogenic bacteria and therefore the diseases associated. On the other hand, infections with pathogenic yeasts, in the observed case, *Candida tropicalis*, appear to pose a greater risk. From the results, no correlation of the occurrence of indicator bacteria in swimming pool water and the incidence of pathogenic species could be observed. However, the results underline the role of skin flora for contaminations with bacteria in swimming pool water. Therefore, they highlight the importance of sufficient showering behavior of bathers, a topic of general concern [338]. Furthermore, the results give an overview on the groups of bacteria covered by heterotrophic plate count approaches used for the assessment of the hygienic status of swimming pool water. Hence, they provide a starting point for discussions on the use of the heterotrophic plate count in swimming pool maintenance.

Final Conclusions and Perspectives

As explained, the present study deals with several aspects of bathing water hygiene. Interlinked, the results presented in the individual chapters allow reappraising of common practices used to maintain hygienic conditions in public swimming pools.

It is the author's opinion that there are diverse open questions associated with current standards in swimming pool maintenance. Foremost, these include the following:

Do the tools applied with the intention to increase the level of safety of swimming pools really provide this assumed increase in safety?

Is the associated cost-value ratio acceptable?

Most importantly, on the meta level, how safe have public swimming pools to be in the first place?

Several approaches were developed under the proposition given by these questions. For instance, one major topic of the present thesis is the assessment of inactivation kinetics of bacteria by hypochlorous acid as a representative of chlorine disinfectants. As described, experimental conditions were designed in a way mimicking the conditions in swimming pool basins, therefore differentiating the experiments performed from those of some comparable studies [113, 174, 186]. Independent of the test species, inactivation curves were characterized by the occurrence of tailing, and, in some rare cases, also initial lag-phases (plateaus). The classic Chick-Watson model succeeded in matching the data obtained, only if the selected conditions were within an ideal range for disinfection (e.g. comparably low pH or comparably high concentrations of free chlorine). In contrast to the conclusions drawn in comparable studies [11, 74], the author deduces from this, that there is an improvement in using alternative models. Inactivation curves apparently were subdivided into two distinct linear phases. Each of these had an individual inactivation rate. Using both mechanistic and vitalistic fitting models, the underlying mechanisms responsible for these phases were investigated. However, comparing the results achieved with these models, neither using the mechanistic, nor the vitalistic models resulted in a significantly better fitting performance (compare chapter 3). In fact, all used models lead to comparably high coefficients of determination.

From the mechanistic perspective, one major factor for the development of tailing presumably was chlorine consumption during disinfection [113]. Although a large volume was chosen for the examination of inactivation kinetics, still a considerable decay in the concentrations of chlorine available for the inactivation of bacteria occurred (compare chapter 5).

From the vitalistic perspective, presumably the individual state of bacteria cells had an impact on disinfection kinetics. The author assumes that two subpopulations existed which had varying resistances to the exposure to chlorine [68, 79]. If this was true, this varying resistance could go back to several factors. One possible explanation is that individual cells varied in their growth state [57, 315]. The used inoculi were produced in a comparably rich medium. Hence, presumably, most of the cells were adapted to conditions ideal for growth, whilst only some cells were more capable of surviving under more harsh conditions. This is in line with the findings of a recent study on the influence of cell growth on chlorine inactivation of microbes [339]. Mentioned study concluded that inactivation rate constants are low for bacteria in stationary phase and comparably higher for cells in exponential growth phase. In the setup

chosen for the experiments of the present study, presumably most cells were in exponential growth phase at the time an experiment was started. If only some cells were in stationary phase, this could explain why they were more resistant, resulting in the occurrence of tailing in the respective experiments.

The results produced using an alternative growth medium (R2a) and the molecular technique FISH indicate that some bacteria survived exposure to chlorine disinfectants for considerably longer periods of time than suggested by the original results of the inactivation experiments. While present, some bacteria cells apparently were not detectable using classical culturing methods. Supposedly, this means that this common tool for monitoring and control may not always be trusted. The implications of this finding become all the more pressing considering the fact that some pathogenic bacteria of relevance for swimming pool hygiene (e.g. *Legionella* spec.) are able to produce virulence factors even if they are in a viable but non-culturable state [340].

As explained, experiments on the inactivation of *P. aeruginosa* revealed a phenomenon of changing colony appearance (compare chapter 4). This phenomenon indicates a potential occurrence of non-culturable bacteria. Therefore, the question arises, whether contaminations with this opportunistic pathogen can be assessed using culture-dependent methods. The results furthermore imply that occurring recontaminations could go back to the presence of cells which are non-cultivable only temporarily, or in other words, cells which recover cultivability, if the respective conditions change towards preferable for their growth. Future examinations should focus on the question, if, in the context of swimming pool disinfection, cells of *Pseudomonas aeruginosa* and other bacteria of interest, are able to regain their ability for growth. Also, it is a very interesting question, if *P. aeruginosa* in a VBNC state can produce virulence factors. Both these topics are also relevant for other fields of research [341]. The author suggests initiating further studies involving molecular techniques such as FISH in order to investigate the role of cultivability on the results of examinations of disinfection success.

In summary, the results of the performed experiments on inactivation kinetics provide information on the general pace of microbial inactivation by hypochlorous acid for three species of interest. The results indicate that chlorine concentrations as currently used in German public swimming pools are at least borderline to insufficient for the inactivation of some bacteria, including *Pseudomonas aeruginosa* and *Staphylococcus aureus*. This finding is supported by the recommendations given by the WHO [2]. Furthermore, the results demonstrate that culture-dependent methods, as commonly used for examination of the hygienic status of swimming pools, are accompanied by uncertainties, which, in turn, may influence the outcome in a considerable dimension. Therefore, the author of this thesis suggests either to increase chlorine concentrations in German swimming pools, or, with respect to disinfection by-products, to put the focus on toxic by-products of disinfecting agents. Considering the increasingly perceived matter of disinfection by-products [179, 346-348], the question of what the greater threat is - pathogenic microbes or the toxic chemicals resulting from their inactivation - should be discussed both by the scientific and the public community.

Apparently, chlorine disinfection is quite effective against most bacteria. However, the currently common focusing on bacteria species should be abandoned. Apart from bacterial pathogens, there is a broad spectrum of viruses and protozoans which are at least equally hazardous to human health and which may be contracted using swimming pools. Some of these pathogens can be assumed to survive exposure to chlorine significantly longer than bacteria. As, most likely, contaminations in swimming pools are not able to be discovered using bacterial

indicators, methods need to be developed and made available, which allow the fast and distinct detection of these threads.

Related to this topic, it is of importance that there is a considerable lack of comprehensive knowledge on the occurrence of pathogenic bacteria in swimming pools, their relevance and the value in assessing their presence by determination of the presence on indicator bacteria, as was introduced in the summary of this thesis. This point will be discussed later.

A similar lack of knowledge also exists regarding the inactivation kinetics of chlorine disinfectants. Consequently, specifications for free chlorine concentrations and pH differ quite remarkably between European countries. For example, in Austria, like in Germany, concentrations of 0.3 to 0.6 mg/L of free chlorine and pH values between 6.5 and 7.2 are mandatory [342]. Greek regulations define concentrations of 0.4 to 0.8 mg/L [46, 343]. In Switzerland, free chlorine concentrations ranging between 0.2 and 0.8 mg/L are allowed, while the pH range is set as 6.8 to 7.6 [344]. Even higher pH values ranging between 7.2 and 8.0 are defined by Spanish regulations [345]. Here, also higher chlorine concentrations are recommended, ranging between 0.2 and 2 mg/L. The results of the present study pinpoint the crucial role of pH maintenance for swimming pool disinfection. Not only chlorine concentration, but also the pH needs to be kept within a range supporting disinfection. Otherwise, the inactivation of bacteria will not proceed in an acceptable manner.

Also of interest for this matter is that most standards for swimming pool maintenance include *Pseudomonas aeruginosa* and *Escherichia coli* as indicators for microbial contaminations [19, 342, 344, 345]. The present thesis provides information on the frequency of detection of these indicators in a sample of cases in German swimming pools, as well as on their sensitivity to chlorine and the occurrence of pathogens in swimming pool water and sand filter material. Combining the findings, the question arises of how reasonable it is to use *Pseudomonas aeruginosa* and, particularly, *Escherichia coli* as indicators for contaminations.

As reviewed by Odonkor and Ampofo [23], perception of how effective indicators are in determining the risk of contaminations and associated infections has changed over time. Traditionally, microbial indicators have been introduced to determine whether pathogens are likely to be present or not. However, the correlation between the occurrence of pathogens and indicators is influenced by diverse factors. According to the mentioned review, there is increasing knowledge on these factors contributing to the parallel presence of indicators during the absence of pathogenic species (or *vice versa*) [23]. Frequently, a direct correlation between numbers of indicator organisms and pathogens cannot be determined [349]. For instance, fluctuating ratios of pathogens to indicators and the varying virulence of pathogens have been identified as such factors leading to failure when relating epidemiologic studies with the incidence of indicators [23, 350].

According to the results of this study, *Escherichia coli* is quite sensitive to chlorine disinfection. This is in line with other studies [42, 43, 113] . Yet, presumably, many other fecally derived pathogenic species are considerably less susceptible to chlorine disinfection (e.g. *Giardia* and *Cryptosporidium* spp.) [2]. On the one hand, this means that if *E. coli* is detected in a swimming pool - a microbe of weak resistance to disinfection procedures - more resistant species are likely to survive or even multiply under the conditions examined. On the other hand, if *E. coli* is not detected, there is no guarantee that other fecally derived microbes are also not present. The results on the incidence of *E. coli* and coliform bacteria indicate that the presence of both these indicators are rare events. However, it remains unclear if this was also the case for pathogenic species associated with the introduction of fecal material.

Already in 1987, Roszak and Colwell described that they doubted the value in indicating public health hazards in general. Referring to an older publication, they concluded that illness was correlated to previous exposure during bathing, even if the respective water was in compliance with existing microbiological guidelines [314, 351]. Likewise, a recent epidemiological study investigated if there was a connection between bathing in public swimming pools and the contracting of infections [352]. The study concluded, that, although there was generally a higher risk for gastrointestinal illness in bathers compared to non-bathers, frequently there was no direct association of these events with water quality, as was tested using fecal indicator bacteria. According to the study, this was especially then the case, if there was no occurrence of a point-source pollution [352].

In a nutshell, considering the high susceptibility of *E. coli* to chlorine disinfection, the rare occurrence in samples taken from swimming pools, and the fact that, when detecting it, the only conclusion can be, that fecal material has been introduced - something which will always occur, as this material will be washed off the anal region of swimmers - the author considers *Escherichia coli* non-appropriate for an indicator used for the assessment of swimming pool water hygiene. The value of using *E. coli* as an indicator is questionable in two ways: If *E. coli* is present, this does not necessarily mean there is a higher risk for bathers to contract gastrointestinal diseases. If, on the other hand, *E. coli* is not present, this does not necessarily mean there is a lower risk for bathers to contract gastrointestinal diseases. Interestingly, Austria currently removed *Escherichia coli* from the list of indicators used to assess microbial swimming pool water quality [353]. With respect to the considerations made here, this step appears reasonable.

Regarding the second common indicator examined in the present study, *Pseudomonas aeruginosa*, the results indicate both a comparably high sensitivity to chlorine (compare chapter 3) and a quite frequent incidence in public swimming pools (compare chapters 2 and 6). At first glance, this may appear as a discrepancy. However, the phenomenon becomes explainable considering the lifestyle of this opportunistic pathogen. Referring to several studies on the significance of *P. aeruginosa* for swimming pool hygiene, a review by Barna and Kádár (2012) considers this species as the most important opportunistic pathogen forming biofilms [4]. The review also states that, although not a typical member of human skin flora, *Pseudomonas aeruginosa* can still be considered a normal constituent of human skin microflora [4, 44, 45]. While shedding of *P. aeruginosa* may be a primary source for this germ in swimming pool water, the more important factor to be considered is the ability of *Pseudomonas aeruginosa* to form persistent biofilms (e.g. in linings and filters; compare chapter 6). As indicated by the results of the present study regarding the frequency of detection in public swimming pools, the occurrence of “contaminations” apparently is not linked to chlorine concentrations. Presumably, incidences of contaminations with *Pseudomonas aeruginosa* do not always go back to an increased introduction of this germ, but to the shedding of biofilms. Yet, according to the mentioned review, there are several studies which indicate that inadequate disinfection is a main factor [4, 354, 355]. Since *P. aeruginosa* also ranks as a prevalent agent of diseases associated with the use of swimming pools [273, 274], use of this species as a process indicator [19] appears reasonable.

Assessing the prevalence of *P. aeruginosa* in swimming pools and spas in Northern Greece, Tirodimos et al. (2018) [46] found that, while *E. coli* was detected in only 0.6% of the samples, *P. aeruginosa* was detected considerably more frequently (15.2 % of samples). The authors of the mentioned study concluded that *P. aeruginosa* should be included in Greek hygienic

regulations. Yet, just like with the correlation of gastrointestinal illnesses and the incidence of *E. coli*, the connection between the occurrence of *P. aeruginosa* and outbreaks of associated diseases is complicated to verify. As diverse factors have an impact (e.g. contact time, virulence of a given strain, personal conditions of the bathers exposed), there is no linear relationship between occurrence of *P. aeruginosa* and infection in bathers [4, 356]. Nonetheless, with respect to the finding that *P. aeruginosa* may switch to non-cultivable but still viable states during disinfection, the author of the present thesis is of the opinion that the use of this species as a process indicator is beneficial.

Next to the mentioned established indicators, *Staphylococcus aureus* was included in the examinations performed in the present study. Among other things, experiments on inactivation kinetics indicate a comparably higher resistance of this Gram-positive species to chlorine disinfection than determined for *E. coli* and *P. aeruginosa* (compare chapter 3). Furthermore, according to the results obtained by identifying colony-forming bacteria using MALDI-TOF, *Staphylococcus* species are a predominant fraction of the microbial population recovered from swimming pool water (compare chapter 6). In turn, according to several other studies on this topic, the majority of total staphylococci isolated from swimming pool water belong to the species *Staphylococcus aureus* [2, 331, 332]. It has been suggested that high densities of bathers could pose a risk for infections comparable to the risks associated with fecal contamination [329]. This means that the shedding of skin particles and associated introduction of skin flora bacteria could pose a considerable health risk. Presumably, *S. aureus* does not survive at concentrations of 1 mg/L of free chlorine or higher [2, 37, 38, 333]. Yet, as the German standard on swimming pool maintenance [19] envisages concentrations of 0.3 to 0.6 mg/L of free chlorine, presumably conditions in German public swimming pools do not prevent contaminations with *S. aureus*. Consequently, using *Staphylococcus aureus* as an indicator for bacterial contamination due to process failures appears reasonable.

Also, with respect to the results on the incidence of indicator bacteria in public swimming pools (chapter 2), the results obtained in inactivation experiments (chapter 3), and the results on the composition of bacteria communities in swimming pools (chapter 6), the heterotrophic plate count appears a good choice for an indicator of swimming pool water quality. Although, and even particularly because, the hpc is not a specific indicator but provides a general measure of microbial contamination, this indicator is suitable for the assessment of water hygiene.

Furthermore, the results obtained identifying bacteria using MALDI-TOF indicate a complexity of the ecosystem in swimming pools, which clearly demonstrates the lack of comprehensive knowledge on this matter. Apparently, both pathogens and bacteria killing other bacteria may be found in filter systems, regions of swimming pools normally not reached by routine control. Efforts should be made to investigate the communities of cultivable bacteria in swimming pools. In this context, although they were not part of the examinations performed for the present study, also the role of *Legionella* spec. in swimming pool water should be reconsidered. As this species is classified as a primary pathogen and the common way infections are contracted is via aerosols, *Legionella* species presumably are more important pathogens in the context of swimming pool hygiene than most of the species covered by this study.

In summary, the results of the present study lead to the following conclusions: Although there are many aspects considered to be established in swimming pool maintenance, reconsideration appears worthwhile. Apart from the question, if the tools used to secure safety regarding microbial hazards are suitable, the more general questions arise if microbial safety is the most important issue regarding safety and how safe swimming pool water can and should

be in the first place. These questions are not easily answered and need to be discussed by the scientific community.

Accordingly, the author of this study has no conclusive answer. However, the author believes that the present study succeeded in highlighting some of the many factors which need consideration if the role of bacteria in swimming pools is to be assessed.

For example, these include a definition of which bacteria are of importance for swimming pool hygiene, regard of bacteria in non-culturable state, understanding of the kinetics of disinfection in swimming pool basins, and the consumption of chlorine during disinfection. For all these topics, many of the underlying mechanisms are still unspecified and continue to be very interesting and promising for future research. The results illustrate that the current requirements on swimming pool safety regarding microbial contaminations are not supportable, as the tools used to guarantee them are insufficient and lack a beneficial cost-value ratio. Therefore, alternative approaches should be developed replacing the established methods for determining the hygienic state of swimming pools.

Providing these hints, the author concludes the present study, hoping that it will contribute to an informed future discussion on swimming pool hygiene and the adjustment of regulations on disinfection and monitoring and therefore to improvements for public health.

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