Faculty of Chemistry
Biofilm Centre

# Protective biofilms to prevent the colonization of household water systems by harmful organisms, exemplified by the washing machine

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*“Οὐ γὰρ ὡς ἀγγεῖον ὁ νοῦς ἀποπληρώσεως ἀλλ' ὑπεκκαύματος μόνον ὥσπερ ὕλη δεῖται.”*

Πλούταρχος (45 μ.Χ. – 125 μ.Χ.)

# Content

# Abstract

For many consumers, washing clothes in a washing machine is an everyday activity. In addition to removing visible dirt and noticeable odors, removing microorganisms is one of the most important functions of a washing machine. As in recent years, and due to the energy crisis of 2022, the quest for higher energy efficiency and energy savings by using lower washing machine programmes (especially 20 °C and 30 °C) has increased, many microorganisms now survive a wash cycle. In addition to inadequate removal of microorganisms, the consequences are above all textile malodors.

The aim of this study was therefore to conduct a comprehensive analysis of the microbial community in washing machines, taking into account the microbial contamination of used textiles, in order to understand the development of textile malodors. So far, neither the composition of biofilms in washing machines that lead to the formation of or protect against malodor has been intensively investigated, nor have microbial communities on used towels after normal use been analysed. Furthermore, no standardised methods exist to transfer a household biofilm into a laboratory model. This would ensure testing under near-consumer conditions.

The results established a correlation between the qualitative and quantitative analysis of microbial communities in washing machines and on used towels and the occurrence of malodor. The evaluation gives an indication of bacterial colonisers of washing machines that can prevent the formation of malodor. It could be shown that especially soil bacteria such as *Rhizobium*, *Agrobacterium*, *Bosea* and *Microbaterium* are present in non-odorous washing machines and that *Rhizobium* species can prevent odor formation in an *in vitro* model.

Furthermore, a method for transferring a household washing machine biofilm to a laboratory model was established. The results showed that although not identical biofilms can be recultivated, they have a high diversity and a micorbial composition similar to a household biofilm. Furthermore, a statistical correlation between the Euclidean distance of the melting curve analysis and the Bray-Curtis dissimilarity of the metagenome analysis could be established. This enables a faster and more cost-effective analysis of the similarity between two DNA samples.

# Zusammenfassung

Das Waschen von Wäsche in einer Waschmaschine ist für viele Verbraucher und Verbraucherinnen eine alltägliche Tätigkeit. Neben der Entfernung von sichtbarem Schmutz und wahrnehmbaren Gerüchen, ist die Entfernung von Mikroorganismen eine der wichtigsten Funktionen einer Waschmaschine. Durch die Energiekrise 2022 hat das Streben nach höherer Energieeffizeinz und Energieeinsparungen sowie das Verwenden niedriger Waschmaschinenprogramme zugenommen. Aus diesem Grund überleben viele Mikroroganismen einen Waschgang mittlerweile. Konsequenzen sind neben unzureichender Abtötung von Mikroorganismen vor allem textile Schlechtgerüche.

Ziel dieser Studie war es daher eine umfassende Analyse der mikrobiellen Gemeinschaft in Waschmaschinen unter Berücksichtigung der mikrobiellen Kontamination gebrauchter Textilien durchzuführen, um die Entstehung von textilen Fehlgerüchen zu verstehen. Bisher wurden weder die Zusammensetzung der Biofilme in Waschmaschinen, welche für die Bildung von oder zum Schutz vor textilem Schlechtgeruch führen, intensiv untersucht, noch wurden mikrobielle Gemeinschaften auf gebrauchten Handtüchern nach normalem Gebrauch analysiert. Des Weiteren sind keine standardisierten Methoden bekannt, um einen Haushaltsbiofilm in ein Labormodell zu übertragen. Dieses würde eine Prüfung unter verbrauchernahen Bedingungen gewährleisten.

Die Ergebnisse stellten einen Zusammenhang zwischen der qualitativen und quantitativen Analyse mikrobieller Gemeinschaften in Waschmaschinen und auf gebrauchten Handtüchern sowie dem Auftreten von textilem Schlechtgeruch her. Die Auswertung gibt einen Hinweis auf bakterielle Besiedler von Waschmaschinen, welche die Geruchsbildung verhindern können. Es konnte gezeigt warden, dass insbesondere Bodenbakterien wie *Rhizobium*, *Agrobacterium*, *Bosea* und *Microbaterium* in nicht riechenden Waschmaschinen vorkommen und dass *Rhizobium*-Arten in einem *in-vitro*-Modell die Geruchsbildung verhindern können.

Es konnte eine Methode zur Übertragung eines Haushaltswaschmaschinenbiofilms in ein Labormodell etabliert werden. Die Ergebnisse zeigten, dass zwar nicht identische Biofilme rekultiviert werden können, diese aber eine hohe Diversität und eine mikorbielle Zusammensetzung aufweisen, welche einem Haushaltsbiofilm ähnelt. Des Weiteren konnte ein statistischer Zusammenhang zwischen der euklidischen Distanz der Schmelzkurvenanalyse und der Bray-Curtis-Dissimilarität der Metagenomanalyse identifiziert werden. Dieses ermöglicht eine schnellere und kostengünstigere Analyse der Ähnlichkeit zwischen zwei DNA-Proben.

# 1. Background

## 1.1 Biofilms

### 1.1.1 Biofilm formation

The formation of a biofilm was graphically illustrated by David Davies in 2011 (Flemming *et al.*, 2011) and can be categorized into five phases (Figure 1) (A. Garnett & Matthews, 2013; Srey *et al.*, 2013).

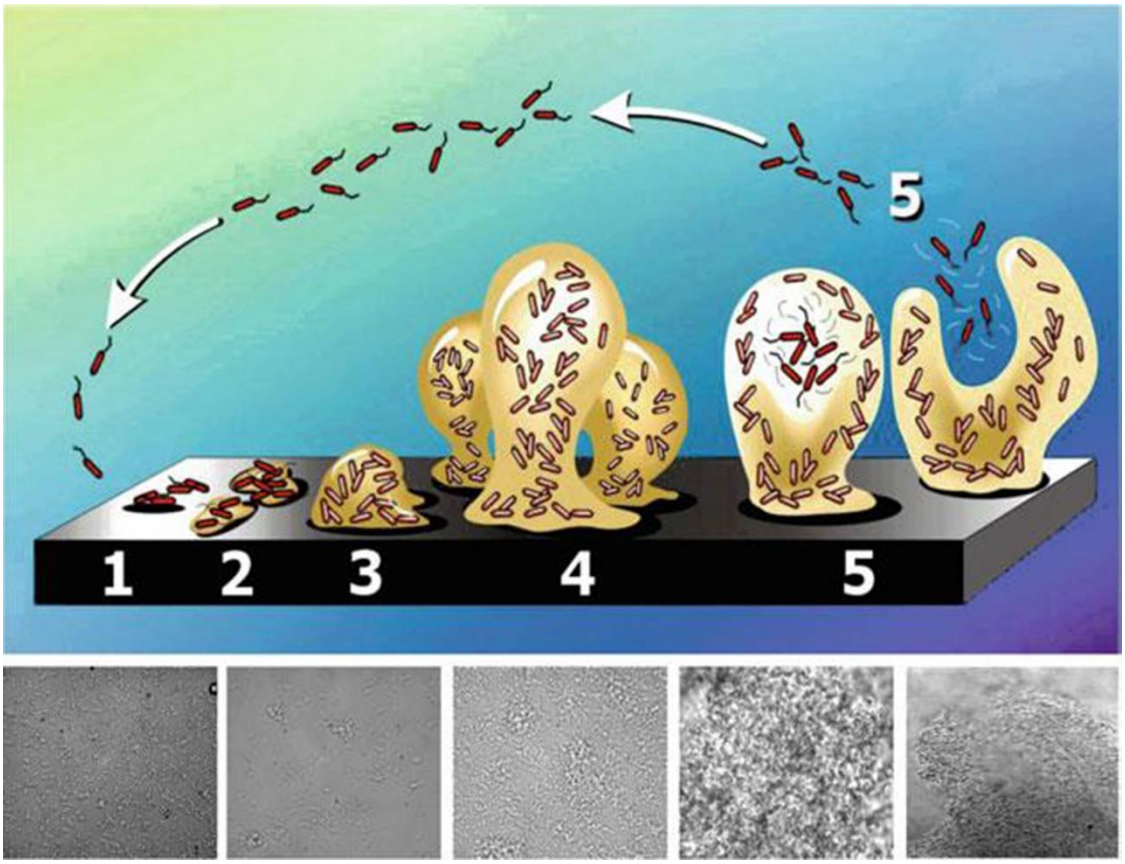



**Figure 1 Five phases of biofilm formation. 1: Attachment of single cells; 2: EPS production, increased adhesion (micro colonies); 3: Formation of biofilm architecture; 4: Further maturation of biofilm, Quorum Sensing; 5: Detachment and spreading of single cells (dispersion) (Davies, 2011)**

Bacteria are generally considered to be planktonic, but reversible attachment to a surface (phase 1) occurs naturally in most bacteria. This adhesion occurs via weak electrostatic or hydrophobic interactions as well as van der Waals forces on a surface that has been coated with polysaccharides or proteins (Ksontini *et al.*, 2013; Phillips, 2016). In 2008, Garrett *et al.* showed that the surrounding medium has a crucial influence on the composition and thickness of the biofilm formed (Garrett *et al.*, 2008)

When the bacteria are firmly attached to the surface, the synthesis of enveloping matrix substances, the extracellular polymeric substances (EPS), which consist of

polysaccharides, proteins, and extracellular DNA (eDNA), begins. This formation results in irreversible attachment to the surface (phase 2) and a change in gene expression pattern and phenotype compared to the first phase (Gomes *et al.*, 2016; Toyofuku *et al.*, 2016).

The bacteria differentiate, form three-dimensional structures (phase 3 and 4) and change their genetic expression patterns to improve their chances of survival. This is the result of a type of communication among bacteria (cell-cell communication) called "quorum sensing". This communication and other physiological and environmental factors initiate the detachment of individual microcolonies, suspended bacteria or biofilm fragments from the biofilm. These can scatter and settle in other locations and form new biofilms (Abdullahi *et al.*, 2016; Flemming *et al.*, 2011; Garrett *et al.*, 2008).

The type and composition of EPS of a mature biofilm is also dependent on environmental factors such as temperature, water availability, or nutrient supply, in addition to the bacteria it contains (Characklis, 1990; Mogha *et al.*, 2014). Previous studies showed that a mature biofilm consists of approximately 85% EPS and approximately 15% cells (Garrett *et al.*, 2008; Phillips, 2016; Toyofuku *et al.*, 2016), and that biofilms occupy niches in the environment (Cappitelli *et al.*, 2014; D. Gutiérrez *et al.*, 2016; Peševska *et al.*, 2016). In addition to implants, catheters, or hulls (Christensen & Characklis, 1990; Cohen *et al.*, 2006; Donlan, 2001; Hall-Stoodley *et al.*, 2004; Kackar *et al.*, 2017; Scotland *et al.*, 2019; Vickery *et al.*, 2004), the niches can also be typical household appliances such as washing machines and dishwashers or even coffee machines and kitchen sponges (Bockmühl, 2017; Brands, Brinkmann, *et al.*, 2016; Brands, Honisch, *et al.*, 2016; Brands & Bockmühl, 2015; Cardinale *et al.*, 2017; Honisch *et al.*, 2015; Raghupathi *et al.*, 2018; Schages *et al.*, 2018).

### 1.1.2 Parameters influencing the formation of biofilms

Various factors are important for the initial attachment as well as the formation of a mature biofilm and the biofilm architecture, which are summarized in Figure 2.

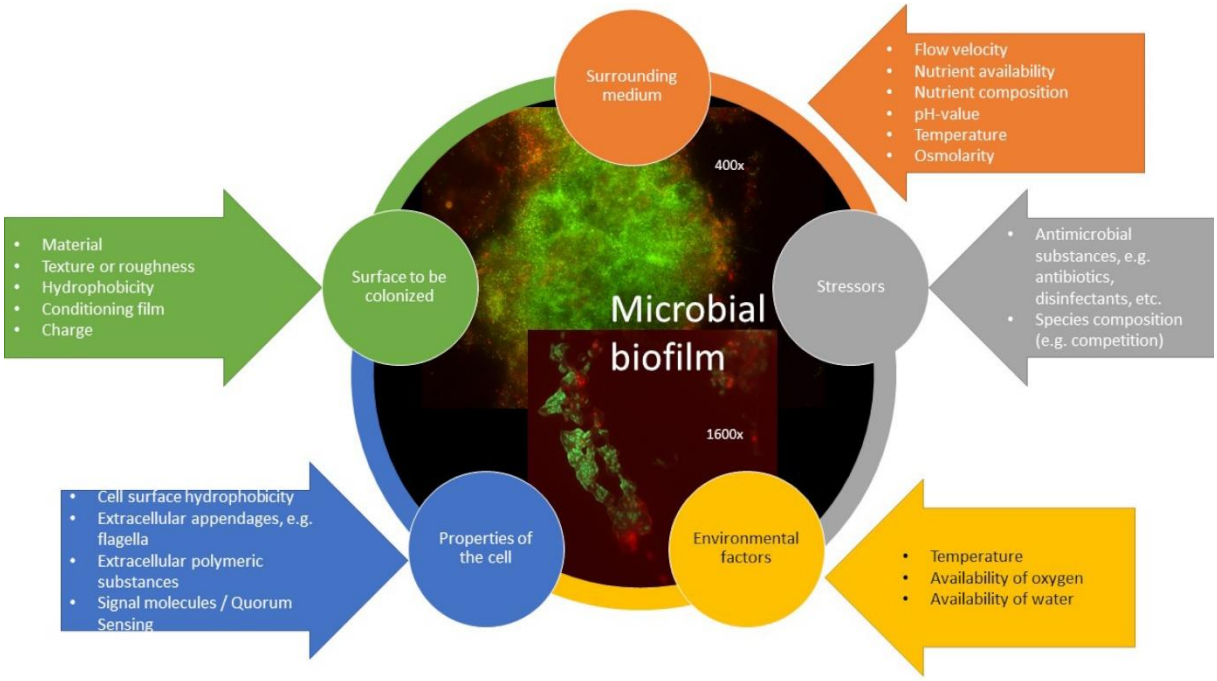



**Figure 2 Factors influencing biofilm formation, architecture and composition. The areas of influence can affect each other in turn (own illustration)**

Most important for the first adhesion of bacteria to surfaces is the physicochemical nature of either the surface to be colonized or the bacterium (Marchand *et al.*, 2012). Hence, the conditioning film can influence the surface charge and surface tension (Garrett *et al.*, 2008). Furthermore, the composition of the medium (NaCl content, nutrient content), pH, oxygen availability, and temperature have a decisive role (Gutiérrez *et al.*, 2016; Marchand *et al.*, 2012; Phillips, 2016). The biofilm structure and density are influenced by the species composition, the environmental temperature and the flow rate.

Generally, hydrophobic surfaces are easier to colonize by microbial cells due to reduced electrostatic repulsion (Abdallah, Benoliel, *et al.*, 2014; Abdallah, Chataigne, *et al.*, 2014). Nevertheless, laminar as well as turbulent flow can promote attachment by increasing the possibility of contact between the bacteria and the surface (Marchand *et al.*, 2012). Notwithstanding, an excessive flow rate (above 2 m/s) can lead to detachment of cells from the biofilm resulting from shear forces (Stoodley *et al.*, 2002; Teh *et al.*, 2014). Bacterial mobilization by flagella can also induce initial contact between bacterial cell and surface (Garrett *et al.*, 2008). Microbial cell contact can be further enhanced by hydrophobic surface structures on microbial cells, such as pili, fimbriae, adhesive proteins, and/or lipopolysaccharides. The hydrophobicity of stainless steel must be considered in

comparison to other materials. For example, stainless steel is more hydrophobic than very hydrophilic glass, but significantly less hydrophobic than polytetrafluoroethylene (PTFE, Teflon) (Phillips, 2016). Moreover, hydrophobicity of stainless steel may vary depending on the age of the material (Kim *et al.*, 2016).

The surface roughness of materials might have an impact on the colonization potential of bacteria as well. In literature, there is a controversial discussion of the assumption that rough surfaces increase the formation of biofilms (Gupta & Anand, 2018; Marchand *et al.*, 2012; Teh *et al.*, 2014). However, the majority agree that a higher surface roughness of the material can reduce the cleaning and disinfection efficiency and thus contribute to increased biofilm formation (Arena *et al.*, 2017; Corcoran *et al.*, 2014; Sadiq *et al.*, 2017).

Increased biofilm formation has been described in some studies in environments with limited nutrients or other circumstances that are unfavorable for the cells (Arena *et al.*, 2017; Van Houdt & Michiels, 2010; Ksontini *et al.*, 2013; Teh *et al.*, 2014). Generally, this is based on the initiation of a stress response and correspondingly modified gene expression.

### 1.1.3 Cooperation and interaction of various biofilm environments

Multi-species biofilms create an optimal environment for the exchange of genetic material, metabolites, and cell-cell communication, which leads to the development of organized structures that can be categorized as cooperative, neutral, or competitive (Flemming *et al.*, 2016; Giaouris *et al.*, 2015). In collaborative environments, primary colonizers facilitate biofilm formation and provide a surface for the attachment of other species with less biofilm-forming potential (Cherif-Antar *et al.*, 2016). Secondary colonizers within the biofilm can produce nutrients or enzymes that benefit all colonizers and degrade toxic metabolites that could limit biofilm proliferation (Cappitelli *et al.*, 2014). This can create regions of reduced oxygen partial pressure and anaerobic regions that can be used by microaerophilic or obligate anaerobic organisms (Giaouris *et al.*, 2015). Competitive interactions occur when microorganisms compete for nutrients, oxygen, and space, which can lead to the production of metabolites that limit or completely inhibit the adherence and reproduction of other microorganisms (Giaouris *et al.*, 2015). Communication between biofilm cells occurs through quorum sensing, which is regulated by the production of soluble autoinducers (Hawver *et al.*, 2016). Increased cell density and retention capacity of autoinducers in the EPS layer cause them to accumulate until they reach a threshold concentration, triggering changes in gene expression, such as increased expression of virulence factors, extracellular enzymes, or EPS production, activation of horizontal gene transfer mechanisms, or dispersion of biofilm cells (Eberl & Riedel, 2011; Giaouris *et al.*, 2015; Phillips, 2016). Quorum sensing occurs through secreted proteins or processes available to the entire biofilm community and is specific to intra-species communication signals (Abisado *et al.*, 2018), although there are some less

specific inter-species quorum sensing systems, such as the autoinducer 2 molecules, encoded by the luxS gene found in both Gram-positive and Gram-negative bacteria (Hawver *et al.*, 2016; Whiteley *et al.*, 2017; Xavier & Bassler, 2003).

## 1.2 Biofilms in the washing machine

### 1.2.1 Washing machine

Nowadays, the washing machine is considered as a main element of hygiene in private households (Jacksch, Kaiser, *et al.*, 2020). The first mention of a simple machine, which, among many other applications, was also to be used for washing textiles, was in 1691 when a patent was granted to John Tizack (Eyre & Spottiswoode, 1871). As early as 1752, a magazine described a wooden tub that could be filled with soapy water and used by hand in smaller households (Cave, 1752). Washing machines, however, were found more often in large laundries and public institutions than in private households until the early 19th century (Orland, 1991). The first automatic household washing machine was introduced and patented in 1937 by Bendix Home Appliances (Chamberlin & Bassett Jr., 1937). The first fully automatic washing machine was launched in 1946 and saved a tremendous amount of time by eliminating the need for human intervention between process steps (König, 2000).

Meanwhile, 94% of households in Western Europe have a washing machine. Nevertheless, there are still some regions, such as China, Turkey or Eastern Europe, where only below 70% of households own a washing machine (Pakula & Stamminger, 2010). Focusing on Western Europe, this results in approximately 170 washing cycles per household per year, accounting for 3.8% of total residential energy consumption in Western Europe (Pakula & Stamminger, 2010).

The focus in washing machine development today is primarily on reducing energy consumption. Some studies and initiatives, such as the International Association for Soaps, Detergents and Maintenance Products (A.I.S.E.), look at consumer behavior with regard to the energy efficiency of a washing machine and make recommendations for low washing temperatures (A.I.S.E., 2014). Between 1972 and 2014, the average washing temperature in private households decreased from 63 °C to 46 °C (Anonymous, 2017; Bockmühl, 2017).

### 1.2.2 Contamination of the washing machine by microorganisms

Contamination of clothing and household appliances with microorganisms frequently occurs in daily life. Various routes of contamination are conceivable. In addition to its introduction by humans, contamination by the environment and by the washing machine itself is possible (Bockmühl, 2017; Lucassen *et al.*, 2014).

Contamination via humans can have different origins. On the skin alone, there are up to $10^6$ cfu/cm² of bacteria (Heeg & Christiansen, 1993). This so-called skin microbiota is divided into residual and transient flora. While the resident flora contains microorganisms that are permanently found on the skin (e.g. *Propionibacterium*, *Staphylococcus*, *Micrococcus*, *Corynebacterium* and *Acinetobacter*), the transient flora contains microorganisms that are only temporarily brought there, e.g. through the environment (such as *Bacillus* or *Pseudomonas aeruginosa (P. aeruginosa*) (Bojar & Holland, 2002). Other sources of contamination are the gastrointestinal tract (Scott, 2013), the genital tract (Costerton *et al.*, 1987) or the oral flora (Blaustein *et al.*, 2021; M. K. Zinn *et al.*, 2020).

Therefore, while the risk of microbial infection from textiles contaminated with microorganisms due to direct skin contact is low, contamination can also originate from bodily excretions (Bockmühl, 2011). Fabrics such as underwear or shirts that are contaminated with bacteria from the axillary areas potentially may be more contaminated with microorganisms and thus may pose a potential risk of infection if not reduced to noninfectious levels (Bockmühl *et al.*, 2019). Furthermore, these microorganisms could represent a greater source of infection for individuals at higher risk of infection, who include the young, the old, pregnant women, and immunocompromised people ("YOPIs") (Bockmühl, 2017). If pathogens such as *Candida* or *Staphylococcus aureus* (*S. aureus*) are present on clothing and textiles that are in direct contact with the skin, they are a significant health risk (Bloomfield *et al.*, 2011; Bloomfield & Scott, 2013; Bockmühl, 2017). If such an infection occurs in private households, it´s important to reduce the pathogen load to a non-infectious level by disinfecting the laundry (Bockmühl, 2011).

In household environments, infected family members, pets, and raw foods are the main sources of infection (Bloomfield, 2013). Pathogens potentially spread through clothing and other textiles include *Salmonella*, *Shigella*, *Campylobacter*, *Escherichia coli*, *Clostridium difficile*, *Vibrio cholera*, viruses, and yeasts such as *Candida albicans (C. albicans)* (Bloomfield *et al.*, 2011; Bloomfield & Scott, 2013; Smith *et al.*, 2012). Studies found bacterial counts of $10^2$-$10^4$ cfu/g (colony forming units per gram) on a woman's blouse, $10^4$ – $10^6$ cfu/g on a man's shirt, $10^5$ – $10^7$ cfu/g on underwear, $10^8$ cfu/g on dish towels, and $10^9$ cfu/g on socks (Stache & Großmann, 1992). Additionally, microbial contamination of approximately $10^3$ cfu/cm was detected on bed sheets and pillowcases after one week of use in the household (Walter & Schillinger, 1975).

Notwithstanding the studies described the available data to date are not sufficient to predict the real microbial load and type of microorganisms in the domestic environment (Honisch, 2017). The occurrence of infections and their impact on the microbial load of textiles and the washing machine in domestic areas is also not yet known. Nevertheless, it has been found that pathogenic bacteria such as *S. aureus* and *Enterococcus faecium*, as well as most

other microorganisms, can survive on textiles such as cotton, polycotton, or polyester (Neely & Maley, 2000; Wiksell *et al.*, 1973), but the period of adherence differs according to the microorganism and influencing factors including temperature and chemistry.

The analysis of domestic laundering procedures should take into account a significant number of microorganisms, particularly pathogens, due to their importance. Important factors for laundry hygiene are microorganism transmission via textiles (e. g. *S. aureus* or *Escherichia coli*), as well as their resistance to the washing process (e.g. *C. albicans*) or the possibility of their presence in the domestic environment, such as *Staphylococcus hominis (S. hominis)*, *Corynebacterium jeikeium (C. jeikeium)* or *Moraxella osloensis (M. osloensis)* (Amichai *et al.*, 2013; Bockmühl, 2011; Bockmühl *et al.*, 2019; Cardinale *et al.*, 2017; Hammer *et al.*, 2011; Honisch, 2017; Honisch, Brands, *et al.*, 2016; Kloos & Schleifer, 1975; Ossowski & Duchmann, 1997; Rintala *et al.*, 2008).

Personal contact with contaminated textiles can lead to transmission of these microorganisms to human skin, and *vice versa* (Gerhardts *et al.*, 2015; Sattar *et al.*, 2001). Therefore, wearing contaminated textiles can potentially cause reinfection or transmission of infections between members of the same household. Microorganisms can be transferred from textile to textile, from washing machine to textile, and reverse during the washing process or during storage as well (Callewaert *et al.*, 2015; Hammer *et al.*, 2011; Moriello, 2016; Sattar *et al.*, 2001).

No cases have been described where infections have been transmitted via laundry in private households. Nevertheless, a previous study by Schmithausen *et al.* investigated the route of transmission in a hospital from a household washing machine to a hospital premature infant department. In this location, preterm infants were colonized with an extended spectrum beta-lactamase-producing strain of *Klebsiella oxytoca* that was also identified in a household washing machine for babies' clothing (Schmithausen *et al.*, 2019).

### 1.2.3 Components influencing the antimicrobial activity

In the past, as today, laundry is washed to remove dust and dirt from clothes, as well as to eliminate bad or unpleasant odors (malodor). In addition to this sensory reduction, clothes should not be a source of infection after washing. For this reason, many washing machine manufacturers aim to reduce bacterial counts on clothing as well as minimize transfer from a contaminated textile to an uncontaminated textile ("cross-contamination"). It was determined that, rather than being inactivated by temperature or surfactants, the reduction of microbial cells on a textile surface following laundry at 20 °C – 40 °C must be seen as a physical removal impact. These actions at higher temperatures or when bleach is used are likely to render microbial cells inactivate as well (Bloomfield *et al.*, 2013; Bockmühl, 2017).

As early as 1960, Sinner described the factors that influence cleaning performance (Sinner, 1960). These include chemistry, mechanics, temperature and time. Likewise, Klapper *et al.* 2018 showed that sinners principle can be applied to the hygiene performance of household cleaning devices (e.g. commercial dishwashers) (Klapper *et al.*, 2018).

#### 1.2.3.1 Chemistry

The great diversity in the composition of detergents makes it difficult to estimate the effects of a particular compound or to compare existing published studies. Two categories of components define the antimicrobial efficacy of detergents: surfactants and bleaching agents (Bockmühl, 2017). The main function of surfactants in the washing process is to remove hydrophobic soils (Bockmühl, 2017).

Available data are not sufficient to assess the role of surfactants in the antimicrobial efficacy of washing, but suspension experiments showed insufficient reduction of the microbial load in active oxygen bleach (AOB) free detergents (Brands, Brinkmann, *et al.*, 2016). The main function of AOB in laundry is to remove stains by oxidizing chromophore groups in the stain molecules, resulting in stain discolouration (Milne, 1998). AOB products oxidize other organic compounds as well, such as odorants, and react with microorganisms (Betz, 2001). AOB is the most important antimicrobial component in laundry detergents (Bockmühl, 2017). Some other common bleaching agents used in modern laundry are chlorine bleach and quaternary ammonium compounds (QACs).

Nevertheless, the range of bleach types and bleaching systems is much wider. In countries that traditionally use cold water for washing, such as Japan, Southern Europe, and South America, chlorine bleach is widely used in the laundry because of its high activity at 20 °C. In Europe, oxygen-based bleaching systems have become established (Wagner, 2017).

Surfactants adhere to surfaces, with the hydrophobic and hydrophilic parts of the molecule aligned to maximize the molecular forces of attraction. They have different properties based on their interfacial activity, which are modified by setting the hydrophobic and hydrophilic parts (Bajpai & Tyagi, 2007): Decreasing the level of surface tension of water, foaming, soil removal and dispersion, formation of micelles in an aqueous solution, and soil carrying capacity. The soil release capacity is described as the primary washing capacity, and the soil dispersing and carrying capacity as the secondary washing capacity (Wagner, 2017). The hydrophobic part is similar in most surfactants and is composed of a long hydrocarbon group, while the linear alkyl benzene sulfonate (LAS) also has phenolic compounds. The hydrophilic part varies among surfactants and is categorized into four groups of anionic, cationic, nonionic, and amphoteric surfactants (Wagner, 2017). Anionic surfactants have a negatively charged group, such as a carboxylate, sulfonate, or sulfate anion (Wagner, 2017). They are the most commonly used surfactants in all detergents, due, *inter alia*, to their low

production costs (Bajpai & Tyagi, 2007). Alkylbenzene sulfonates represent a major group of anionic surfactants. In addition to low production costs, other attributes such as cleaning effect, solubility in cold water or foaming at all temperatures determine the choice of surfactant (Wagner, 2017). Another important consideration in the choice of a surfactant ingredient is biodegradability. In Germany, there has been a law regulating the placing of detergents and cleaning agents on the market since 1975. In addition to the prohibition of certain ingredients, manufacturers must provide information on the substances used and the water hardness range (Altmaier, 2013).

Cationic surfactants are derived from quaternary ammonium salts, wherein a hydrophobic portion is linked to a hydrophilic, positively charged nitrogenous group. The combination of cationic and anionic surfactants is ineffective in the washing process due to their lack of adsorption on the surface. Cationic surfactants have a positive charge, while anionic surfactants are negative. When these two types of surfactants are combined, their charges neutralize and a balanced state is created. This means that the surfactants can no longer adsorb to the surface of dirt particles and fabrics in the washing machine, which implies that they can no longer effectively remove dirt and grease. Neutral salts are formed by the reaction of anionic and cationic surfactants (Wagner, 2017). Therefore, cationic surfactants are used in fabric softeners. QACs such as benzalkonium chloride (BAC), dimethyldidecylammonium chloride (DDAC) or esterquats are usually not applied to detergents but to rinse aids or fabric softeners and are subsequently added to the wash cycle after the main wash. The antimicrobial activity of BAC and DDAC differs according to the type of microorganisms and the product formulation. They are very effective against Gram-positive bacteria when used at low concentrations, whereas higher concentrations are required to inactivate Gram-negative bacteria or fungi (Basiry *et al.*, 2022; Chojecka *et al.*, 2019; Fredell, 1994; Ioannou *et al.*, 2007; Loiko *et al.*, 2022; Pereira & Tagkopoulos, 2019; Yoshimatsu & Hiyama, 2007).

Additionally, the pH of wash water plays a critical role in the effectiveness of surfactants, which are the active ingredients in laundry detergents responsible for cleaning. Surfactants have hydrophobic and hydrophilic ends that attach to dirt and oil and carry them away in water. However, the effectiveness of surfactants is dependent on the pH of the wash water. In acidic conditions, the hydrophilic end of the surfactant can become protonated, reducing its ability to attract water and carry away dirt. In basic conditions, the hydrophobic end of the surfactant can become ionized, reducing its ability to attach to dirt and oil. Therefore, it is crucial to maintain the pH of the wash water within a specific range to ensure optimal cleaning. A study published in the Journal of Surfactants and Detergents reported that the optimal pH range for laundry detergents is between 9 and 10.5 (Bajpai & Tyagi, 2007; Sajitz & Grohmann, 2011).

The pH of wash water also affects the condition of the items being washed. For instance, acidic wash water can damage delicate fabrics like silk and wool, while alkaline wash water can damage cotton and linen. Therefore, it is important to choose detergents with the appropriate pH for the type of fabric being washed (Abbott, 2015).

#### 1.2.3.2 Temperature and time

Temperature is a critical factor affecting the effectiveness of domestic laundering processes. It affects the LR of microorganisms on wash fabrics, the mechanical removal of cells and the activation of detergents containing AOB. Studies have shown that increasing the wash temperature results in a significant reduction of microorganisms during washing, with an increase in LR of 2.5 orders of magnitude when the temperature is increased from 24 °C to 68 °C (Wiksell *et al.*, 1973). Many other studies have also shown a significant effect of temperature on microbial reduction (Arild *et al.*, 2003; Bellante *et al.*, 2011; Honisch, 2017; Honisch *et al.*, 2014). High washing temperatures of at least 60 °C have been found to ensure high efficacy of household washing processes (Bellante *et al.*, 2011; Bloomfield & Scott, 2013; Bockmühl, 2017; Fijan *et al.*, 2007; Hammer *et al.*, 2011; Honisch, 2017; Honisch *et al.*, 2014; Lichtenberg *et al.*, 2006; Linke *et al.*, 2011; Lucassen *et al.*, 2013; Ossowski *et al.*, 1999; Walter & Schillinger, 1975; Wiksell *et al.*, 1973).

Lowering the temperature of the washing process leads to a significant reduction in its effectiveness, with the greatest loss of effectiveness observed when the temperature is lowered from 60 °C to 40 °C (Bloomfield *et al.*, 2013). However, decreasing temperatures can be compensated by increasing the duration of the wash cycle according to the Sinner's circle theory (Bockmühl *et al.*, 2019; Boonstra *et al.*, 2020; Müller-Kirschbaum *et al.*, 2020; Sinner, 1960). By extending the duration of the washing process, the LR may increase due to the prolonged effect of the elevated temperature or combination of parameters on the microorganisms. Thus, a shorter duration of the washing process can be compensated by increasing the temperature, and hygienic efficacy and textile cleanliness can be ensured at lower temperatures by extending the duration of the washing process (Janczak *et al.*, 2010).

#### 1.2.3.3 Mechanics

Mechanics in the washing machine drum is another factor that is known to affect washing performance and can be anticipated to affect the antimicrobial efficacy of washing as well (Moriello, 2016). Western Europe primarily employs drum washing machines with a horizontal axis (Wagner, 2017), in which the detergent solution only partially covers the laundry. By rotating the drum and the ribs in the drum, the laundry is lifted out of the liquor and then falls back in.

Moreover, the intensity of the mechanical action in the washing process is limited by the fact that the textiles should not be damaged, so the mechanical impact during washing is probably not lethal to the microbial cells (Bockmühl, 2017; Hasan *et al.*, 2022). Nevertheless, this factor significantly affects the reduction of microbial load on textiles (Betz, 2001; Block *et al.*, 2001; Wiksell *et al.*, 1973). Most likely, this factor relates to the physical detachment of the microbial cells. This detachment is dependent on the other factors of Sinner´s circle. The dilution of the microbial load, which is determined by the amount of used water, could also be a factor in connection with this detachment (Bloomfield *et al.*, 2013).

A study by Ossowski *et al.* focused on the effect of mechanical action during the washing process upon the survival of different *Candida* species on textiles (Ossowski *et al.*, 1999). There was a significant difference between the treatment of contaminated textiles in a water bath and washing in a washing machine at 60 °C. The exact magnitude of this effect could not be estimated because the microbial contaminants were evaluated semi quantitatively. Furthermore, the construction of the washing machine could strongly influence the mechanical removal of the microbial cells.

## 1.3 Laundry-associated odor

A laundry-associated bad odor (malodor) problem has increased for many consumers in recent years, especially as washing temperatures have steadily decreased due to energy savings (Bockmühl, 2017; Bockmühl *et al.*, 2019; Callewaert *et al.*, 2014; Munk *et al.*, 2001). Several types of odor are associated with laundry odor (Van Herreweghen *et al.*, 2020), whereas this study focuses on the "wet-and-dirty-dustcloth-like" or "wet fabric" malodor (Kubota *et al.*, 2012; Nagoh *et al.*, 2005; Takeuchi *et al.*, 2012). This particular type of malodor occurs on laundry when it´s hung to dry indoors, is already dried, or is stored under humid conditions (Kubota *et al.*, 2012; Munk *et al.*, 2001; Nagoh *et al.*, 2005).

Many factors influence the formation of malodor. Being a microbial product, malodor depends on the temperature, nutrients present, humidity and time, as well as the microorganisms present. In addition to human skin (resident and transient skin flora) and clothing, the washing machine has been identified as a source of bacteria transmitted by water (Van Herreweghen *et al.*, 2020). Previous studies showed that bacterial counts on textiles are as high as $10^6$ cfu/cm², of these, the most abundant genera are *Staphylococcus*, *Corynebacterium*, and the *Betaproteobacteria* group (Costello *et al.*, 2009; Cundell, 2018; Grice & Segre, 2011).

Hence, the development of body odor has to be considered as one of the factors that influence the formation of laundry odor as well (Troccaz *et al.*, 2015). A study (Troccaz *et al.*, 2015) reported that the most important species contributing to the formation of body odor are *Corynebacterium tuberculostearicum*, *S. hominis*, and *Anaerococcus spp.*

The typical volatile compounds associated with sweat odor, 3-methyl-2-hexenoic acid (3M2H) and 3-hydroxy-3-methylhexanoic acids, have been found to beproduced by corynebacteria in a reaction with the enzyme N-acylglutamine aminoacylase.

Despite the fact that the formation of body odors takes place directly on the skin, textiles may have a significant role in the formation and storage of odorants. Various volatile compounds such as short-chain fatty acids and branched-chain fatty acids appear on unwashed textiles in a distribution that is dependent on the textile (Chung & Seok, 2012; Teufel *et al.*, 2010). Nevertheless, it´s also possible to detect various odorants after washing. Next to fatty acids and 3-methylbutanoic acid (Munk *et al.*, 2001), steroids (e.g. 5-a-androst-2-en-17-one) (Munk *et al.*, 2001), ketones (e.g. 1-octen-3-one) (Munk *et al.*, 2001), esters (e.g. ethyl-2-methylpropanoate) (Munk *et al.*, 2001), aldehydes (e.g. hexanal) (Chung & Seok, 2012) and alcohols (e.g. oct-1-en-3-ol) (Hammond, 2013) have also been found. In addition to these compounds, sulfur compounds such as dimethyl disulfides (DMDS) and dimethyl trisulfides (DMTS) were also detected on odorous textiles (Denawaka *et al.*, 2016; Stapleton & Dean, 2013). Another substance associated with malodor is 4-methyl-3-hexenoic acid (Takeuchi *et al.*, 2012). This compound is a carboxylic acid or short-chain saturated fatty acid. As 4-methyl-3-hexenoic acid (4M3H) has a low molecular weight, it is volatile at room temperature (O´Brian, 2004). The bacterium *M. osloensis* has been identified as the producer of this substance (Kubota *et al.*, 2012). Particularly in Japan, a correlation between the presence of *M. osloensis* on textiles and the occurrence of malodors in the form of 4M3H has been demonstrated (Kubota *et al.*, 2012). The capacity of *M. osloensis* to resist scarring, as well as its metabolic route for producing 4M3H, are thought to be critical elements for survival and odor generation in washing machines and on washed clothes (Kubota *et al.*, 2012). Additionally, *M. osloensis* was discovered as an abundant coloniser of washing machines in a recent research in German households (Jacksch, Thota, *et al.*, 2020). The antecedents for the conversion to 4M3H are unknown and are being researched further.

There were also attempts to analyze microorganisms on odorous textiles in some studies and predominantly *Staphylococcus sp.* and *Micrococcus sp.* and in smaller numbers *Bacillus sp.*, *Enterobacteriaceae* and *Acinetobacter sp.* were identified (Callewaert *et al.*, 2014; Mcqueen *et al.*, 2007; Teufel *et al.*, 2010).

## 1.4 Investigation of biofilms

### 1.4.1 Microscopic methods

Fluorescence microscopy is a valuable tool for investigating the composition of biofilms and other materials. One example of a useful fluorescent dye is 4',6-diamidino-2-phenylindole (DAPI), which selectively binds to regions rich in adenine-thymine DNA (Flemming *et al.*, 2016; Guilhen *et al.*, 2019; Hansen *et al.*, 2019; Moritz *et al.*, 2010; Wilson *et al.*, 2017). DAPI can also bind to RNA, although it is not as bright as with DNA (Kapuscinski, 1990; Omelon *et al.*, 2016). When DAPI is bound to double-stranded DNA, it absorbs ultraviolet light at a rate of 358 nm and emits blue light at a rate of 461 nm. Consequently, in fluorescence microscopy, DAPI is excited by ultraviolet light and detected by blue-and-yellow filters. The emission peak is wide.

Another method for assessing biofilm formation is the live/dead staining assay (Oliveira *et al.*, 2015; Reichhardt & Parsek, 2019; Welch *et al.*, 2012). This fluorescent test is based on membrane integrity and bacterial survival. SYTO® 9 is a green fluorescent nucleic acid dye that labels intact cell membrane bacteria, while propidium iodide is a red fluorescent nucleic acid dye that only penetrates damaged membrane bacteria (Mountcastle *et al.*, 2021).

Previous internal investigations conducted at Rhine-Waal University of Applied Sciences (data not published) have demonstrated the difficulty of analyzing worn textiles using fluorescent dye-based methods due to the intrinsic fluorescence of the textiles themselves, which is caused by the presence of fluorescent whitening agents (FWAs) or optical brighteners (OBAs) (Salas *et al.*, 2019; Smulders & Sung, 2011). These compounds are often included in detergents to enhance the brightness and whiteness of clothes by absorbing ultraviolet light and emitting it as blue light, resulting in the clothes appearing brighter and whiter (Salas *et al.*, 2019; Smulders & Sung, 2011). However, FWAs can accumulate on textiles over time, particularly if they are not rinsed properly during the washing process. These accumulations can cause the fabric to appear to glow under particular lighting conditions, as the FWAs can still absorb and re-emit light following the washing process.

Furthermore, organic substances such as dirt can also contribute to the fluorescence of textiles. This is because organic fabrics may contain fluorescent compounds such as chlorophyll, which can absorb and re-emit light in a similar way to FWAs.

For example, DAPI is a fluorescent dye that specifically binds to DNA and is commonly utilized in microscopy to visualize DNA in cells and tissues. However, the fluorescence from optical brighteners and dirt residues may overlap with the wavelengths of light used to detect DAPI fluorescence, resulting in false positives or complicating the interpretation of results.

In addition to distinguishing living and dead cells, there are also viable but non-cultural bacteria (VBNCs) with reduced metabolic activity (Kirschner *et al.*, 2021; Oliver, 2005).

These bacteria are not dividing, but they have the ability to grow after resuscitation. VBNCs can enter into this state due to harsh nutrient, temperature, or acid conditions. The cells are morphologically small and have reduced nutrient transport rates and respiration (Ayrapetyan *et al.*, 2018; Dietersdorfer *et al.*, 2018; Kirschner *et al.*, 2021; Schrammel *et al.*, 2018).

### 1.4.2 Cultural test methods

For the detection of biofilm colonization via cultivation-based methods, the essential prerequisite is their ability to grow on the nutrient media used under the selected incubation conditions. Biofilms have nutrient, pH, oxygen, and perhaps inhibitor/antibiotic gradients (Flemming *et al.*, 2016; Hall & Mah, 2017; Olsen, 2015), which allow a wide diversity of organisms to grow in the corresponding niches. In the laboratory, these can never be mapped simultaneously. Furthermore, the metabolic rates of microorganisms are very low, especially in the lower layers of the biofilm, which can lead to the underestimation of the real microbial number and species diversity caused by the lack of detection of viable but nonculturable (VBNC) organisms (Flemming *et al.*, 2016; De la Fuente-Núñez *et al.*, 2013). An advantage of cultural methods is the ability to isolate and characterize isolates for microbial spoilage potential, temperature range of growth, to identify specific species and biofilm formation capacity under various conditions.

### 1.4.3 Molecular biological test methods

The independence of cultivation steps is a benefit of molecular biological methods. In the case of direct DNA extraction from the sample, there is theoretically no possibility of the selected incubation conditions discriminating against individual bacterial groups, and VBNC cells can also be detected. However, a critical point is cell disruption, which proceeds more efficiently in most cases for Gram-negative bacteria than for Gram-positive bacteria with more robust cell walls, so that an overrepresentation of Gram-negative organisms may occur in molecular biology procedures (Weber *et al.*, 2014).

Another challenge with direct methods is the inability to distinguish between DNA from proliferating cells and DNA from lysed cells, as well as eDNA in the biofilm matrix, which is also largely composed of lysed cells (Aldecoa *et al.*, 2017; Flemming *et al.*, 2022). This can be circumvented with live-dead discrimination by treating the sample with propidium monoazide (PMA) using a dye before cell disruption. This dye can selectively pass through only damaged cell membranes and modify DNA inside the cell. The PMA-modified DNA molecules are discriminated during DNA extraction by reduced water solubility, or they can no longer be amplified by PCR (Arena *et al.*, 2017; Banihashemi *et al.*, 2012; Chen *et al.*, 2011; Soejima *et al.*, 2008).

If PCR amplification of the gene sequences to be analyzed is necessary after DNA extraction, its success represents another critical point. Especially in DNA extracts from mixed cultures of species with different contents of the bases guanine (G) and cytosine (C) (GC content), DNA sections with low GC content might be preferentially amplified due to the higher melting temperature of GC-rich sequences (Khandelwal & Bhyravabhotla, 2010; Liu *et al.*, 2007).

## 1.5 Next-Generation-Sequencing (NGS)

Next Generation Sequencing has become increasingly common for the technique of automated simultaneous sequencing of large amounts of nucleic acid. This method has meanwhile been very successful in researching the genome. Different procedures have been developed in the meantime. Most of these new methods have in common that the DNA to be sequenced is prepared, usually fragmented and then attached to a solid matrix. This is followed by a base-bound matrix amplification with a special polymerase chain reaction. In this so-called "bridge PCR", a "bridge" is created by simultaneous matrix attachment of the 3' and 5' ends of the amplified nucleic acid, which is then denatured after synthesis of the complementary strand in such a way that one DNA strand remains attached to the 3' end and one to the 5' end (Khan *et al.*, 2008). Sequencing technology provides qualitative information by reading the nucleotide sequence of DNA or RNA molecules. Although the number of reads for a particular sequence can be determined, this metric does not directly reflect the abundance or quantity of the original molecule in the sample due to technical limitations and biases. As a result, sequencing is more suitable for qualitative or semi-quantitative analyses rather than precise quantification of nucleic acid molecules (Mardis, 2017; Min *et al.*, 2020).

Hence, three-dimensional clusters of identical DNA segments are created, and each of them is then attached to the matrix as a forward and backward strand in a small spatial extent. The DNA fragments amplified in this way can be analyzed in different ways. Methods from Illumina (Illumina; San Diego, CA, USA), which are currently highly regarded among sequencing methods, use fluorescent dyes bound to different nucleotides, for example. Additionally, these are provided with an inhibitor molecule so for each cycle only one nucleotide binds (Hagar *et al.*, 2022; Wang *et al.*, 2017).

Each time labelled nucleotides are added (binding process), their fluorescence is detected by a camera and the inhibitor molecule at the 3' end of the DNA is subsequently separated to allow the next nucleotide to bind. Each pixel produces a specific sequence of fluorescence signals that is used to reconstruct the base sequence of the attached DNA fragment. As many fragments are sequenced simultaneously on the same matrix, a data set of partially overlapping base sequences is generated, which are reconnected according to automated algorithms based on their overlap. Ideally, there are several copies of each section

of the genome to be sequenced, so the procedure offers a high degree of certainty in the reconstruction of the overall sequence through the high redundancy of the sequencing result for each section. The number of identical sequences of covered bases is described as "coverage" (Danko *et al.*, 2021; Sims *et al.*, 2014), a sequence of nucleotide signals across a pixel as "read" (Chaisson *et al.*, 2009). As well as analyzing an entire genome in a short time with high cost efficiency, known as Whole Genome Sequencing (WGS) (Caspar *et al.*, 2021; Schwarze *et al.*, 2018), targeted methods can also be used to analyze the exome (Whole Exome Sequencing, WES) (Schwarze *et al.*, 2018; Weissenkampen *et al.*, 2019) or transcriptome at the RNA level of a sample (Cardoso-Silva *et al.*, 2014; Kukurba & Montgomery, 2015).

# 2. Objectives

Washing is one of the most important activities in the domestic environment today. Due to the increasing importance of saving energy, the use of low temperature wash programmes has increased in recent years. As a result, not all microorganisms are killed and an unpleasant odor (malodor) develops. The importance of malodor and the growing consumer dissatisfaction with it, which is likely to be exacerbated by the current energy crisis, necessitated a more detailed analysis of the problem.

To determine potential transfer routes and health risks, a comprehensive investigation and comparison of different habitats within the washing machine is essential. In order to improve the understanding of the microbial composition of various habitats, not only the microbial composition but also the microbial composition is investigated.

On the basis of this data, a laboratory model will be established and developed, which allows identified microorganism groups with a potential protective effect to be tested for their effect in biofilms as close as possible to the consumer. 16S sequencing and a metagenome analysis (ITS region for fungi) improve the evaluation and interpretation of the *ex situ* biofilms.

The third part of the study aims at evaluation of the identified microorganisms in an established laboratory model, which is able to reproduce the typical unpleasant laundry odor with the help of bacterial combinations. In addition, the existing model is to be adapted on the basis of the results obtained and *ex situ* biofilms are to be used as a basis instead of bacterial combinations.

Based on this, the thesis aims to possibly identify microorganisms that might act as protective shield. Furthermore, it is intended to investigate the microbial composition of the washing machine and the transfer of a household biofilm into a laboratory model.

# 3. Methods

## 3.1 Conducting an initial assessment of laundry odor using a questionaire

Using the web application "SoSci Survey (SoSci Survey GmbH, Munich, Germany), a questionnaire for an initial assessment of the topic was conducted in the period from March 21 to August 21, 2020 (see Appendix). A total of 359 people completed the survey, and 60 agreed to continue supporting the study.

In a questionnaire on washing practices and washing machine use, it may be important to include questions on the household composition, age and model of washing machine, typical washing cycles and detergents, drying of laundry, previous experience with laundry odor, and willingness to support this project in the future for several reasons.

The number of people in the household can provide important information about washing practices and washing machine use. Larger households may require more frequent washing, which can affect the efficiency and longevity of the washing machine. In addition, larger households may have more complex laundry needs, such as separating different types of clothing or fabrics, which can affect washing machine usage and efficiency. Pets in the home can affect laundry practices and washing machine usage. Pets shed hair and dander, which can build up in clothes and fabrics and require more frequent washing. In addition, pet hair can clog washing machine filters and reduce their efficiency. Including questions about pets in the household can help identify these potential problems and inform strategies for dealing with them. The age of the washing machine can provide important information about its efficiency and potential problems. Older washing machines may require more energy and water to operate, which can have environmental and cost implications. In addition, older machines may be more prone to breakdowns or malfunctions, which can affect laundry practices and laundry cleanliness.

Wash routines can provide important information about how often laundry is washed and the types of items included in each wash. This information can inform strategies to improve efficiency, reduce environmental impact and address potential wear and tear issues. The type of detergent and wash additives used can affect the cleanliness and longevity of garments, as well as the efficiency and environmental impact of the washing machine. Including questions about these factors can help identify potential problems with the hygiene quality of the washing machine and inform strategies for addressing them. The use of hygienic rinsers can have an important impact on laundry hygiene. Hygienic rinsers are products that claim to add disinfectant or antibacterial properties to the washing machine. However, their efficacy and safety may be unclear and their use may contribute to the development of antibiotic-resistant bacteria. Including questions about the use of sanitizers can provide important insights into the

potential risks and benefits of these products. Washing temperatures can affect the neatness and durability of garments, as well as the efficiency and environmental impact of the washing machine. Different types of fabrics and items may require different washing temperatures. Including questions about washing temperatures can help identify potential problems with wash quality and possible hygiene gaps in microorganism reduction.

Inquiring about individuals' previous experiences with malodour in a questionnaire that pertains to laundry practices or washing machine usage can provide valuable insights into the possible sources of malodour and help guide interventions. The information gathered can facilitate the identification of the origin, gravity, and occurrence frequency of the issue, as well as the evaluation of the efficacy of the current approaches employed to address it. This knowledge can inform future intervention strategies by informing what has and has not been effective in the past. Moreover, previous experiences with malodour can reveal potential health hazards related to the problem, such as the transmission of skin infections or other illnesses.

## 3.2 Microbiological investigations

### 3.2.1 Sampling

A total of 48 households were investigated further from the 60 respondents who indicated their intention of participating in the study in the future. Each household received a second questionnaire, asking for the number, age and gender of the persons in the household. Also included were questions about the keeping of pets, existing illnesses in the last four weeks, and the number of visitors who shared the test towel during the one-week test period.

Each of these households received a sterile 30 x 50 cm towel to be used for hands in the bathroom for one week. In addition to this, an area of 4 $cm^2$ was sampled in the washing machine at two locations (see Figure 3). With the help of a template, four different locations of 1 $cm^2$ each were sampled in the detergent drawer and the rubber sealant.

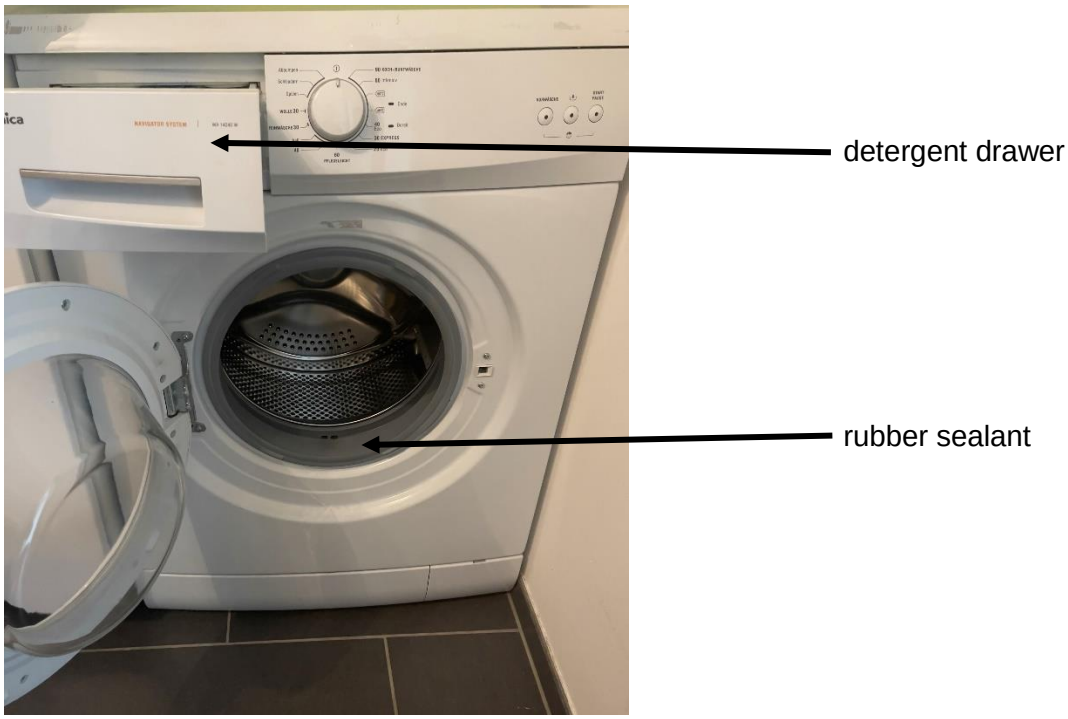



**Figure 3 Sampling sites (detergent drawer and rubber sealant) inside the washing machine**

Using a sterile swab, this area was sampled for one minute and then placed in a sterile 2 mL reaction tube filled with 1.5 mL of buffered peptone water (PBS). The samples were stored at 4 °C until further use.

In a second series of experiments, 20 of these households were also instructed to use a sterile 30 x 50 cm towel for body post-shower use and a sterile 45 x 60 cm dish towel for kitchen use. These were additionally used for hands for one week.

Households were instructed not to wash any of these towels or allow them to dry over the heater.

### 3.2.2 Sample preparation

The subsequent processing of the towels was carried out following Teufel *et al.* (Teufel *et al.*, 2008). A 4 x 4 cm (16 cm²) sample was taken from the contaminated towels, transferred to a sterile 50 mL reaction tube, and superfused with 20 mL of sterile 0.9% sodium chloride solution (NaCl). The reaction tubes were placed on a shaker for 20 min and first vortexed for one minute and then centrifuged for 20 min at 8 °C and 4696 g. The sample was removed from the reaction tube. After centrifugation, the supernatant was discarded and the remaining pellet resuspended in 700 µL of sterile 0.9% NaCl. This was used for the DNA extraction, as well as for the determination of the bacterial count.

Samples from the detergent drawer and the rubber sealant of the washing machines were extracted as descripted above.

### 3.2.3 Calculation of the microbial count

Microbial counts were determined using a total of one general-purpose medium for total aerobic mesophilic microbial count (TSA) and four selective media for yeasts and moulds (MEA), gram-negative bacteria (MacConkey), *Pseudomonadaceae* (Cetrimid), and staphylococci (MSA).

A decimal dilution series was prepared and 100 µL of each dilution level was pipetted onto all five agars and homogeneously distributed using a sterile drigalski spatula. Subsequently, the agar plates were incubated at 30 °C (MEA, MSA) or 37 °C (TSA, Cetrimid, MacConkey) for 24 h (TSA, MacConkey, MSA) or 48 h (Cetrimid, MEA) in an incubator.

Colony forming units (cfu/cm²) were calculated by counting surface cultures on each agar plate. Using the following formula (Equation 1), the colony forming units (cfu/mL) in the extraction fluids (cw) were determined (Bast, 2014):

**Equation 1 weighted mean of the live cell count**

$$c_w = \frac{10^x}{V} * \frac{\sum c_x + \sum c_{x+1}}{n_x + 0.1 n_{x+1}}$$

$_w$ weighted mean of the live cell count in 1 cm² undiluted sample

$10^x$ Dilution factor of the lowest dilution

V Sample volume added to the agar plate (mL)

$\sum c_x$ Sum of colonies on all plates of the lowest countable dilution level $10^{-x}$

$\sum c_{x+1}$ Sum of colonies on all plates of the next highest dilution level $10^{-(x-1)}$

Agar plates with less than 10 cfu or with more than 300 cfu were excluded. If not specified all experiments were performed in triplicate.

### 3.2.4 *Ex situ* model for investigating microbial communities isolated from household washing machines

#### 3.2.4.1 Biofilm sampling

The biofilm was collected with a sterile cottonswab for 120 s with constant rotation of the swab. An area of 4 cm² was sampled at the corresponding sampling site. The head of the swab was transferred to a sterile 2 mL reaction tube containing 1.5 mL phosphate buffered saline (PBS) and separated from the neck of the swab. The sample was stored in a refrigerator at 4 °C until further processing within the next 24 h.

For extraction of cells from the swabs, the reaction tubes were shaken comfortably for 10 min at 15 °C and 1400 rpm in a Thermomixer. The head of the swab was removed from the

solution and the cell suspension was centrifuged for 15 min at 8 °C and 14,800 rpm following Schages *et al.* (2020) for washing (L. Schages *et al.*, 2020). The supernatant was discarded and the cell pellet resuspended in 2 mL PBS. This cell suspension served as inoculum for the cultivation experiments (see section 3.2.4.2) and as starting solution for DNA extractions at time t = 0. Remaining inoculum was stored as glycerol stock (1:1) in 1.5 mL reaction tubes for later recultivation experiments at -80 °C (see section 3.2.4.3).

#### 3.2.4.2 Culture of biofilms in 6 well culture plates

To simulate biofilm formation, 50 µL of the original inoculum was pipetted in a multiplicate of 6 onto a coverslip (round, Ø 18 mm) placed in a 6-well plate and allowed to attach initially for 30 min under the sterile bench. After the time expired,various media (see Table 1) were added. The ingredients were chosen to mimic nutrient addition in the form of food, fatty acid and surfactant residues in a washing machine. After gentle horizontal shaking to disperse the media components, the cell culture plates were incubated at room temperature and the medium was changed every 48 - 72 h. The medium was then changed to a new medium. The biofilms were grown for 7 d and 10 d, respectively. On day 7 resp. 10 the cells were extracted from the coverslips followed by DNA extraction (see chapter 3.2.4.4 and 3.3.1).

**Table 1 Different media composition for the establishment of the *ex situ* biofilm**

| number | composition (in %) | number | composition (in %) |
|---|---|---|---|
| 1 | 0.1% TSB + 0.1% MEB | 5 | 0.001% meat extract + 0.001% potato starch + 0.1% APG |
| 2 | 0.1% TSB + 0.1% MEB + 0.1% APG | 6 | 0.001% meat extract + 0.001% potato starch + *Rhizobium* DSM 102134 (2.23 x $10^8$ cfu/mL) |
| 3 | 0.1% TSB + 0.1% MEB + 0.1% APG + 0.002% potato starch | 7 | 0.001% meat extract + 0.001% potato starch + *Rhizobium* DSM 106839 (1.61 x $10^8$ cfu/mL) |
| 4 | 0.001% meat extract + 0.001% potato starch | | |

#### 3.2.4.3 Recultivation of biofilms from glycerol stocks & pooling of samples for independent reproducibility

To establish a reproducible model independent of continuous sampling, different methods for biofilm recultivation were investigated. Glycerol stocks from biofilms whose melting characteristics after 7 d or 10 d resembled those at t = 0 and showed a comparably complex course were defrosted and a batch in 6-well plates identical to the initial biofilm

cultivation was performed. Since a 1:1 dilution with glycerol was available, 100 µL of cell suspension was used as inoculum in this case.

In addition, it should be tested whether further recultivation of new stocks is possible. For this purpose, the stocks from t = 7 or t = 10 were used as inoculum, the biofilms were grown for 10 d as before, and the biofilms thus obtained were frozen again as glycerol stocks. After checking that the melting profile still resembled that of t = 0, they were used for new experiments. These further cultivations were performed three times in multiple determinations with biofilms from original samples.

After confirming the possibility of successful recultivation from glycerol stocks after 7 d or 10 d with a constant melting curve of the biofilms, a basis for using always the same biofilm for different studies should also be established. Therefore, in order to use uniform initial suspensions, stocks of biofilms that had melting curves similar to that at t = 0 were used as inoculum for a 6-fold approach for 7 d and 10 d, respectively. Cell extraction from these wells was performed as described in 3.2.4.4.

#### 3.2.4.4 Sample extraction

For the extraction of biofilms from the coverslips, the medium was first removed from the wells and these were rinsed with 2 mL PBS to remove superficial cells from the coverslip. Following DIN EN 13697:2019-10 (chapter 5.5.2.1), the coverslip was then removed and covered with 2 mL PBS in a new 6-well plate. After addition of 2.5 g glass beads per well, the cells were detached from the surface by using Thermomixer comfort with MTP exchange block for 5 min at 650 rpm and 15 °C (Anonymous, 2012).

The resulting germ suspension was removed from the well, used as a basis for DNA extraction and, if necessary, stored at -80 °C for further experiments to create a glycerol stock as previously described.

## 3.3 Molecular biological analyses

### 3.3.1 DNA extraction

To compare the biofilm composition of different time points or after exposure to the test substances with the original composition using HRMA, DNA was extracted at time t0. Further sampling took place to investigate the stability of the biofilm after 7 d resp. 10 d. Accordingly, when recultured biofilms were used, the original DNA of the sample from the stock was used as a reference value for t0.

DNA extraction took place using the FASTDNA Spinkit for Soil (MP Biomedicals). The extraction was performed according to the manufacturer's instructions. The addition of 250 µL cell suspension instead of solid material sample for lysis in the matrix E-tube was modified.

Furthermore, mechanical lysis was performed in the FastPrep-24™ instrument twice for 60 s at 6.0 m/s. The resuspension step of the binding matrix with 500 µL SEWS-M also took place twice (L. Schages *et al.*, 2020). An incubation step (5 min, 55 °C) was performed to optimize DNA recovery. Finally, the DNA was eluted in 50 µL DES water. The concentration [µg/mL] of the DNA extracts was determined by adsorption at 260 nm using a photometer. The DNA extracts were stored at -20 °C until further use.

### 3.3.2 Production of qPCR standards for absolute quantification of amplicons

Absolute quantification by means of a standard curve describes quantification of the copy number of a gene under investigation in samples using standards with known absolute concentration (Nolan, Huggett, and Sanchez, 2013). For this reason, PCR standards with known copy number of the corresponding gene were previously prepared for measuring DNA copies/mL of genes to be detected. For the detection of bacterial ITS DNA, *P. aeruginosa* DSM 939 was used to prepare the standards. The amplified PCR product of the intI1 gene was previously amplified within the working group by the "Hygiene and Microbiology" working group, confirmed and made available for use as a standard.

The gene is originally derived from the extracted gDNA of an environmental isolate. In addition to their quantification function, the standards serve as positive controls for successful amplification of the target genes.

For the preparation of the ITS standards, an overnight culture of *P. aeruginosa* was prepared by adding a 1 mL glycerol stock (1:1) in approximately 40 mL TSB and the culture was incubated for 24 h at 37 °C in a shaker incubator at 150 rpm. From the overnight culture, 1 mL was transferred to a reaction tube and the remaining medium was separated from the cells by centrifugation for 15 min at 14,000 rpm and 8 °C. The supernatant was discarded and the cell pellet resuspended in 1 mL of PBS. 500 µL of this cell suspension was used for DNA extraction using FASTDNA Spinkit for Soil. The extraction was carried out with the previously explained modifications according to Schages *et al.* (2020). Using this DNA extract as a template, a PCR approach was created to amplify the target gene. The materials used and their volumes for the master mix with a total of 220 µL including template DNA can be seen in Table 2. PCR primers were purchased from Eurofins Genomics Germany GmbH. The primer sequence can be found in the appendix.

**Table 2 Composition of the PCR master mix for the preparation of DNA standards of the bacterial ITS gene in quadruplicate. The gDNA of *P. aeruginosa* DSM 939 was used as template.**

| Material | Volume (µL) |
|---|---|
| DNA Green Master Roche | 100 |
| PCR grade water | 96 |
| Forward Primer | 2 |
| Reverse Primer | 2 |
| Template-DNA | 20 |
| Total volume | 220 |

For PCR, 4 x 55 µL of the master mix was added to multiply-µ strips and PCR was started. The PCR program can be seen in Table 3.

**Table 3 PCR program used to amplify the bacterial ITS sequence for PCR standard production.**

| | **Number of cycles** | **Stage** | **Temperature [°C]** | **Duration [s]** |
|---|---|---|---|---|
| **Pre-incubation** | 1 | - | 95 | 600 |
| **PCR-cycle** | 35 | denaturation | 95 | 30 |
| | | annealing | 55 | 30 |
| | | elongation | 72 | 30 |
| **Final elongation** | 1 | - | 72 | 180 |

The PCR products obtained after PCR were pooled and purified using the High Pure PCR Product Purification kit. Purification took place according to the manufacturer's instructions. The PCR product was eluted in Elution Buffer with a volume of 80 µL.

To check the PCR, gel electrophoresis of the PCR products was performed. For this, a 2% (w/v) agarose gel was prepared from agarose standard and TAE buffer. 100 mL of the batch was heated to dissolve the agarose and 10 µL of Roti-Safe Gelstain was added. The gel was poured into an electrophoresis apparatus with appropriate combs and waited until it was solidified. The electrophoresis chamber was filled up to the mark with TAE- buffer. 10 µL of the purified PCR products or marker were mixed with 2 µL of Gel Loading Buffer and pipetted into the pockets. The DNA Ladder 50 bp ready-to-use from GeneOn was used as the marker. After gel electrophoresis, the bands were detected by UV light.

After confirmation of successful PCR based on amplicon length in the gel, decimal dilutions were prepared using PCR grade water up to a dilution of $10^{-10}$ as DNA standards. In addition, the adsorption of the undiluted product was measured at 260 nm. Using the DNA Copy Number and Dilution Calculator from Thermo Fischer Scientific (Thermo Fisher Scientific, o. J.) and the known amplicon length, the initial concentration of DNA copies/mL of the undiluted standards could be determined. The concentrations of the dilutions were derived

from this value by multiplying by the appropriate dilution factor. The purified DNA and the dilutions were stored at -20 °C until use.

By adding multiple dilutions (in the case of ITS, dilutions $10^{-3}$ to $10^{-8}$) to qPCR runs, these standards allow the concentration of specific genes in unknown samples to be ranked. The initial concentration of the ITS sequence was 2 x $10^{14}$ copies/mL.

### 3.3.3 Performance and evaluation of real-time polymerase chain reaction (qPCR)

The qPCR was performed in the QuantStudio 3-Lightcylcer (Thermo Fisher Scientific) with the integrated Instrument Operating Software version v1.4.1. The associated Quant Studio Design & Analysis Software v.1.5.2 was used to set up the qPCR and HRMA programs and to analyze the data obtained. By using standards, the absolute copy number in copies/mL of the detected and amplified genes was automatically calculated by the software using the obtained standard curve.

For the PCR set-up, a master mix was prepared for each of the samples with all the necessary components of the qPCR except the DNA template. The required volumes per sample can be found in Table 4. The primer sequence can be found in the appendix. In each case 10 µL of the master mix was placed in a 96-well light cycler plate according to the own sample scheme and then 1 µL of the respective template DNA was added. The PCR plate was sealed with PCR film, centrifuged and placed in the PCR instrument. After entering the sample data and pipetting scheme into the computer system, qPCR was started.

**Table 4 Composition of the PCR master mix for qPCR. All components except the template were prepared as master mix for all samples and submitted in the wells.**

| Material | Volume (µL) |
|---|---|
| DNA Green Master Roche | 5.0 |
| PCR grade water | 4.8 |
| Forward Primer | 0.1 |
| Reverse Primer | 0.1 |
| Template-DNA | 1.0 |
| Total volume | 11.0 |

The cycler program for amplification of the ITS sequences was adapted from the procedure of Andini *et al.* (2017) and can be seen in Table 5 (Andini *et al.*, 2017).

**Table 5 qPCR program for amplification and absolute quantification of ITS sequences. Temperature increases and decreases occurred with an adjustment of 1.6 °C/s. Final elongation and maintenance of a constant temperature after PCR cycles were not required for qPCR to detect the bacterial ITS gene, as melting curve analysis was performed directly afterwards.**

| | Number of cycles | Stage | Temperature [°C] | Duration [s] |
|---|---|---|---|---|
| **Pre-incubation** | 1 | - | 95 | 300 |
| **PCR-cycle** | 33 | denaturation<br>annealing<br>elongation | 95<br>55<br>72 | 15<br>30<br>60 |

The transition to the melting curve after ITS amplification took place by heating to 95 °C for 28 s and then cooling to 28 °C for 30 s. The melting curve was then followed by a cooling step.

### 3.3.4 Performance and evaluation of the High Resolution Melting Analysis

High Resolution Melting Analysis is used to characterize the melting behavior of DNA samples. Composition, sequence length, and GC content play a major role (Anonymous, 2009; Nolan *et al.*, 2013). For melting curve analysis, DNA double-stranded binding (saturation) fluorescent dyes, which can also be used in qPCR to quantify DNA, are used (Reed *et al.*, 2007). In the analysis, the temperature in the well is increased by degrees following a PCR. When a melting point of the DNA is reached, the double strand present separates. This releases the previously bound fluorescent dye, yields a fluorescent signal, and plots this as the rate of change of fluorescence as a function of temperature (Nolan *et al.*, 2013). The decrease in fluorescence is thus proportional to the amount of single-stranded DNA in the preparation (Ruskova & Raclavsky, 2011). The result corresponds to a melting profile characteristic of the DNA sample (Anonymous, 2009). Typically, melting curve analyses are used for mutation screening e.g. for virus subtypes, genotyping or for the detection of methylation (Lin *et al.*, 2008; Thermo Fisher Scientific, 2009).

Furthermore, it is possible to use databases to identify isolates of important bacterial representatives in clinical diagnostics on the basis of their melting curve (Cheng *et al.*, 2006; Šimenc & Potočnik, 2011). These include species from the *Chlamydiaceae* and *Enterobacteriaceae* families (Cheng *et al.*, 2006; Robertson *et al.*, 2009). Hjelmsø *et al.* (2014) presented the use of HRMA to detect compositional changes in a microbial community (Hjelmsø *et al.*, 2014).

Melting curve analysis was performed automatically following qPCR. The Melting behavior between 50.0 °C and 95.0 °C was recorded. The temperature increased at a rate of 0.02 °C/s. The raw data were exported, each data set was normalized separately between $y = 0$ and $y = 1$, and the melting curves were mapped graphically using GraphPad Prism software, by taking the negative derivative of the fluorescence (-d(RFU)/dt) as a function of

temperature in °C was compared. When the values are compared in this form, a clear large peak indicates the melting temperature (Tm) of the corresponding sample. At this temperature, 50% of the DNA is present as a double strand and 50% of the DNA is already dissociated as a single strand. Other smaller peaks with lower intensity can be caused by existing DNA strands with shorter length (*Life Technologies Corporation, 2009*). In the analysis, only relevant temperature ranges (81 °C - 91 °C) were considered as active melting ranges in the data processing. Normalized values were calculated using Equation 2. The normalization of the data was performed in Microsoft Excel.

**Equation 2 Calculation of values normalized between 0 and 1 of the melting curves**

$$I_{norm} = (I - I_{min})x \left(\frac{i_{max} - i_{min}}{I_{\max - I_{min}}}\right) + i_{min}$$

$I_{norm}$ normalized values
I original value
$I_{min}$ minimum value in the data set
$I_{max}$ maximum value in the data set
$i_{min}$ lower limit of normalization (in this case: 0)
$i_{max}$ upper limit of normalization (in this case: 1)

### 3.3.5 Metagenomic analysis

The DNA samples were analyzed by an external laboratory (Cegat GmbH, Tübingen, Germany). The method of shotgun metagenomic sequencing was used. An advantage of this method is that all DNA fragments are sequenced and thus functional genes related to specific metabolic pathways are also analyzed. The amount used for analysis was 0.1 ng and samples were prepared using the Nextera XT kit (Illumnia Inc, San Diego, USA). Assays were performed using the NovaSeq 600 System (Illumnia Inc, San Diego, USA) and a flow cell type of 2 x 100 bp.

### 3.3.6 Bioinformatics analysis

Demultiplexing of sequencing reads was performed using Illumnia bcl2fastq (2.20). Adapters were trimmed using Skewer (version 0.2.2) (Jiang *et al.*, 2014). Quality trimming of the reads was not performed. For taxonomic and functional data analysis, 10 million of the adapter trimmed raw forward reads were aligned to the filtered RefSeq protein database (version 94) using Diamond in BLASTX mode (Buchfink *et al.*, 2014). Taxonomic classification was performed using the Lowest Common Ancestor (LCA) algorithm implemented in MEGAN6 Ultimate Edition (version 6.15.2) (Huson *et al.*, 2016). Only taxa with relative sequence

abundances above 0.01% were considered. Functional classification was performed in MEGAN6 Ultimate Edition (version 6.15.2) by assigning reads to KEGG, SEED, VFDB, and Interpro identifiers (Huson *et al.*, 2016).

## 3.4 Statistical analysis

Statistics were performed using the GraphPad Prism program (GraphPad Software, 2020). All data were presented as mean values (± standard deviation). The alpha diversity (Shannon diversity exp(H'), Equation 3), which indicates the microbial diversity within a sample, was not normally distributed, so the Mann Whitney test (Equation 4) ($p \leqslant 0.05$) was used to determine significant differences in the origins of the samples (Magurran, 2004; Shannon & Weaver, 1949).

**Equation 3 Shannon Diversity**

$$H' = \sum \left| \left(\frac{n_1}{N}\right) x \left(\ln \frac{n_1}{N}\right) \right|$$

H´ Shannon diversity
$n_1$ Number of species/genera in sample 1
N Sum of all species/genera

**Equation 4 Mann-Whitney U test**

$$U = n_1 n_2 + \frac{n_2(n_2+1)}{2} - \sum_{i=n_1+1}^{n_2} R_i$$

U number of times observations in one sample precede observations in the other sample in the ranking
$n_1$ sample 1
$n_2$ sample 2
$R_i$ ranks

Microbial community composition was performed using Principle Component Analysis (PCA) and associated heat map. For this, the open-source program ClustVis was used (Metsalu & Vilo, 2015). To identify dissimilarities between two samples or environments, Bray-Curtis Dissimilarity (beta diversity, Equation 5) (Bray & Curtis, 1957) was performed. Two-way analysis of variance was used to identify significant differences in microbial counts.

**Equation 5 Bray-Curtis Dissimilarity**

$$BC_{ij} = 1 - \frac{(2\ x\ C_{ij})}{(S_i + S_j)}$$

$BC_{ij}$ Bray-Curtis Dissimilarity

$C_{ij}$ The sum of the lower values for the species found at each sampling site

$S_i$ The total number of individuals counted at site i.

$S_j$ The total number of individuals counted at site j.

To compare the similarity of samples based on melting curves and genomic data, the median Euclidean distance between the individual data points of two active melting regions to be compared from a PCR run was calculated in parallel. Equation 6 was used for the calculation:

**Equation 6 Calculation of the Euclidean distance between two data points (Weisstein, 2022)**

$$d = \sqrt{(x_2 - x_1)^2 + (y_2 - y_1)^2}$$

d Euclidian distance

$x_1/y_1$ point 1

$x_2/y_2$ point 2

## 3.5 Malodor model according to Zinn *et al.* 2021

### 3.5.1 Preparation of the biomonitors

In the beginning, 1 g beef tallow and 1.5 g TEGO Care PS were weighed into 25 mL 0.9% NaCl in a 50 mL reaction tube. The tube was heated in a water bath at a temperature of 80 °C to 90 °C for 30 min. Cooling down to 40 °C was done for 15 min in a shaking incubator (20 °C, 200 rpm). For further use, 10 mL of beef tallow solution was used for each test microorganism.

For the preparation of the germ carriers, the microorganisms were completely dissolved from three completely covered agar plates and transferred into 30 mL Trypton Soja Bouillon (TSB). Incubation was performed in a shaker incubator for 24 h at 37 °C and 200 rpm. The overnight culture was transferred to a 50 mL reaction tube and centrifuged at 4,800 rpm for 10 min. To wash the resulting pellet, the supernatant was first discarded, the pellet resuspended with 10 mL of 0.9% NaCl and centrifuged again for 10 min at 4,800 rpm. At the end of the washing step, the supernatant was discarded and the pellet resuspended in 10 mL of the beef tallow solution. Then, 1 mL of each microorganism-bovine tallow solution

was first pipetted onto a 2 x 2 cm piece of textile placed in a petri dish. In addition, the bacterial combination of *Micrococcus luteus (M. luteus)*, *C. jeikeium* and *S. hominis* was prepared. For this purpose, 333 µL of the microorganism-bovine tallow solution was pipetted into a 1.5 mL reaction vessel, so that a final volume of 999 µL of the microorganism combination could be pipetted onto the textiles. To test the odor reduction of selected rhizobial strains, overnight cultures of each strain were prepared and incubated for 72 h at room temperature. This was followed by the same steps as described above (centrifugation, washing and resuspended in 10 mL PBS). Finally, 1 mL of this solution was pipetted onto the sterile textile, followed by the normal microorganism/bovine tallow solution. For incubation, the prepared samples were stored in a constant climate chamber at 27 °C and a humidity of 84% RH.

To investigate the influence of *Bradyrhizobium* and *Rhizobium* strains on malodor households or the influence of *M. osloensis, M. luteus, S. hominis* and *C. jeikeium* on non-objectonable households, an existing washing machine biofilm was taken from the corresponding household (see Section 3.2.4) and dissolved in 1 mL of beef tallow. This solution was added to the textile together with 1 mL of the teststrain-solution and also incubated for seven days in a constant clima chamber at 27 °C and 84% RH.

### 3.5.2 Sensory evaluation

A sensory panel trained on the odor attribute "malodor", consisting of up to 10 people, evaluated the different samples, including a positive control and a negative control (0.01% benzalkonium chloride (BAC). The panelists consisted of employees of the Rhine-Waal University of Applied Sciences and were trained in advance on the odor attribute "malodor" (Zinn *et al.*, 2021).The evaluation was done on a scale from 1 to 5 (see Table 6).

**Table 6 Rating scale for malodor model from Zinn *et al.*.**

| **Rating scale** | 0 | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|---|
| **Description** | not detectable | very faintly detectable | faintly detectable | clearly recognisable | strongly recognisable | very strong recognisable |

The evaluation of the sensory data was analyzed using an Excel file specially developed for this purpose (see appendix). In this file, the values of the positive control were first set to 100% for each panelist and the values of the samples adjusted according to the positive control. In the second step, the values of the individual samples were subtracted from the positive control for each panelist and combined into a mean value. These mean values show the reduction of the odor in percent compared to the positive control.

# 4. Results

At the beginning of the experiments, a questionnaire was used to get an overview of the importance of textile malodor. Subsequently, various household consumer washing machines as well as towels used over a week were examined for existing microbial counts and the existing microbiome.

With the help of the findings, a method was developed to transfer as diverse washing machine biofilms as possible into a laboratory model. After successfully developing this method, it was used to verify the new findings from the analysis of household washing machines in the laboratory model. In addition, verifying experiments were conducted in an established odor model according to Zinn *et al.* (2021).

## 4.1 Analysis of the microbial communities in the laundering cycle

### 4.1.1 Investigating household compositions, existing washing routines and textile malodor experiences using questionnaires

359 people were asked about general household information, existing washing routines and previous experiences with malodor using an online questionnaire. The following figures show the results of the survey for the sections ´general information about the household´ (Figure 4), existing washing routines (Figure 5) and previous experiences with malodor (Figure 6).

The evaluation of the questionnaire with regard to general information about the household was fulfilled by a group of 359 households. The majority of households consisted of 2-person households (about 150 households), large families of more than 4 persons participated the least in the survey (39 households). Among the respondents, about 55% did not own any pets. With regard to the existing washing machine, it was found that the majority of households owned a washing machine between the ages of 4 and 8 years (155 households). Moreover, it was noted that 118 households had bought a new washing machine in the last 3 years. In contrast, only about 1.4% of the surveyed households own a washing machine older than 20 years.

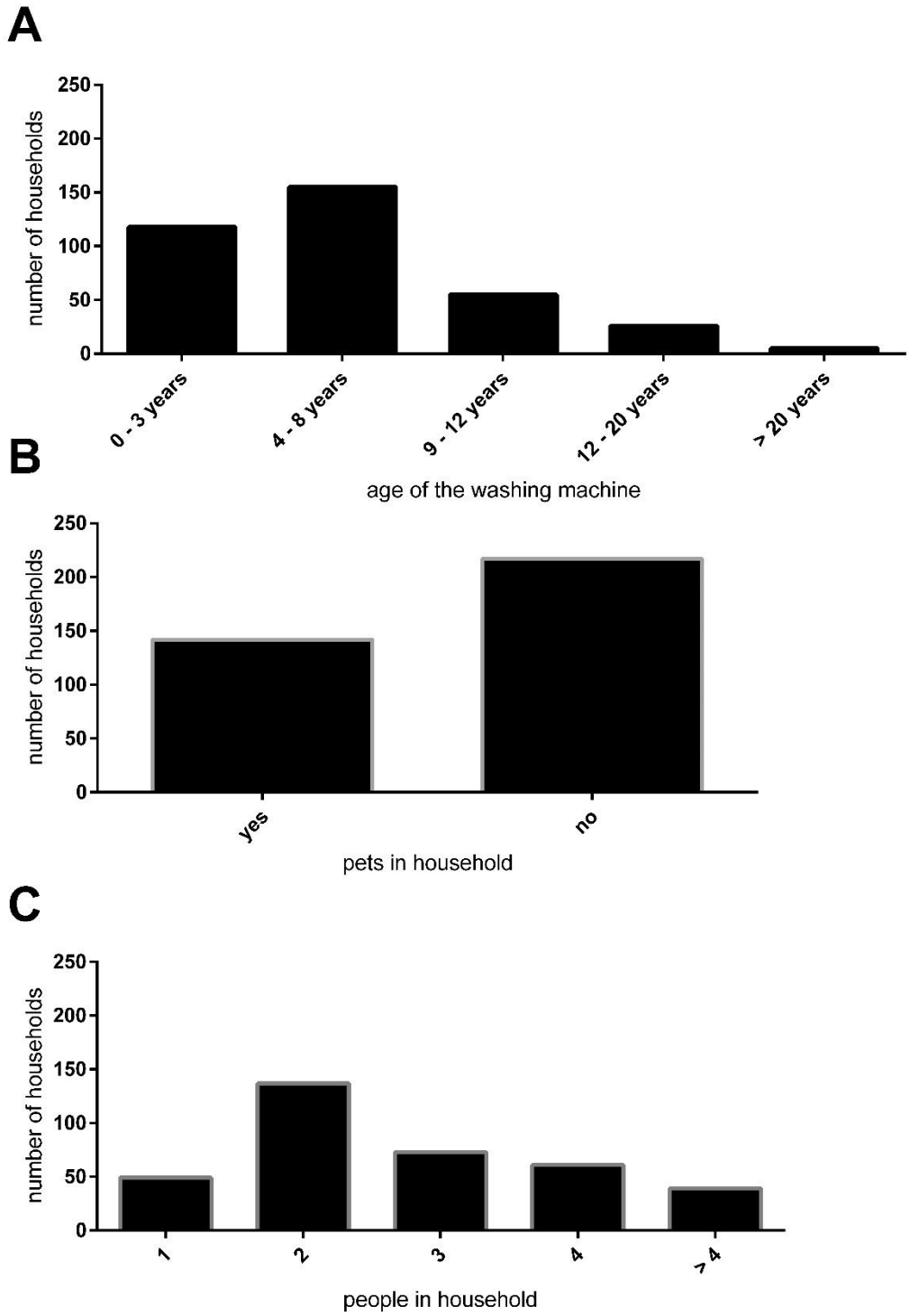


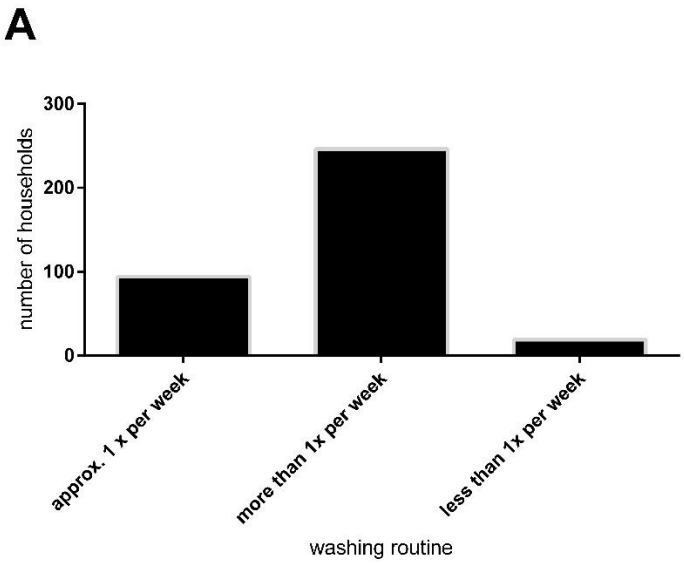

**Figure 4 Self-assessment of the surveyed households (n = 359) with regards to general information about the age of the washing machine (A). Households were asked about the presense of pets (B) and number of people in household (C).**

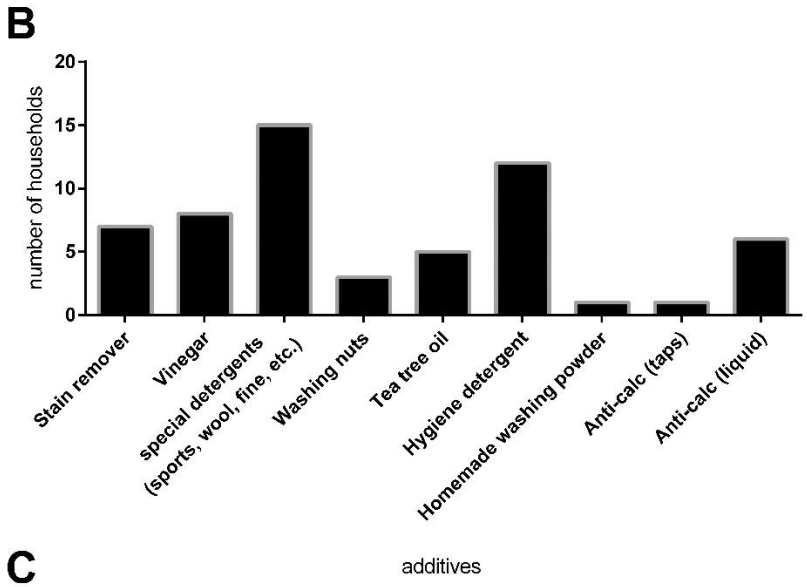
Figure 5 shows that most households wash more than once a week (246 out of 359 households) and primarily use heavy-duty detergents in solid form for this purpose (197 out of 359 households). Likewise, colour detergents in solid (120 households) and liquid (135 households) form and fabric softeners (140 households) are used very frequently. Sixty households stated that they use other washing additives. The most commonly reported additives are special detergents (e.g., sports, wool, fine wash) (15 households), vinegar (8 households), and stain remover (7 households). In particular, the use of heavy-duty powder detergents is an important factor in washing machine hygiene due to the presence of Active Oxygen Bleach (AOB). AOB is a type of bleach that contains oxygen-releasing compounds. It is used to disinfect and bleach laundry and can effectively reduce the number of bacteria present. However, prolonged and repeated use of AOB can damage textiles and shorten their life.

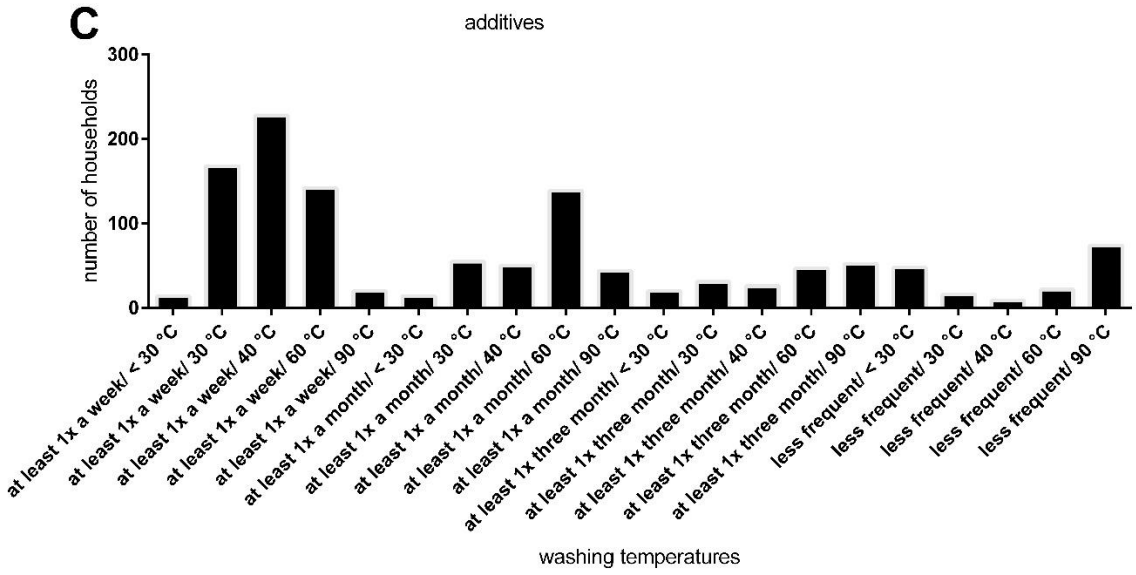
A minority only used hygiene rinsers, on the other, in every wash cycle (28 households). The majority did not use hygiene rinsers at all (199 households). Hygiene rinses, as mentioned above, are designed to remove detergent residues and prevent the build-up of biofilm in the washing machine, which can contribute to microbial contamination and odour. They can effectively reduce the number of bacteria in the washing machine and improve overall hygiene, but are suspected of contributing to antibiotic resistance.

In addition to the detergent and the washing additives, the survey also included questions about the washing programs. The results show that the 60 °C (350 households) and 40 °C (313 households) programs are mainly used. The 30 °C programs followed with 270 households. Programs with a temperature of < 30 °C are used least (96 households).

Another question on drying showed that most households dry indoors on the line in a heated room (218 of 685 mentions).


A
number of households
300
200
100
0
approx. 1 x per week
more than 1x per week
less than 1x per week
washing routine


B
number of households
20
15
10
5
0
Stain remover
Vinegar
special detergents (sports, wool, fine, etc.)
Washing nuts
Tea tree oil
Hygiene detergent
Homemade washing powder
Anti-calc (taps)
Anti-calc (liquid)
additives


C
number of households
300
200
100
0
at least 1x a week/ < 30 °C
at least 1x a week/ 30 °C
at least 1x a week/ 40 °C
at least 1x a week/ 60 °C
at least 1x a week/ 90 °C
at least 1x a month/ < 30 °C
at least 1x a month/ 30 °C
at least 1x a month/ 40 °C
at least 1x a month/ 60 °C
at least 1x a month/ 90 °C
at least 1x three month/ < 30 °C
at least 1x three month/ 30 °C
at least 1x three month/ 40 °C
at least 1x three month/ 60 °C
at least 1x three month/ 90 °C
less frequent/ < 30 °C
less frequent/ 30 °C
less frequent/ 40 °C
less frequent/ 60 °C
less frequent/ 90 °C
washing temperatures

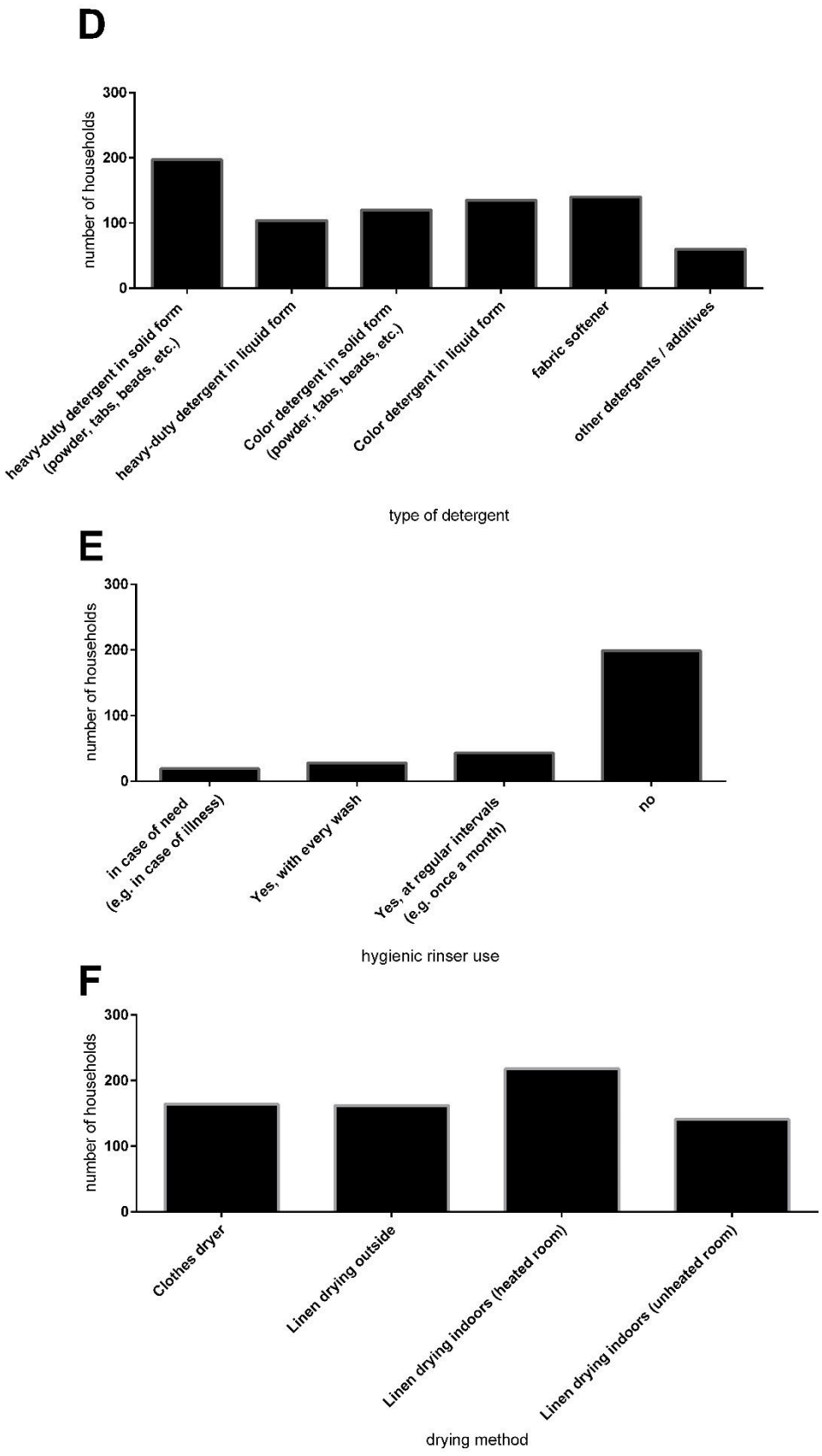



**Figure 5 Self-assessment of the surveyed households (n = 359) with regards to washing routine (A). Households were asked about possible washing additives (B; supported by keywords), used washing temperatures (C), the type of detergents that are used (D), if the household use hygienic rinsers (E) and the type drying their laundry (F).**

The last section of the questionnaire referred to malodor and previous experience with this issue. The results are shown in Figure 6.

Of the 359 households, less than half (143 households) reported no experience with malodor. 110 households experienced malodor on laundry before washing, 91 households after washing on wet laundry, and 73 households experienced malodor on laundry after drying. Drying processes can also affect the reduction of bacteria. For example, drying laundry at high temperatures in a clothes dryer can effectively reduce the number of bacteria present, while line drying in direct sunlight can also help to kill bacteria.

The majority of households described this malodor as musty (87 households). The affected textiles are mainly towels (19 households), T-shirts (18 households), sportswear (17 households) and trousers (15 households) and are mainly made of cotton (40 households) and polyester (29 households).

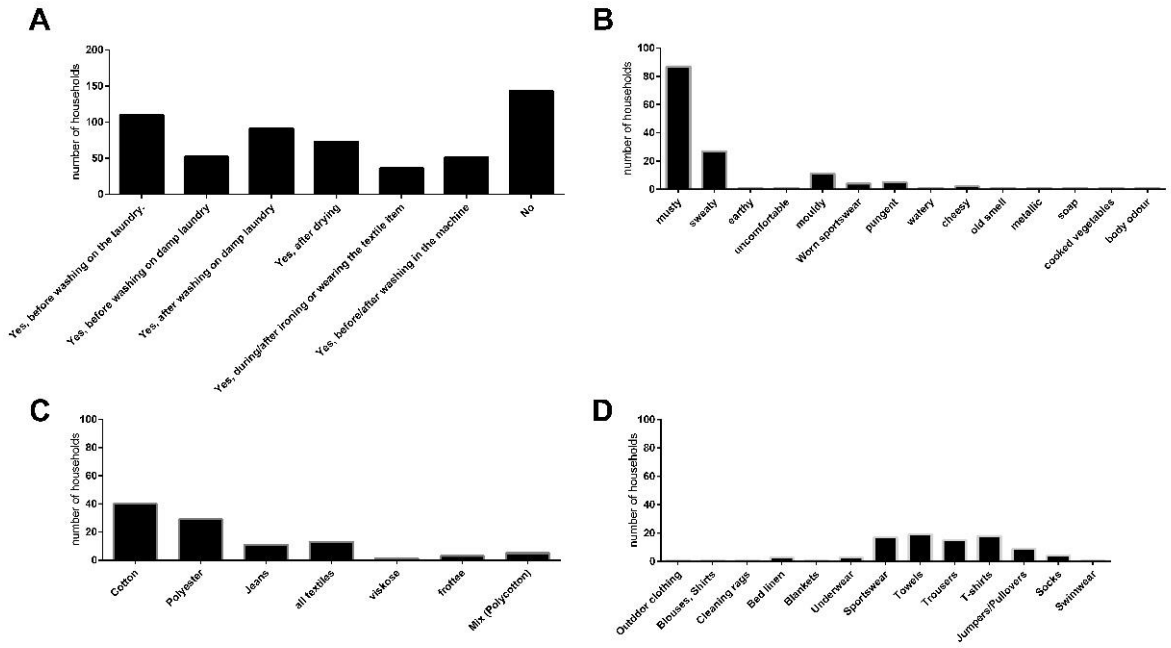



**Figure 6 Self-assessment of the surveyed households (n = 359) with regards to laundry-related malodor experience (A). Households with malodor experience were asked to provide a description of the malodor (B; supported by keywords), the textile mainly associated with malodor (C) and the type of garment or textile mainly associated with malodor (D).**

Of the 359 households that completed the questionnaire, 48 households made themselves available for sampling.

The orientation questionnaire, which was intended to provide an initial overview of the topics of washing, detergents, malodor, etc., was completed by 359 households. Besides collecting information, the questionnaire also served the purpose of finding households for sampling.

### 4.1.2 Assessing microbial counts and diversity in detergent drawers, rubber sealants, and used towels

In households, which agreed to sampling (n = 48), the detergent drawer and the rubber sealant of the washing machine as well as a hand towel in the bathroom, which was provided and used for seven days, were sampled for analysis of the microbial communities by plating on selective media as well as next generation sequencing after DNA extraction. In addition, in 21 households, a body towel (for use after showering) and a kitchen towel were distributed and analyzed accordingly. The aim of this study was to get an overview of the live germ counts in the household as well as the microbial composition of different parts of the washing machine. In addition, a possible relationship between these results and the questionnaire in section 4.1.1 regarding malodorous and non-odorous households was to be identified.

The results show that the microbial counts, of all test households, for the total aerobic mesophilic bacteria as well as on all selective media were similar for the detergent drawer and the rubber sealant, except for *Pseudomonas spp.*, which showed slightly higher counts in the detergent drawer (Figure 7). The aerobic mesophilic bacterial count for these sampling sites was about $10^5$ cfu/cm², while approx. $10^3$ cfu/cm² of yeasts and moulds as well as gram negative bacteria were present. At genus level, *Pseudomonas spp.* were present at less than 10 cfu/cm² while approx. $10^2$ cfu/cm$^2$ staphylococci could be detected. The bacterial counts on the towels and the kitchen cloth were on average much lower than those found in the washing machine, but again similar for the different sample types. Here, out of the approx. $10^2$ cfu/cm$^2$ resembling the total mesophilic count, $1x10^1$ cfu/cm$^2$ turned out to be fungi or staphylococci, respectively, while gram negative bacteria could be found to an even lesser extent.

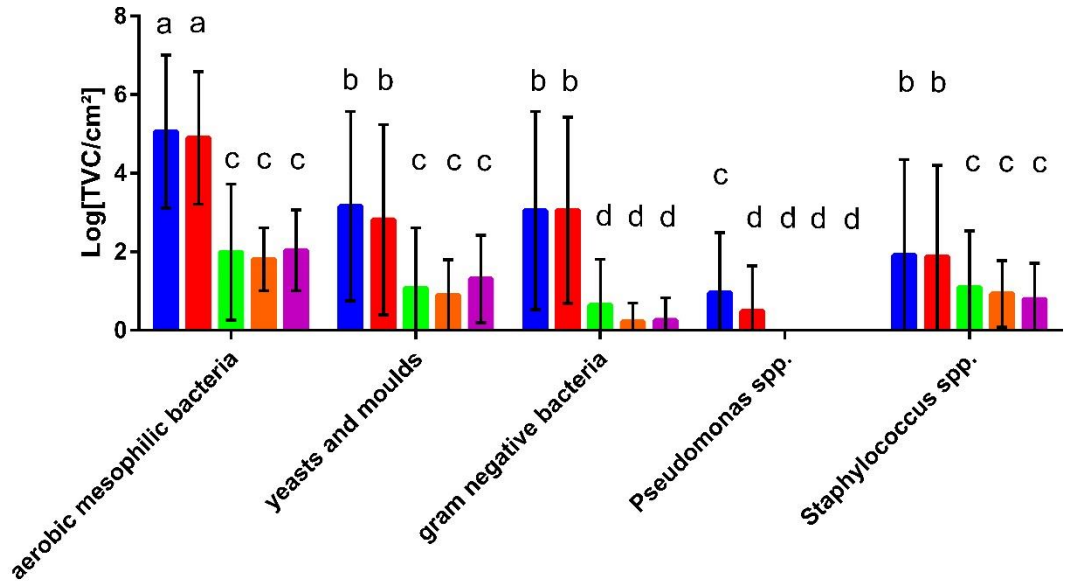



**Figure 7 Microbial counts in the detergent drawers (blue) and rubber sealants (red) of the sampled washing machines (n=48) as well as on used, unwashed hand towels (green; n = 48), body towels (orange; n=21) and kitchen cloths (purple; n = 21). Different letters indicate significant differences calculated by two-way ANOVA.**

The results show that the microbial counts for the total aerobic mesophilic bacteria as well as on all selective media were similar for the hand towel, body towel and kitchen cloth, except for gram negative bacteria, which showed slightly higher counts in the body towel (Figure 8).

The aerobic mesophilic bacterial count for these sampling sites was about $10^2$ cfu/cm², whereas approx. $10^1$ cfu/cm² of yeasts and moulds as well as *Staphyloccus spp.* were present. On genus level, gram negative bacteria were present at less than 10 cfu/cm². The comparison between people under 35 (dark columns) and people over 60 (light columns) did not show significant differences.

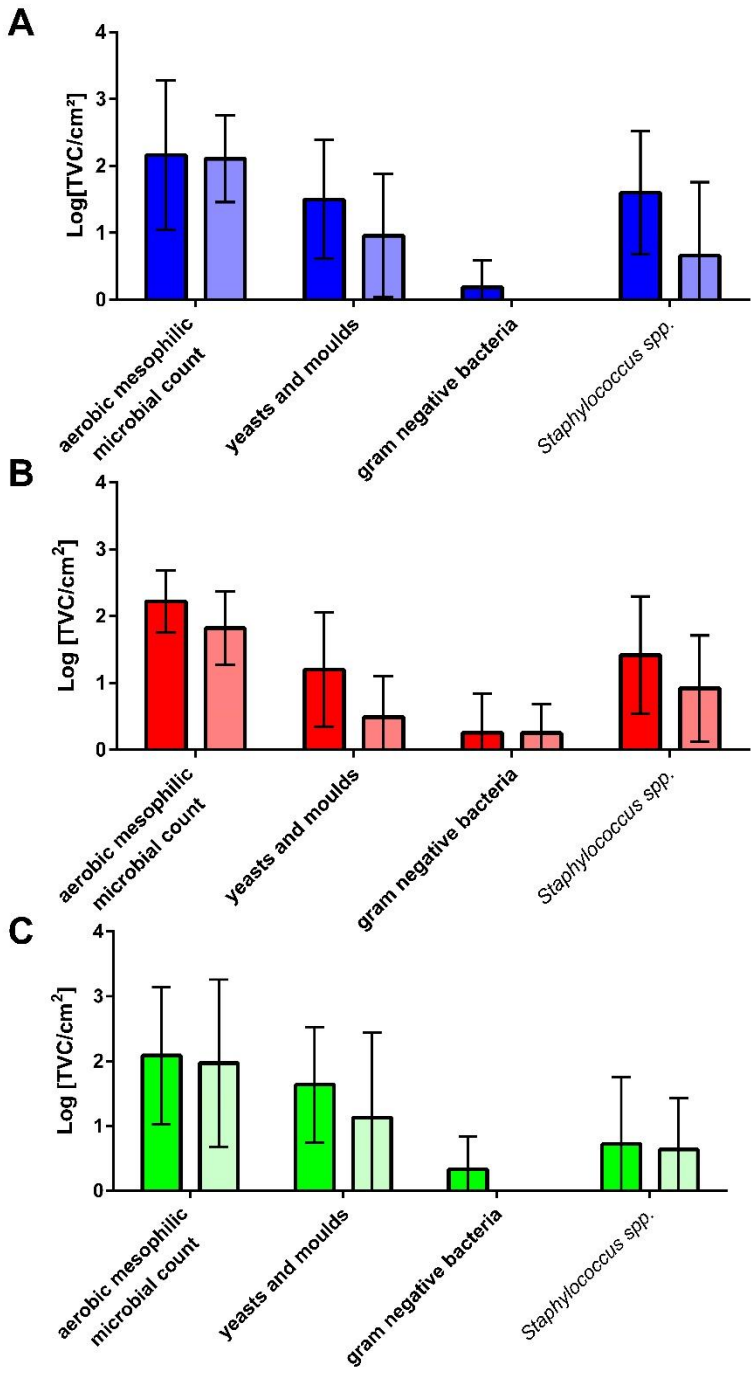



**Figure 8 Comparison of microbial counts on used and unwashed hand towels (A), body towels (B) and kitchen cloth (C) of households with young people (under 35; dark columns, n = 6) and old people (over 60; light columns, n = 7). No significant differences**

Figure 9 shows the comparison of microbial counts on used and unwashed hand towels, body towels and kitchen cloths of households with (dark columns) and without (light columns) children. The microbial counts for the aerobic mesophilic microbial count as well as yeasts and moulds and *Staphylococcus spp.* were similar for the hand towel and the kitchen cloth. The yeasts and moulds on body towels, in contrast, showed that households with children had slightly higher microbial counts. In summary, no significant differences in microbial counts were identified between households with and without children.

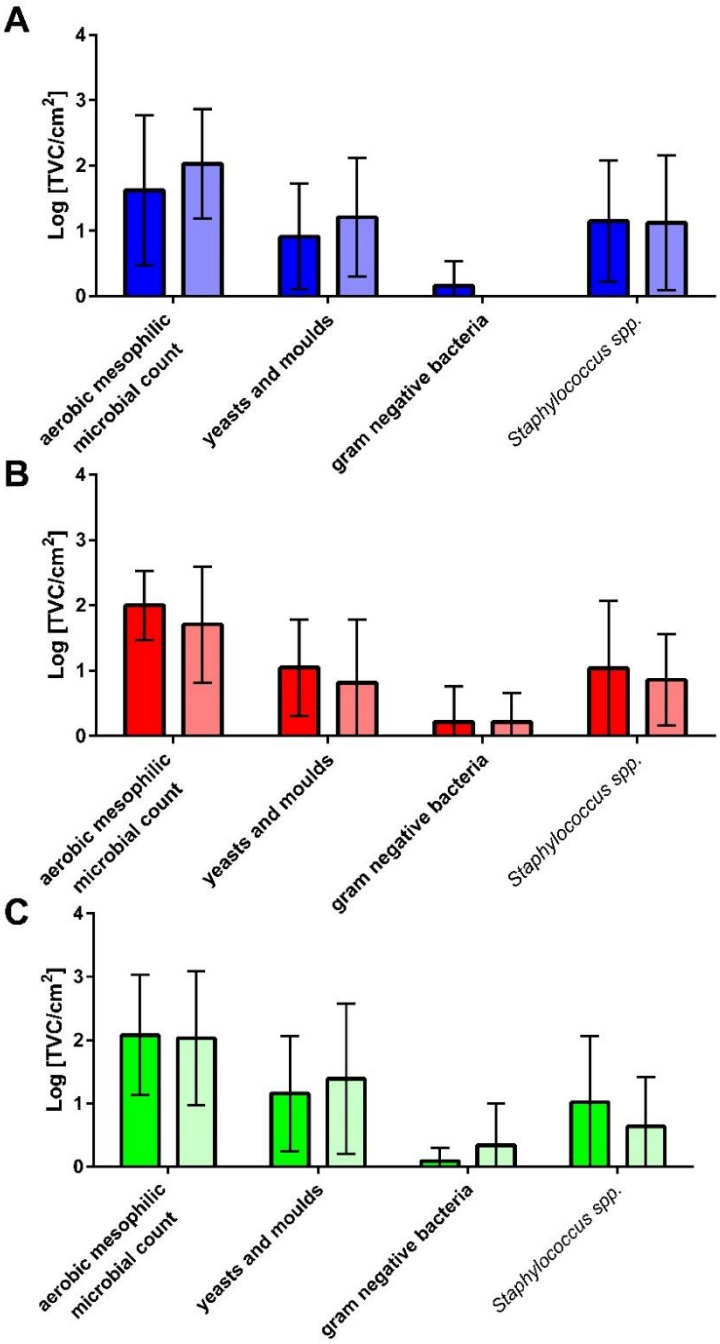



**Figure 9 Comparison of microbial counts on used and unwashed hand towels (A), body towels (B) and kitchen cloth (C) of households with (dark columns, n = 16) and without children (light columns, n = 32) households. No significant differences**

The comparison of the bacterial counts of used and unwashed hand towels, body towels and kitchen cloth from households with and without pets shows that households without pets tended to have higher microbial counts than households with pets (Figure 10). A significantly higher microbial count was identified in kitchen cloths in yeasts and moulds than in the hand and body towels. The aerobic mesophilic microbial count of petless households was approx. $1x10^2$ cfu/cm², the number of yeasts and moulds as well as *Staphylococcus spp.* was approx. $5 \times 10^1$ cfu/cm². Gram-negative bacteria were only found in small numbers in both cases (<10 cfu/cm²).

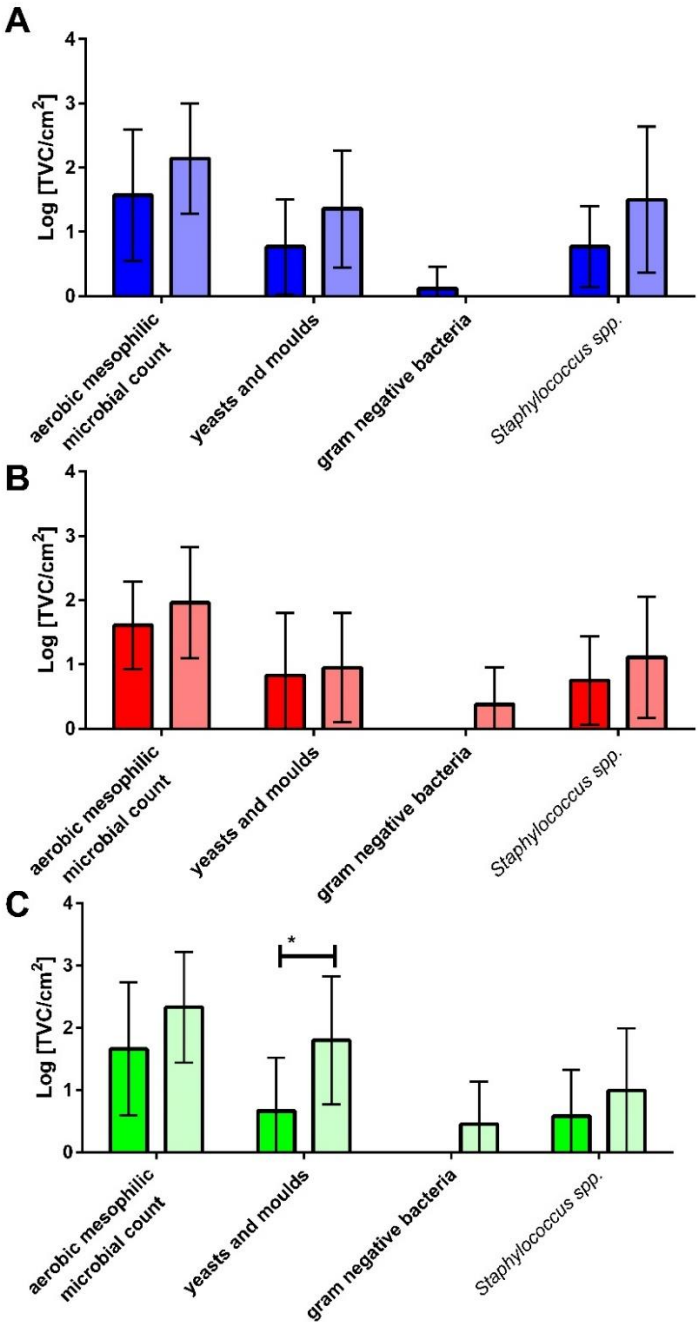



**Figure 10 Comparison of microbial counts on used and unwashed hand towels (A), body towels (B) and kitchen cloth (C) of households with pets (dark columns, n = 21) and without pets (light columns, n = 27). * ≤ 0. 05**

A heatmap was used to display the metagenome data. This is a graphical representation of data where colors are used to indicate the value of a variable in a matrix. In metagenomics, a heatmap is used to visualize and compare the relative abundance of genes or taxonomic groups in different samples. Heatmaps can thus help reveal differences in microbial community composition between different environments or conditions and investigate relationships between different parameters or properties of the metagenomic data (Lozupone *et al.*, 2006; Segata *et al.*, 2011).

The results of the cultural analysis was partly confirmed and complemented by the metagenome data (Figure 11), revealing the presence of additional genera, many of them belonging to the family *Pseudomonadaceae* or other gram-negative bacteria. Moreover, gram-positive genera, such as *Microbacterium* and *Paracoccus* were identified. Most strikingly, the Genus *Rhizobium* was found as well. The most frequently found bacteria, however, belonged to the genus *Pseudomonas* and *Acinetobacter*. The Shannon diversity of bacterial communities was significantly (according to Man-Whitney-U-Test) lower ($p < 0.05$) in towels ($\exp(H') = 19.97 \pm 5.32$) and rubber sealants ($\exp(H') = 28.96 \pm 6.61$) compared to the detergent drawer ($\exp(H') = 33.06 \pm 8.66$). In contrast, there were no significant differences in species richness (towels: $n = 591 \pm 357.9$; detergent drawer: $n = 742 \pm 257.1$; rubber sealant: $n = 645 \pm 335.6$). Beta diversity showed a high degree of variation among the different sampling sites. In particular, detergent drawer samples were found to have more species in common with rubber sealant samples (Bray-Curtis index=0.36) than towel samples with detergent drawer (Bray-Curtis index=0.68) or rubber sealant (Bray-Curtis index=0.52).

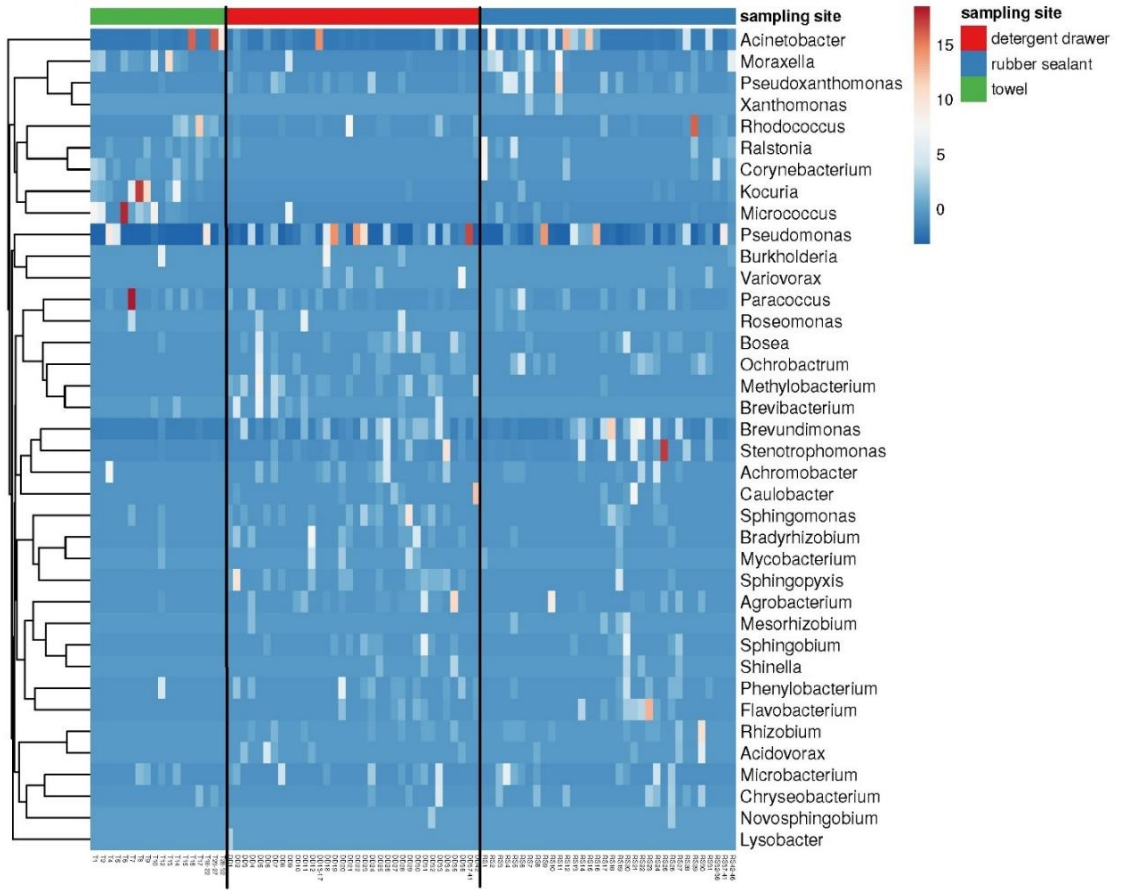



**Figure 11: Heatmap showing the main genera present at the different sampling sites. Figure shows "best hits" (i.e. 50% rel. frequency) of the metagenome analysis (n = 32 towels, 42 detergent drawer and 46 rubber sealants). The number of samples shown may differ from the true sample quantity, as individual samples were pooled in the metagenome analysis.**

The evaluation of the questionnaire with regard to existing experiences with laundry-associated malodor showed that 54.17% of the households did not have an existing laundry odor problem (Figure 12). 33.33% indicated and described problems with malodor, as described in the literature. Equally, 12.5% of the respondents reported a problem with laundry odor, but described other odor attributes.

This classification of households allowed a more detailed comparison of microbial counts and microbial composition.

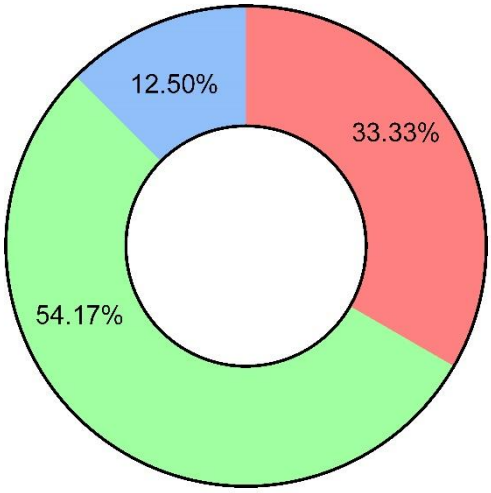



**Figure 12 Classification of households by questionnaire in households without odor problems (light green, n = 26), in households with laundry related malodor problems (light red, n = 16) and in households with other descriptive attributes for their laundry odor (light blue, n = 6)**

In general, the microbial counts for all sampling sites and all selective media showed no differences between malodor and non-objectionable households (Figure 13) except for the rubber sealant where the mean values tended to be slightly higher and *Staphylococcus spp.* counts were significantly higher in malodor households.

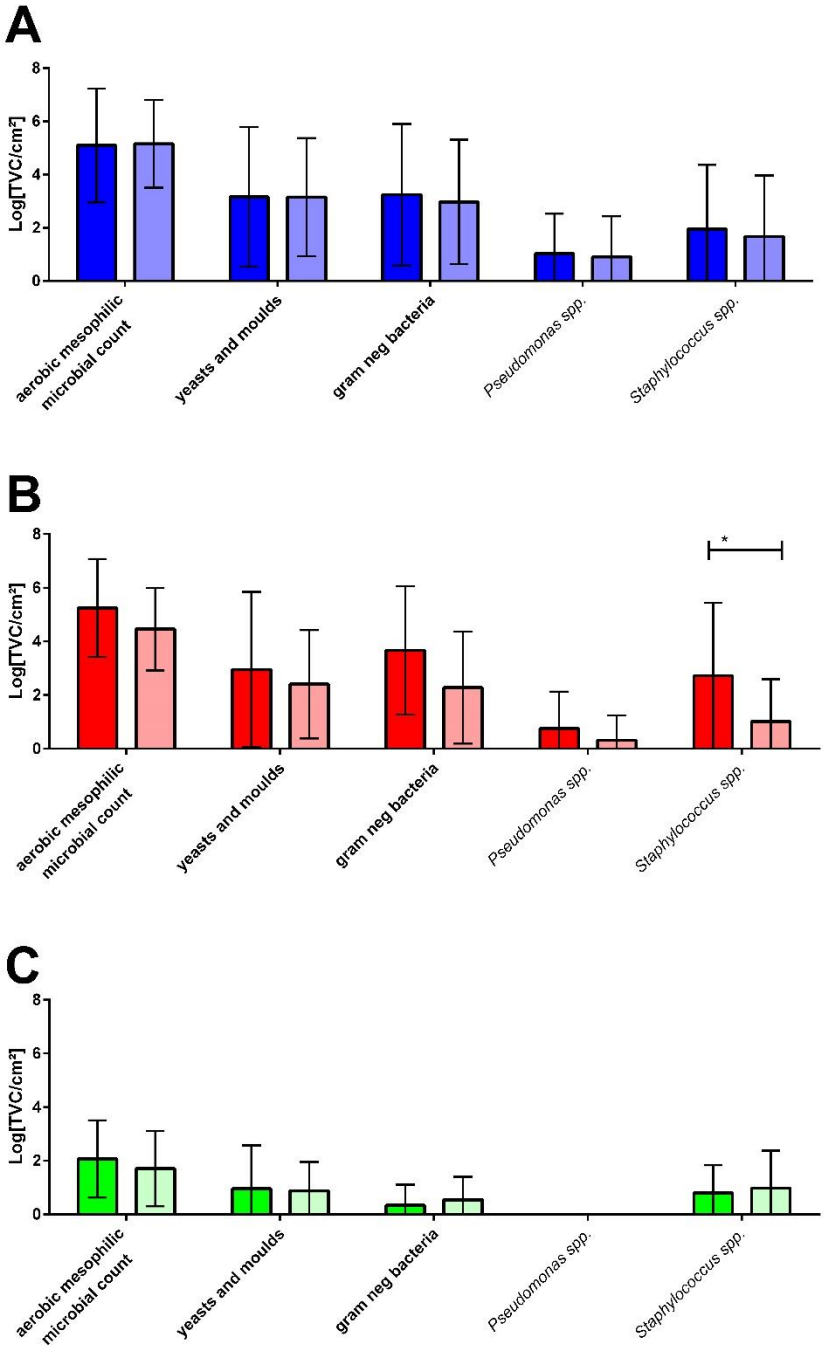



**Figure 13 Comparison of microbial counts in the detergent drawer (A), rubber sealants (B) and on used and unwashed towels (C) of laundry related malodor (dark columns, n = 16) and non malodor (light columns, n = 26) households. * ≤ 0.05**

Considering the presence of different genera in the malodour and no-malodour sampling sites, there was no clear microbial pattern that could be associated with either environment. For the detergent drawer, similar species were present in malodour and no-malodour machines, with an emphasis on members of the *Pseudomonadaceae*, such as *Pseudomonas*, *Pseudoxanthomonas, Sphingomonas and Brevundimonas* (Figure 14). Interestingly, several soil bacteria such as *Agrobacterium*, *Bosea* and *Methylobacterium* were found, particularly in the non-objectionable machines, of which only *Bosea* was also present in the malodour machines.

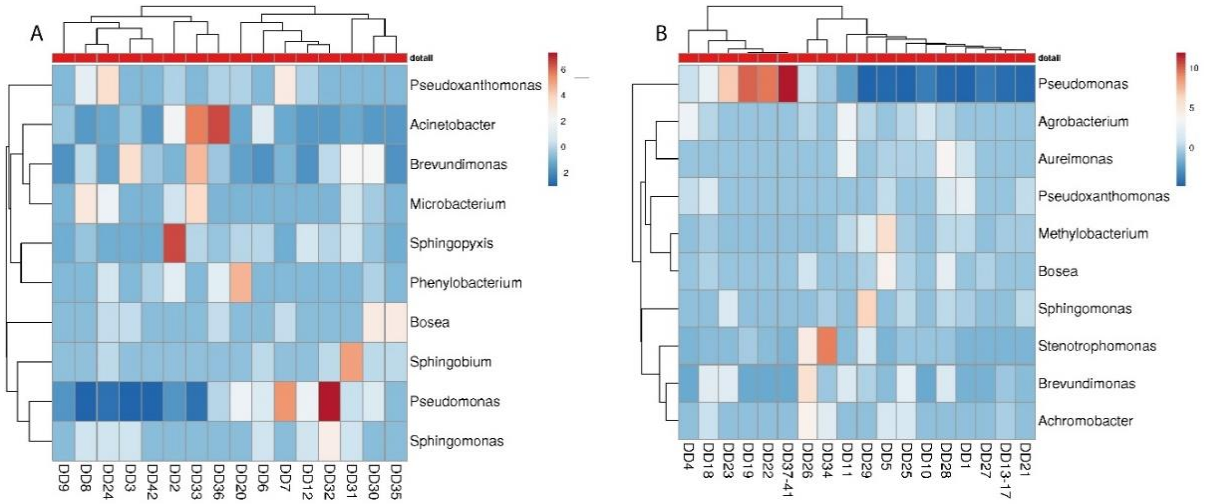



**Figure 14 Heatmap of the "best hits" (fractions >1% of the total quantity) of the bacterial genera identified in the metagenome analysis in different detergent drawers of malodor (A; n = 16) and non-objectonable (B, n = 26) households. The number of samples shown may differ from the true sample quantity, as individual samples were pooled in the metagenome analysis.**

For the rubber sealant *Pseudomonadaceae* again turned out to be the most common bacterial colonizer (Figure 15). However, other microbial genera could be identified, which were not that abundant in the detergent drawer. *Inter alia*, *Cutibacterium* and *Moraxella* were present in malodor machines, while *Rhizobium* and *Agrobacterium* could be found only in non-objectionable machines. Again, other soil bacteria, such as *Rhodococcus* could be found in both malodor and non-objectionable machines.

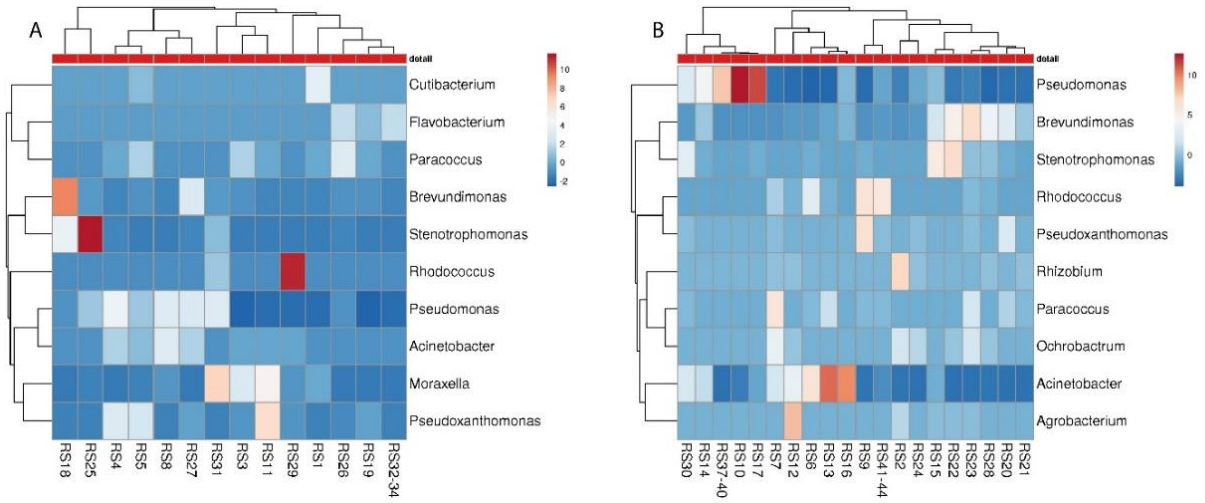



**Figure 15 Heatmap of the "best hits" (fractions >1% of the total quantity) of the bacterial genera identified in the metagenome analysis in different rubber sealants of malodor (A; n = 16) and non-objectonable (B, n = 26) households. The number of samples shown may differ from the true sample quantity, as individual samples were pooled in the metagenome analysis.**

Although towels harbour microbial communities that are different from the sampling sites inside the washing machine, there were some considerable consistencies in terms of malodor-relations (Figure 16). Again, different soil bacteria were identified on the non-objectionable towels: *Rhodococcus*, *Blastococcus* and *Phenylobacterium*, whereas on the malodor towels, the genera *Moraxella*, *Staphylococcus*, *Corynebacterium* and *Micrococcus* were present which have been previously described to be associated with malodor (Kubota *et al.*, 2012; M. Zinn *et al.*, 2021).

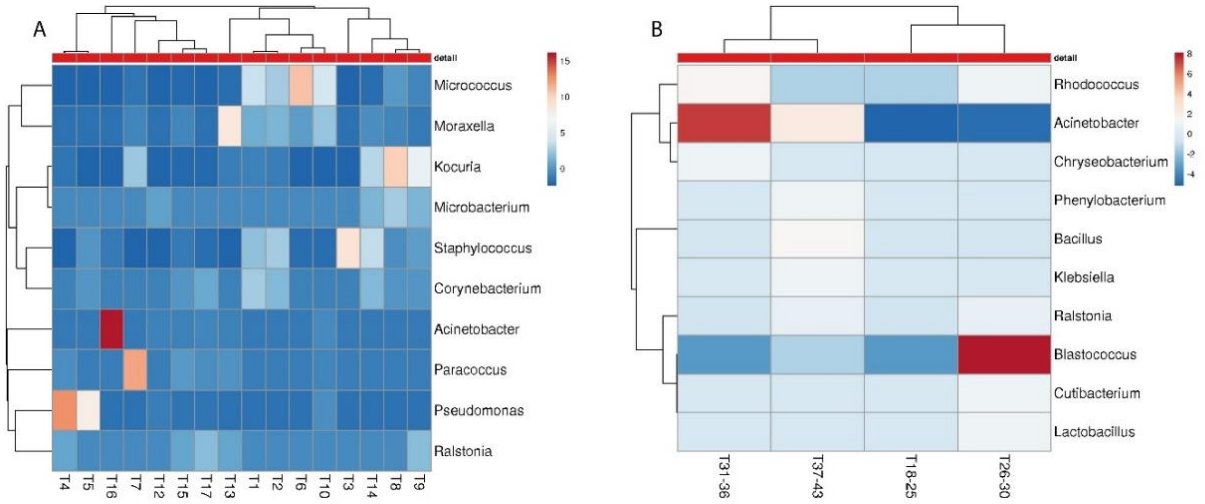



**Figure 16 Heatmap of the "best hits" (fractions >1% of the total quantity) of the bacterial genera identified in the metagenome analysis in different towels of malodor (A; n = 16) and non-objectonable (B, n = 26) households. The number of samples shown may differ from the true sample quantity, as individual samples were pooled in the metagenome analysis.**

In total, thirteen facultative pathogenic strains could be identified in this study (Table 7 - Table 8). Amongst other bacteria associated with humans, staphylococci and *Cutibacterium acnes* in particular were regularly detected on the towel samples. In contrast, the dominant species in the washing machine were water-borne bacteria such as *P. aeruginosa,* and *Stenotrophomonas maltophilia (S. maltophilia)*, which have been found in washing machines before (Schages *et al.*, 2021).

**Table 7 50 most common species in detergent drawer, rubber sealant and on various towels presented as rel. frequency in % of total microbial composition (nd= not defined in (Anonymous, 2015a, 2016)). Part 1**

| species | risk group (according to (Anonymous, 2015a, 2016) | detergent drawer (in %) | rubber sealant (in %) | hand towel (in %) | body towel (in %) | kitchen cloth (in %) |
|---|---|---|---|---|---|---|
| *Actinomycetospora succinea* | 1 | 0.21 | 0.02 | 0.02 | 0.00 | 0.00 |
| *Agrobacterium tumefaciens* | 1 | 0.51 | 0.36 | 0.02 | 0.00 | 0.00 |
| *Aspergillus glaucus* | 1 | 0.00 | 0.00 | 0.73 | 0.00 | 0.00 |
| *Aureimonas altamirensis* | 1 | 0.56 | 0.03 | 0.01 | 0.00 | 0.00 |
| *Bacillus subtilis* | 1 | 0.01 | 0.00 | 2.89 | 0.00 | 0.00 |
| *Brevundimonas bullata* | 1 | 0.22 | 0.27 | 0.00 | 0.00 | 0.00 |
| *Brevundimonas diminuta* | 1 | 0.17 | 0.30 | 0.01 | 0.00 | 0.00 |
| *Brevundimonas sp DS20* | 1 | 0.13 | 0.23 | 0.00 | 0.00 | 0.00 |
| *Brevundimonas sp SH203* | 1 | 0.35 | 0.31 | 0.01 | 0.00 | 0.01 |
| *Flavobacterium lindanitolerans* | 1 | 0.05 | 0.26 | 0.00 | 0.00 | 0.00 |
| *Homo sapiens* | 1 | 0.03 | 0.24 | 0.24 | 2.17 | 0.15 |
| *Kocuria rhizophila* | 1 | 0.01 | 0.07 | 1.60 | 0.07 | 0.01 |
| *Methylorubrum extorquens* | 1 | 0.23 | 0.00 | 0.01 | 0.00 | 0.00 |
| *Micrococcus luteus* | 1 | 0.22 | 0.16 | 2.54 | 0.02 | 0.01 |
| *Mycolicibacterium tusciae* | 1 | 0.19 | 0.05 | 0.01 | 0.00 | 0.00 |
| *Pseudomonas fluorescens* | 1 | 0.20 | 0.15 | 0.14 | 0.03 | 0.02 |
| *Pseudomonas fragi* | 1 | 0.01 | 0.12 | 0.01 | 6.57 | 4.49 |
| *Pseudomonas oleovorans* | 1 | 0.04 | 0.27 | 0.00 | 0.00 | 0.00 |
| *Pseudomonas veronii* | 1 | 0.29 | 0.05 | 0.00 | 0.00 | 0.00 |
| *Pseudoxanthomonas spadix* | 1 | 0.02 | 0.58 | 0.00 | 0.00 | 0.00 |
| *Pseudoxanthomonas suwonensis* | 1 | 0.11 | 0.39 | 0.00 | 0.00 | 0.00 |
| *Rhizobiales bacterium* | 1 | 0.16 | 0.21 | 0.01 | 0.00 | 0.01 |
| *Rhizobium sp ACO-34A* | 1 | 0.00 | 0.34 | 0.00 | 0.00 | 0.00 |
| *Rhodococcus erythropolis* | 1 | 0.04 | 0.10 | 0.18 | 0.17 | 0.20 |
| *Skermanella aerolata* | 1 | 0.22 | 0.10 | 0.00 | 0.00 | 0.00 |

**Table 8 50 most common species in detergent drawer, rubber sealant and on various towels presented as rel. frequency in % of total microbial composition (nd= not defined in (Anonymous, 2015a, 2016)). Part 2**

| species | risk group (according to (Anonymous, 2015a, 2016) | detergent drawer (in %) | rubber sealant (in %) | hand towel (in %) | body towel (in %) | kitchen cloth (in %) |
|---|---|---|---|---|---|---|
| *Staphylococcus warneri* | 1 | 0.00 | 0.00 | 0.66 | 0.02 | 0.00 |
| *Stenotrophomonas rhizophila* | 1 | 0.06 | 0.20 | 0.00 | 0.00 | 0.07 |
| *Xanthobacter autotrophicus* | 1 | 0.37 | 0.08 | 0.00 | 0.00 | 0.00 |
| *Xanthobacter tagetidis* | 1 | 0.04 | 0.26 | 0.00 | 0.00 | 0.00 |
| *Acinetobacter johnsonii* | 2 | 0.17 | 0.47 | 0.16 | 0.00 | 3.00 |
| *Acinetobacter ursingii* | 2 | 0.26 | 0.32 | 0.03 | 0.00 | 0.55 |
| *Brevibacterium casei* | 2 | 0.57 | 0.03 | 0.11 | 0.01 | 0.00 |
| *Cutibacterium acnes* | 2 | 0.02 | 0.15 | 0.54 | 12.26 | 0.21 |
| *Moraxella osloensis* | 2 | 0.42 | 2.75 | 3.26 | 0.05 | 0.60 |
| *Paracoccus yeei* | 2 | 0.11 | 0.37 | 0.74 | 0.00 | 0.03 |
| *Pseudomonas aeruginosa* | 2 | 1.04 | 0.35 | 2.84 | 0.07 | 0.05 |
| *Pseudomonas alcaligenes* | 2 | 0.20 | 0.07 | 0.00 | 0.00 | 0.00 |
| *Pseudomonas stutzeri* | 2 | 0.64 | 0.50 | 0.08 | 0.00 | 0.01 |
| *Roseomonas gilardii* | 2 | 0.21 | 0.03 | 0.05 | 0.00 | 0.00 |
| *Staphylococcus aureus* | 2 | 0.00 | 0.02 | 1.40 | 0.16 | 0.04 |
| *Staphylococcus epidermidis* | 2 | 0.02 | 0.01 | 0.47 | 0.44 | 0.02 |
| ~~*Stenotrophomonas maltophilia*~~ | 2 | 0.70 | 1.54 | 0.04 | 0.01 | 0.02 |
| *Aquabacterium sp SJQ9* | (nd) | 0.64 | 0.03 | 0.00 | 0.00 | 0.00 |
| *Blastococcus sp CCUG 61487* | (nd) | 0.02 | 0.20 | 0.05 | 0.00 | 0.00 |
| *Janibacter indicus* | (nd) | 0.14 | 0.23 | 0.04 | 0.00 | 0.00 |
| *Micavibrio aeruginosavorus* | (nd) | 0.01 | 0.63 | 0.00 | 0.00 | 0.00 |
| *Paracoccus salipaludis* | (nd) | 0.00 | 0.02 | 0.52 | 0.00 | 0.00 |
| *Phenylobacterium sp Root700* | (nd) | 0.36 | 0.19 | 0.25 | 0.00 | 0.00 |
| *Rahnella inusitata* | (nd) | 0.00 | 0.00 | 0.01 | 3.03 | 1.28 |

Statistical analysis of the bacterial genera found at different sampling sites in malodor and non-objectionable households shows that more genera tend to be identified in malodor households than in non-objectionable households. Furthermore, more genera are found in the washing machine (5-15 genera) than on the towels (2-7 genera) (Table 9).

**Table 9 Shannon diversities of the bacterial genera identified in the metagenome analysis in different towels of malodor (A; n = 16) and non-objectonable (B, n = 26) households.**

| Sample | Shannon-diversity | Standard deviation |
|---|---|---|
| malodor detergent drawer | 15.03 | 7.28 |
| non-objectionable detergent drawer | 10.56 | 9.38 |
| malodor rubber sealant | 10.57 | 8.02 |
| non-objectionable rubber sealant | 5.03 | 4.09 |
| malodor towel | 7.25 | 5.56 |
| non-objectionable towel | 2.75 | 1.24 |

The PCAs (Figure 17) show that malodor samples in all three sampling sites formed clusters, which, for the detergent drawer and the rubber sealant lied within the non-objectionable samples' distribution, suggesting a distinct, yet not completely different colonization pattern for malodorous machines. In contrast, the malodor samples from the investigated towels differentiate more clearly from the non-objectionable samples. Species that are only present in non-objectionable machinery may exert their protective properties by suppressing odour-producing species or by metabolising odour-producing substances.

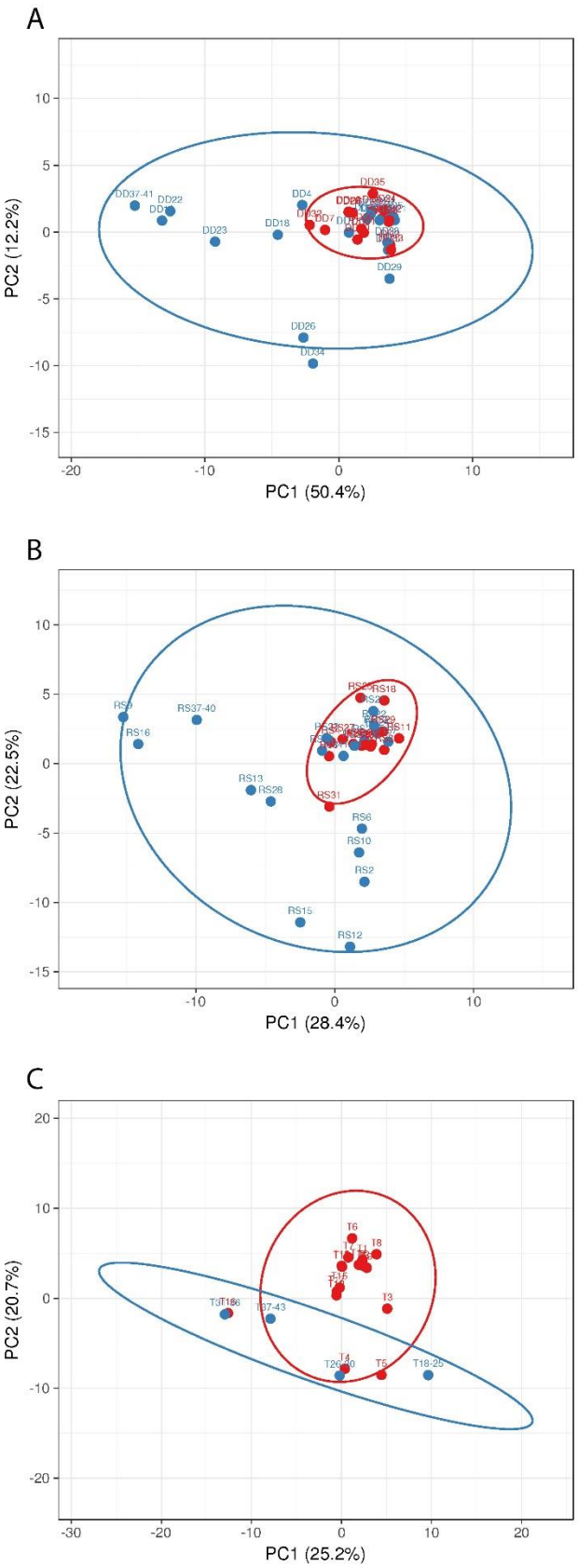



**Figure 17 Principle Component Analysis (PCA) of the different sampling sites (A: detergent drawer; B: rubber sealant; C: towel malodor and (red, n = 16) and non-objectionable (blue, n=26) households). The number of samples shown may differ from the true sample quantity, as individual samples were pooled in the metagenome analysis.**

## 4.2 *Ex situ* model for investigating microbial communities isolated from household washing machines

Since the conditions for bacterial growth in a washing machine are very diverse and complex, it has not yet been possible to establish a household biofilm in the laboratory. In order to carry out experiments in the laboratory that are even closer to the consumer in the future, a method for transferring a household biofilm to the laboratory is to be developed.

### 4.2.1 Development of a stable *ex situ* biofilm model and the recultivation of biofilms from glycerol stocks

Biofilms are complex communities of microorganisms commonly found in natural and engineered environments. To study the biology and ecology of biofilms, it is often necessary to develop models that can replicate their behaviour in a controlled laboratory environment. *Ex situ* biofilm models are particularly useful because they allow researchers to manipulate environmental conditions and test the effects of different variables on biofilm growth, stability and function.

However, developing a stable *ex situ* biofilm model for domestic biofilms can be challenging. Biofilms are dynamic communities that are influenced by a wide range of factors, including nutrient availability, temperature, pH and microbial interactions. These factors can affect biofilm structure and function, making it difficult to replicate biofilm behaviour in the laboratory.

A stable *ex situ* biofilm model can overcome these challenges by providing a controlled environment in which to study biofilm behaviour. Such a model can be used to investigate the mechanisms underlying biofilm formation, growth and stability, and to test the efficacy of different treatments or interventions to control or manipulate biofilm communities.In addition to establishing an *ex situ* model, it will be investigated whether it is possible to store a successful transfer via glycerol stocks and to use it again after a storage period. This would avoid time-consuming preparatory work.

#### 4.2.1.1 Biofilm cultivation from original washing machine biofilm

Different biofilms from different washing machines were grown for 7 or 10 days in 6-well cell culture dishes on coverslips. The initial melting curves for the tested washing machines are shown in Figure 18. The results of the establishment experiments are shown in melting curves in Figures 19 to 23 for the detergent drawer and in Figures 24 to 27 for the rubber sealant. Figure 18 shows the melting curves of the gDNA of the initial biofilms from three different washing machines. The active melting region of the melting curve analysis showed a high diversity between the machines. Likewise, slight shifts of the peaks within a machine can be observed (A: the main peak of machine 1 (orange) is at approx.

86 °C, resp. (purple) at approx. 86.6 °C). Comparing the washing machines rubber sealants, machine 1 showed a main peak at approx. 86 °C. Machine 2, on the other hand, showed a main peak at approx. 86.6 °C and a smaller peak at approx. 83.5 °C. Machine 3 had a main peak at approx. 87.7 °C and two smaller shoulders at 86.5 °C and 88.6 °C. There were also two smaller peaks at 83.5 °C and 84.7 °C.

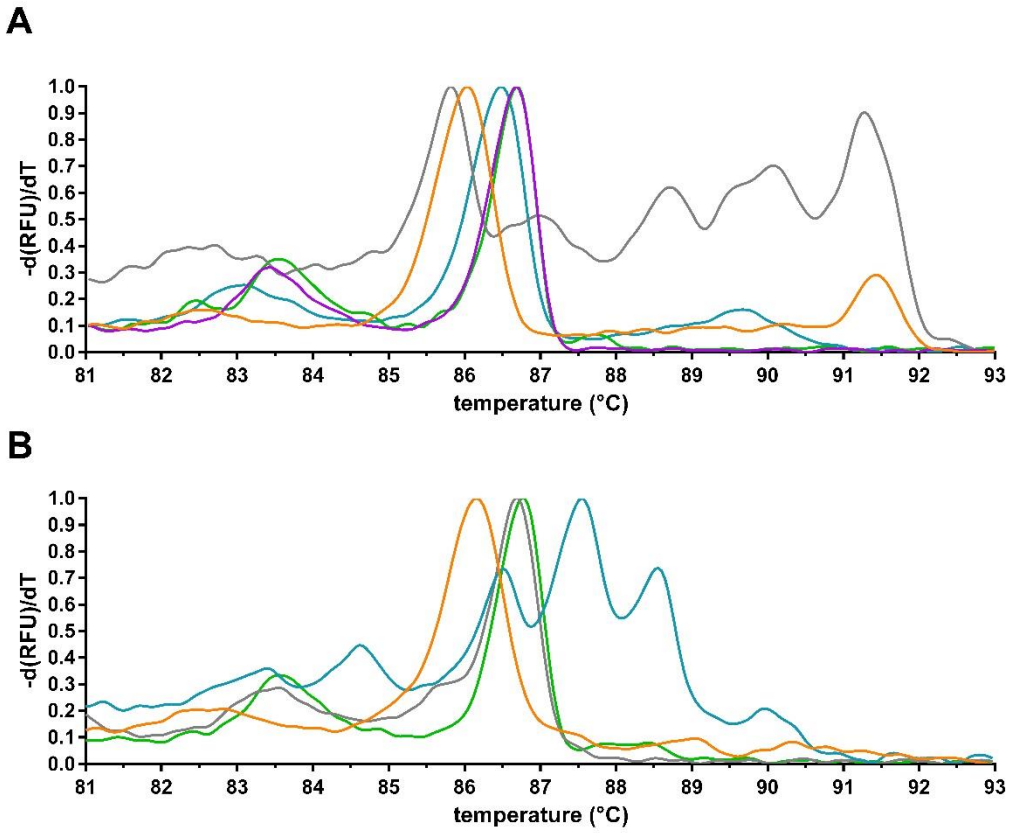



**Figure 18 Normalized melting curve of the active melting region of the gDNA of the biofilm samples from three independent washing machines at time t = 0 with the addition of medium 4. The different colours represent the three machines (machine 1[malodor machine]: orange and purple; machine 2 [non-objectionable machine]: gray and green; machine 3 [non-objectionable machine]: turquoise). A shows the melting curves for the detergent drawer, B for the rubber sealant**

Detergent drawer

Figure 19 illustrates the melting curve of the gDNA of the biofilm sample from the detergent drawer of machine 1 at the beginning (grey curve), after 7 d (A, C, E) and after 10 d (B, D, F) in each case in the quadruplicate assay. The multiple measurements all showed very similar curves, but some of them deviate significantly from the initial t=0 curve. The largest main peak of the melting curve from t=0 was at approx. 86 °C, a second peak could be seen at approx. 91.5 °C. The main peak was also almost reached with media 1 and 2 (A-D). Medium 1 additionally showed a characteristic shoulder at approx. 85 °C and a small additional peak at approx. 88 °C (A + B). The characteristic shoulder of medium 1 could also be seen

with medium 2 (C + D). The additional peak at 88 °C was missing here, however. Medium 3 (E+F) showed a shifted main peak (approx. 88 °C) compared to t = 0 and two additional peaks at approx. 85 °C and 86 °C. Medium 4 (G, H) also showed three additional peaks at 82 °C, 83 °C and 88 °C in addition to the main peak. Medium 5 (I, J) showed two additional peaks at 82 °C and 82.5 °C in addition to the main peak at 86.5 °C.

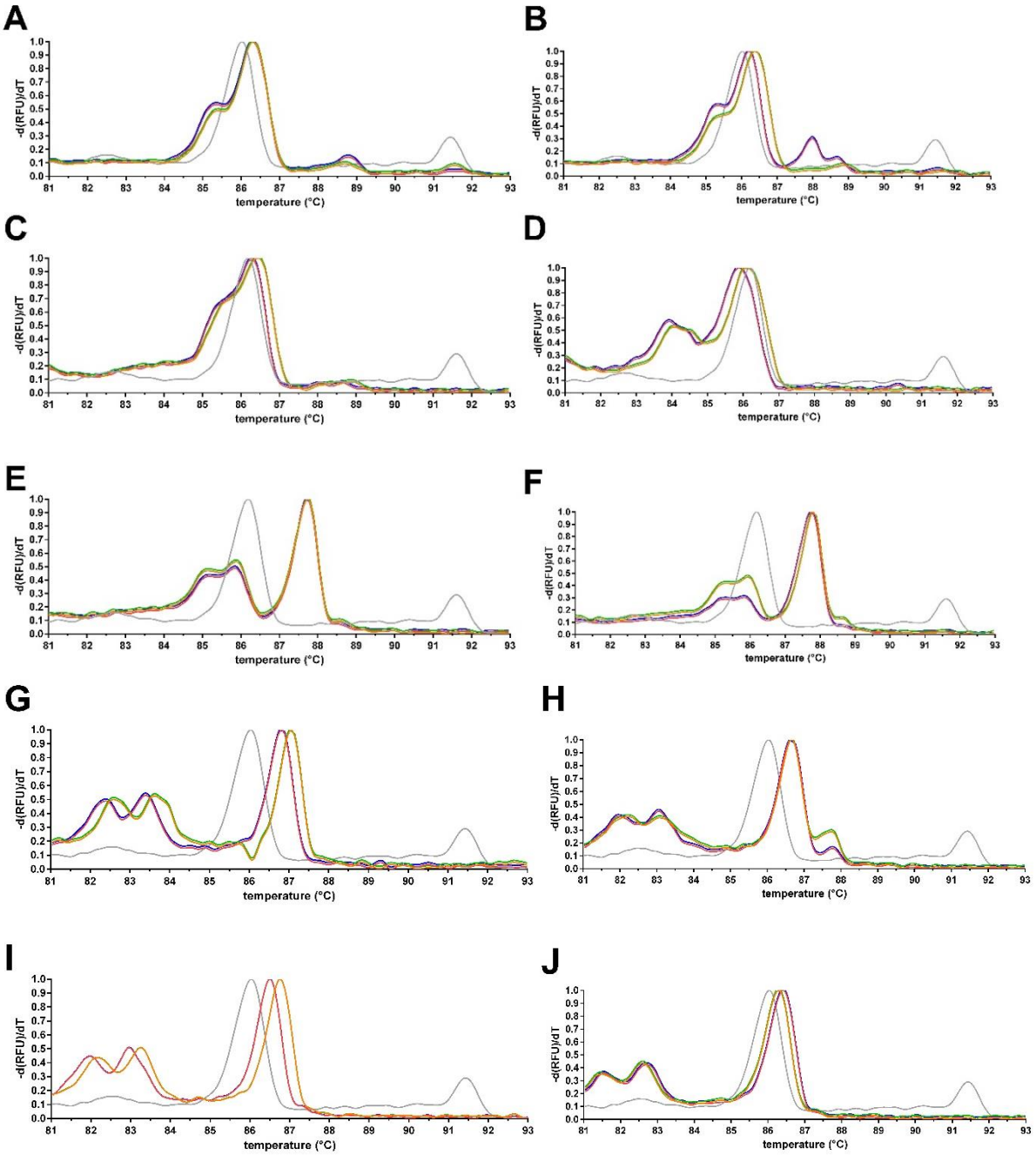



**Figure 19 Normalized melting curve of the active melting region of gDNA of the biofilm sample from the detergent drawer of machine 1 [malodor machine] at time point t = 0 (gray curve) and time points t= 7 (A, C, E) and t = 10 (B, D, F) with the addition of different media (medium 1: A, B; medium 2: C, D; medium 3: E,F; medium 4: G, H; medium 5: I, J). The various colours (orange, red, green and blue) show the four independent replicates. n = 4**

As depicted in Figure 20 the first gDNA fusion curve of the biofilm sample from machine 2's detergent drawer (grey curve), 7d (A, C, E) and 10d (B, D, F) by quadrupling. Except for B, the multiple determinations show very similar curve progressions, but in some cases strongly deviate from the initial curve t=0. The first curve had a main peak at 86.5 °C and two smaller peaks at 82.5 °C and 83.5 °C. Media 1 and 2 (A-D) have a central peak at 86 °C, while in B, two of the four repetitions have significant deviations and the central peak is 88 °C. All samples also showed fewer peak (grey) than the original samples. The medium 2 (C, D) also showed two shoulders at 84 °C and 87.3 °C, as well as the main peak. The 3rd medium (E, F) shows a shift in the main peak to 87.5 °C, and two smaller peak to 85 °C and 85.5 °C.

After 7d, medium 4 also showed a peak of 83.5 °C and another peak of 84.7 °C (B). After 10d, it is consistent with the first curve's three peaks and the additional 87.5 °C (C) peaks. The melting curve with medium 5 (D, E) is also consistent with the initial sample, with the peak slightly shifting to the left (81.7 °C, 83 °C, and 86 °C). The peak was 87.5 °C.

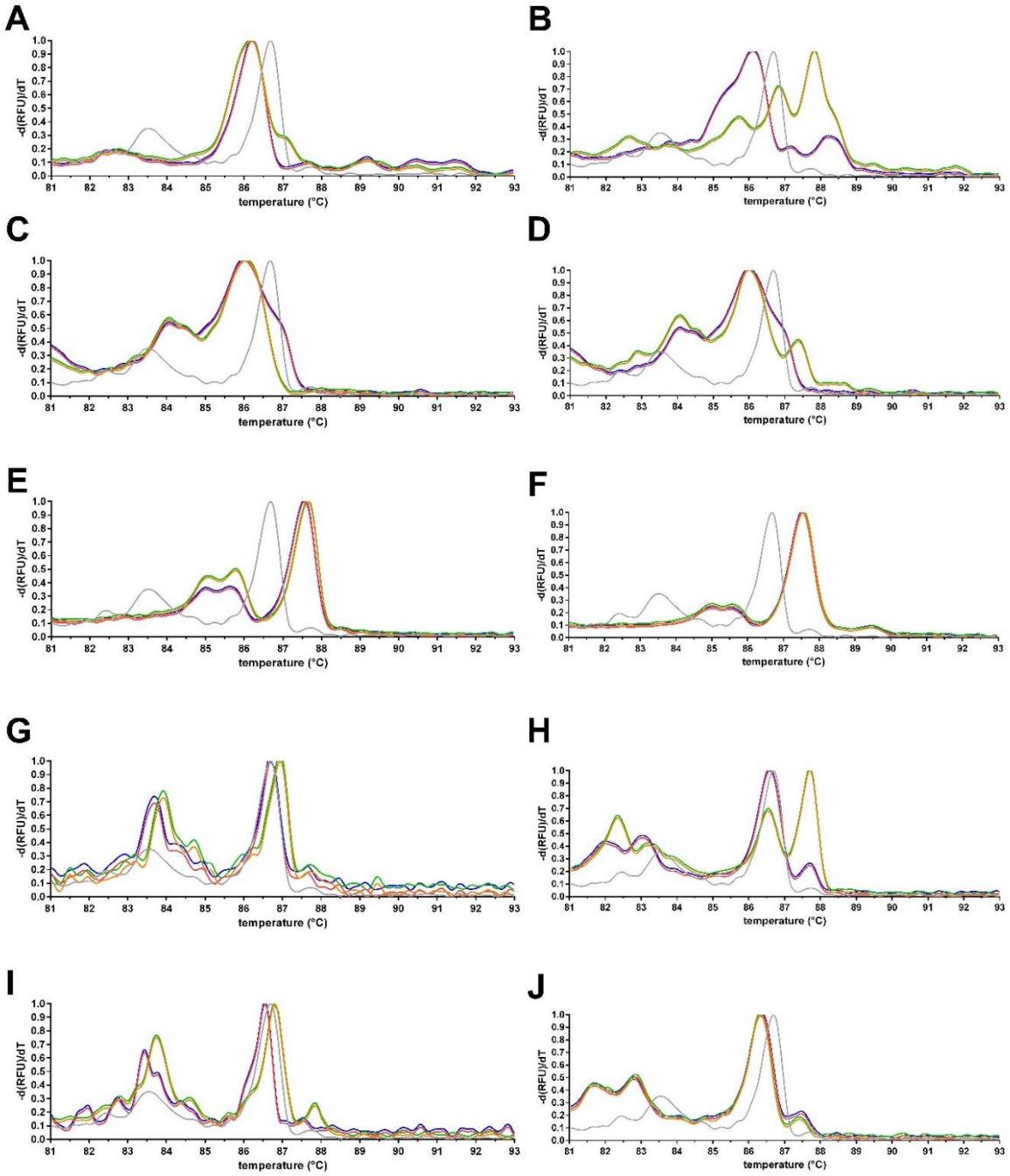



**Figure 20 Normalized melting curve of the active melting region of gDNA of the biofilm sample from the detergent drawer of machine 2 [non-objectionable machine] at time point t = 0 (gray curve) and time points t= 7 (A, C, E) and t = 10 (B, D, F) with the addition of different media (medium 1: A, B; medium 2: C, D; medium 3: E,F; Medium 4: G, H; medium 5: I, J). The various colours (orange, red, green and blue) show the four independent replicates. n = 4**

The data in Figure 21 reveals the gDNA melting curves of the biofilm sample from the initial machine 3 detergent drawer (grey curves) and the subsequent 10d quadruple tests. With the exception of B, all multiple determinations show a very similar curve progression, but the initial curve is t=0. The main peak of the initial curve is 86.5 °C and two smaller peak is 83 °C and 89.5 °C. The curves of medium 1 (A) and medium 5 (C) were similar, but were significantly different from the original samples. There were three main peaks of 82 °C, 84 °C and 87 °C, and two shoulders of 82.5 °C and 85.5 °C. This curve can also be seen in two of four repetitions of medium 2 (B). However, the other two replicates showed similarities with the original sample. The main peak was 86.5 °C, and the two smallest peaks were 82 °C and 84 °C. Medium 4 (D) showed very good conformity with the initial sample in two of the four copies. These characteristic peaks occur simultaneously. The other two reproductions showed a similar curve, but were moved to the left at about 0.5 °C.

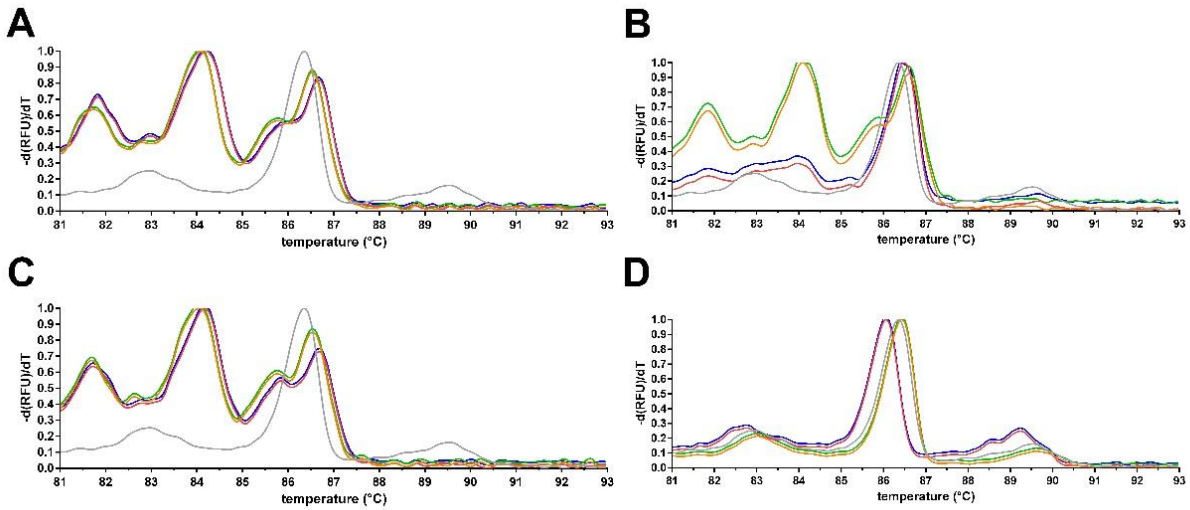



**Figure 21 Normalized melting curve of the active melting region of gDNA of the biofilm sample from the detergent drawer of machine 3 [non-objectionable machine] at time point t = 0 (gray curve) and time point t = 10 (A, B, C, D) with the addition of different media (medium 1: A; medium 2: B; medium 5: C; medium 4: D). The various colours (orange, red, green and blue) show the four independent replicates. n = 4**

Rubber sealant

Figure 22 illustrates the gDNA fusion curves of biofilm samples extracted from machine 1 rubber sealants (grey curves) and 7d (A, C, E, G, I) resp. 10d (B, D, F, H, J) in quadruple measurement. The multiple determinations are all similar, but some of them are significantly different from the initial curve t=0. The initial curve had a peak of 86 °C and a small shoulder of 82.5 °C. Biofilms with medium 1 (A, B) also showed an important peak at 86 °C in the melting curve. Furthermore, two other peaks could be observed at 88 °C and 90.5 °C. Overall, the values were slightly higher than in the original sample. The same applies to medium 2 (C, D). The melting curve showed two main peaks at 86 °C and 87 °C, as well as another peak at 88 °C. Medium 3 (E, F), on the other hand, deviates from the main peak (87.5 °C) of the first sample and shows two other small peaks of 85 °C and 85.5 °C. After 7 d, medium 4 (B, C) showed a large-scale melting curve called "noise", while at 10 d, two samples each showed similar curves. One peak is 86.2 °C, two other peak is 81.5 °C, and two other peak is 86.7 °C, two other peak is 82 °C and 83.5 °C. Average 5 (D, E) showed a main peak of 86.5 °C after 7 d and two other peaks of 82.3 °C and 83.5 °C, and after 10 d the curve shifted 0.5 °C to the left.

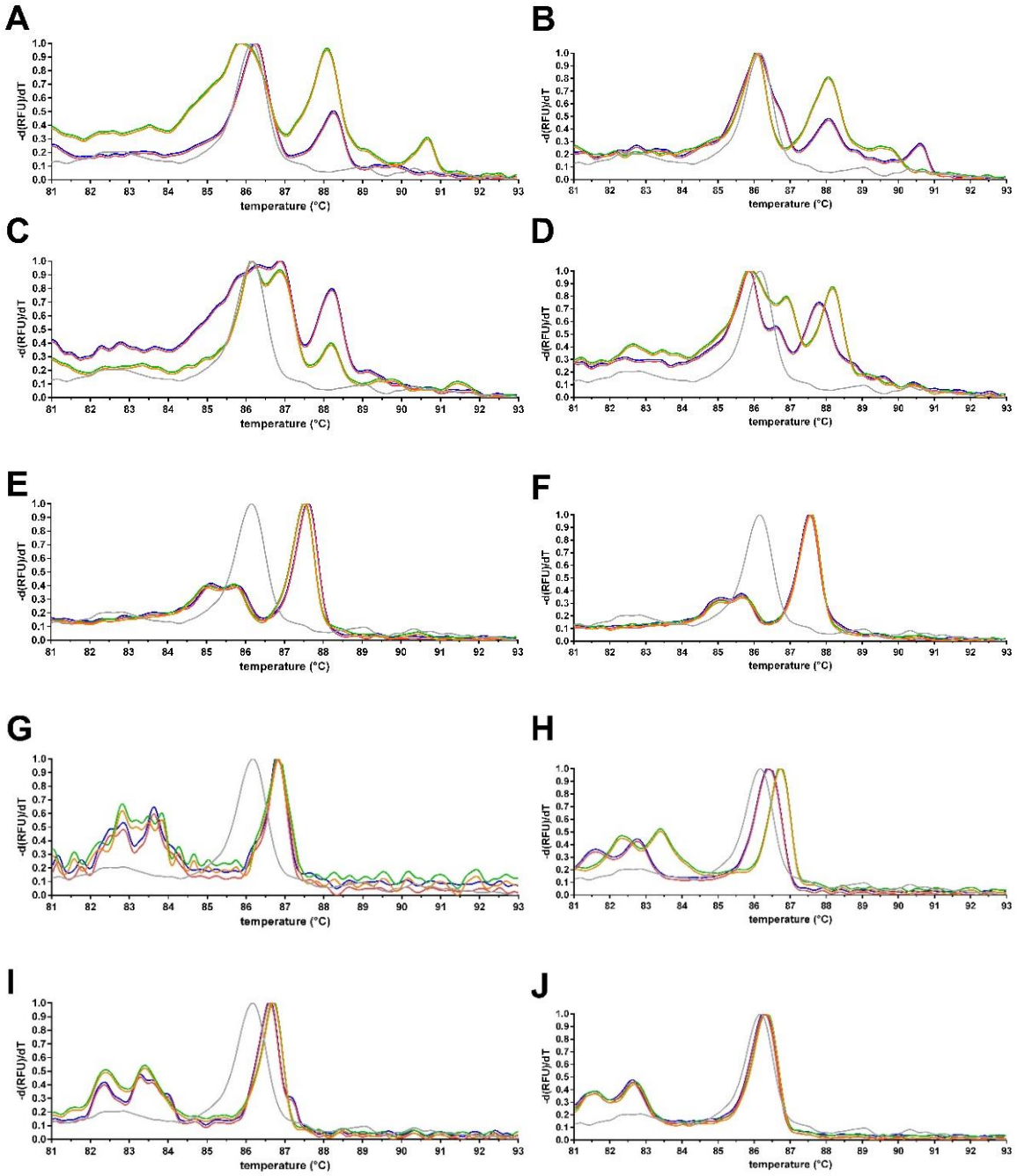



**Figure 22 Normalized melting curve of the active melting region of gDNA of the biofilm sample from the rubber sealant of machine 1 [malodor machine] at time point t = 0 (gray curve) and time points t= 7 (A, C, E, G, I) and t = 10 (B, D, F, H, J) with the addition of different media (medium 1: A, B; medium 2: C, D; medium 3: E, F; medium 4: H, H; medium 5: I, J). The various colours (orange, red, green and blue) show the four independent replicates. n = 4**

Figure 23 shows the gDNA melting curve of the biofilm sample of machine 2 rubber sealant at the beginning (grey curve) and after 7d (B, D) resp. 10d (A, C, E) in a quadruple setup. Multiple determinations showed a similar curve after 10 d, but in part they deviated considerably from the initial curve t=0. The initial curve had a major peak of 86.5 °C and a smaller peak of 83.5 °C. Medium 1 (A) shows two samples with similar characteristics. The first two had a main peak of 85°C and two smaller peaks of 86.5 °C and 87.7 °C, while the other two also had a main peak of 86.5 °C. In addition, a few small peaks of 82.5 °C, 84 °C, 84.7 °C, and 87.7 °C can be seen. The fusion curve of medium 4 (B, C) also shows that after 7 d (B), two samples each have a different curve shape. One peaks with a major peak at 87°C and two other peak at 82.5 °C and 83.5 °C, and the other shows a major peak at 87.3 °C and many other small peaks, which can be described as "noise". After 10 d, all four repetitions behaved the same, with two additional peaks at 82 °C and 83 °C, the main peak being above the initial curve. After 7d and 10d, medium 5 (D, E) behaved similarly to medium 4.

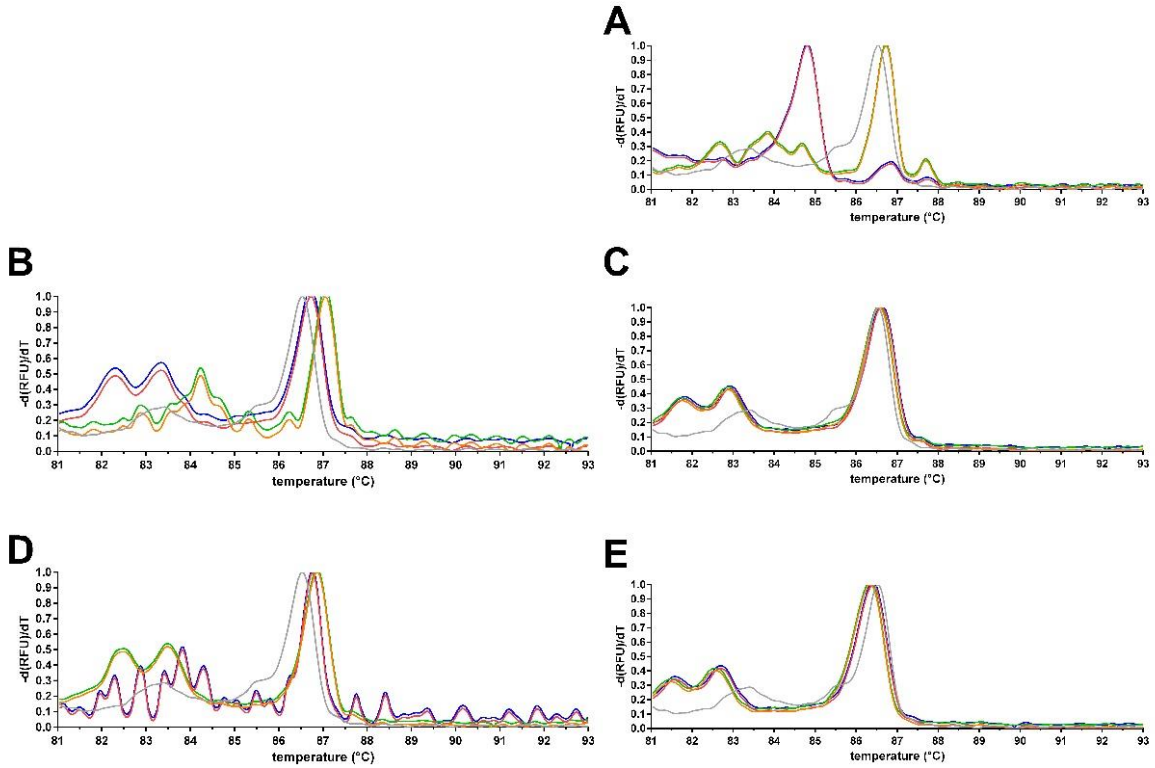



**Figure 23 Normalized melting curve of the active melting region of gDNA of the biofilm sample from the rubber sealant of machine 2 [non-objectionable machine] at time point t = 0 (gray curve) and time points t= 7 (B, D) and t = 10 (A, C, E) with the addition of different media (medium 1: A; medium 4: B, C; medium 5: D, E). The various colours (orange, red, green and blue) show the four independent replicates. n = 4**

As depicted in Figure 24 the gDNA melting curve of the biofilm sample in the detergent drawer of machine 3 at the beginning (grey curve) and after 10 d in the quadruple experiment. With the exception of A and B, multiple determinations show a very similar curve progression, but in part differ considerably from the initial curve t=0. The first curve had a main peak of 87.5 °C, two shoulders of 86 °C and 88.3 °C, and a small peak of 84.5 °C. Middle 1 (A) shows two different curves. One curve has a main peak of 84 °C, several small peaks of 81.5 °C, 85.5 °C, and 86.5 °C, while the other curve has two main peaks of 88 °C and 90 °C. In addition, there were small peaks at 81.5 °C, 85.5 °C and 87 °C. Medium 2 (B) also showed two different curves. On the one hand, there is a main peak of 84 °C and three other peaks of 82 °C, 85.5 °C and 86.5 °C, on the other, there is a main peak of 86.5 °C and two other peaks of 82 °C and 84 °C. The upper peak of medium 4 (C) was 87 °C, and other peaks were 82 °C, 84 °C, and 86 °C. Medium 5 (D) showed a similar course to medium 8 with a main peak of 87 °C and other peaks of 82 °C, 84 °C and 85.5 °C.

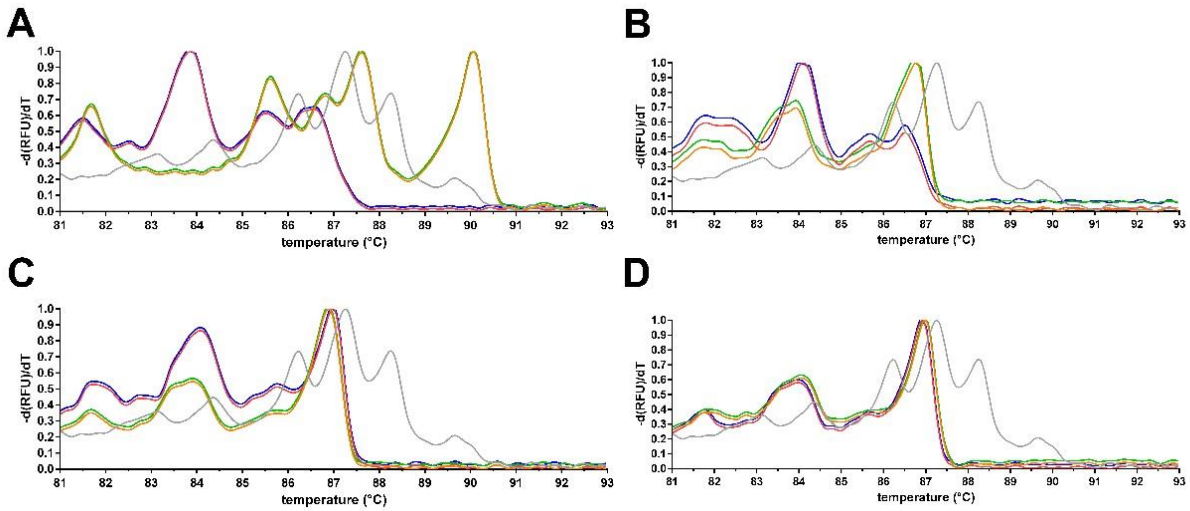



**Figure 24 Normalized melting curve of the active melting region of gDNA of the biofilm sample from the rubber sealant of machine 3 [non-objectionable machine] at time point t = 0 (gray curve) and time point t = 10 (A, B, C, D) with the addition of different media (medium 1: A; medium 2: B; medium 4: C; medium 5: D). The various colours (orange, red, green and blue) show the four independent replicates. n = 4**

### 4.2.1.2 Recultivation of biofilms from glycerol stocks

In order to be able to examine washing machine biofilms independently of continuous sampling, the original biofilm was mixed 1:1 with glycerol (80%) and frozen at a temperature of -80 °C. The biofilms were then used as a stock. New experiments were prepared from these stocks and their biofilms were frozen again after 10 d as a stock. The results of these cultivations can be seen in Figure 25 (A-H).

The data in Figure 25 reveals the active melting range of g DNA of various biofilm samples after 6-fold determination of glycerol recovery from the stock. The replicates showed

high levels of agreement, but in some cases they are strongly different from the original (grey) sample. The two samples grew in medium 5 (A, C) and clearly showed a change in the melting curve of the original sample. Compared to grow that medium 4 (B, D), the curve was clearly less harmonious. In particular, sub-figure B showed a high degree of agreement between there plica and the original sample. The highest temperature was 86 °C and the lowest was 82.5 °C. The curves of machine 2 (E, F) and machine 3 (G, H) are similar in that the main peak of the reactivation sample moved to the right between 1 °C and 0.5 °C compared to the original sample. It could also be seen that the four runs had an additional peak of 81.5 °C.

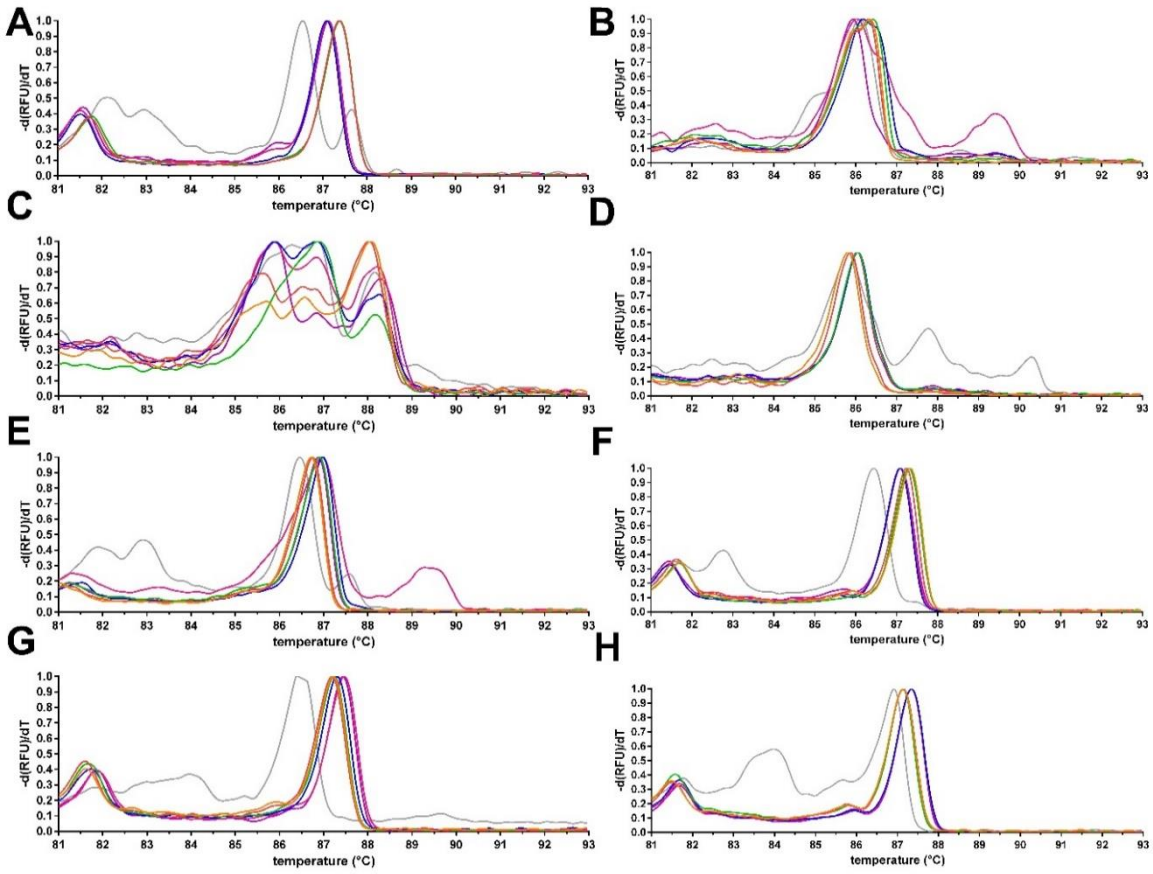



**Figure 25 Normalized melting curve of the active melting region of gDNA of biofilm samples (after 10d incubation and stored in glycerin stocks; A, B: machine 1 malodor detergent drawer; C, D: machine 1 malodor rubber sealant; E: machine 2 non-objectionable detergent drawer; F: machine 2 non-objectionable rubber sealant; G: machine 3 non-objectionable detergent drawer; H: machine 3 non-objectionable rubber sealant) at time point t = 0 (gray curve) and time point t = 10 (A-H) with the addition of different media (medium 4: B, D, E, F, G, H; medium 5: A, C). The various colours (orange, red, green, purple, pink and blue) show the six independent replicates. n = 6**

Table 10 shows the Euclidean distances of the standardised melting curves. The Euclidean distance was determined for each sampling site (rinsing chamber and rubber seal of three washing machines), each incubation time (t7 and t10) and five different media (composition see Table 1).

Evaluation of the Euclidean distances showed that of the media tested, the composition of 0.001% meat extract and 0.001% potato starch had the lowest Euclidean distances (medium 4: 0.34). Media 1 (0.1% TSB + 0.1% MEB), 3 (0.1% TSB + 0.1% MEB + 0.1% APG + 0.002% potato starch) and 5 (0.001% meat extract + 0.001% potato starch + 0.1% APG) follow with Euclidean distances of 0.41 to 0.42. The transfer of biofilm was least successful with medium 2 (0.1% TSB + 0.1% MEB + 0.1% APG: 0.57).

**Table 10 Evaluation of the Euclidean distances of the standardised melt cures. The Euclidean distances were determined between the original biofilm and the corresponding *ex situ* biofilm (t7 or t10). A low value (green fields) indicates a high similarity to the original sample, a high value (red fields) indicates a high dissimilarity. X means that no value was detected.**

| sampling site | incubation time | Medium 1 | Medium 2 | Medium 3 | Medium 4 | Medium 5 |
|---|---|---|---|---|---|---|
| Machine 1 detergent drawer | t7 | 0.15 | 0.24 | 0.37 | 0.32 | 0.46 |
| | t10 | 0.32 | 0.44 | 0.43 | 0.26 | 0.60 |
| Machine 1 rubber sealant | t7 | 0.41 | 0.51 | 0.44 | 0.36 | 0.29 |
| | t10 | 0.33 | 0.56 | 0.39 | 0.45 | 0.62 |
| Machine 2 detergent drawer | t7 | 0.26 | 0.69 | 0.40 | 0.26 | 0.20 |
| | t10 | 0.36 | 0.43 | 0.41 | 0.40 | 0.39 |
| Machine 2 rubber sealant | t7 | 0.45 | 0.61 | 0.54 | 0.51 | 0.56 |
| | t10 | 0.69 | 0.66 | 0.41 | 0.20 | 0.20 |
| Machine 3 detergent drawer | t10 | 0.68 | 0.65 | x | 0.50 | 0.68 |
| Machine 3 rubber sealant | t10 | 0.51 | 0.92 | x | 0.11 | 0.11 |
| mean | | 0.42 | 0.57 | 0.42 | 0.34 | 0.41 |

### 4.2.2 Effect of *Rhizobium sp.* on the melting behavior of gDNA of biofilms

The presence of rhizobia is one factor that can affect the stability of gDNA in biofilms. rhizobia can interact with other microorganisms in the biofilm and affect the overall stability of the community. Understanding the effect of *Rhizobium sp.* on the melting behaviour of gDNA in biofilms can provide important insights into the biology and ecology of these complex microbial communities. Based on the results of chapter 4.1, two different *Rhizobium* strains were added to medium 4, which was identified as the ideal recultivation medium (Table 10).

Figure 26 demonstrates the melting curve of the gDNA of the biofilm sample from the rubber sealant of machine 2 (after 10 d incubation and storage in glycerol stocks) at the beginning (grey curve) and after 10 d in the quadruplicate experiment. The multiple determinations shoed a very similar curve progression, but deviate considerably from the initial curve t=0. The initial curve had a main peak at 86.5 °C and two smaller peaks at 81.5 °C and 82.5 °C. By adding medium 6, the main peak shifted to 87 °C and 87.5 °C, respectively. In addition, there were two further peaks at 81.2 °C and 85.5 °C. Medium 7 led to the formation of two main peaks at 86 °C and 87.3 °C. There were also two smaller peaks at 82.5 °C and 83.5 °C. Neither of the two media showed an approximate agreement with the initial sample.

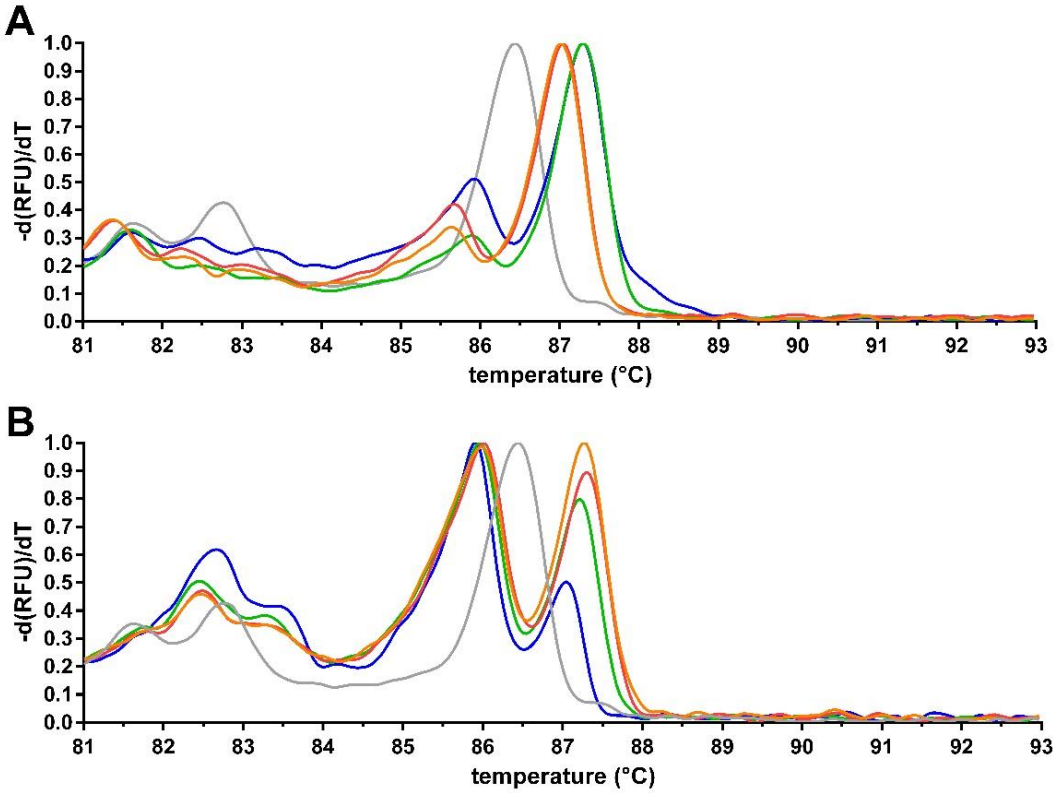



**Figure 26 Normalized melting curve of the active melting region of gDNA of the biofilm sample from the rubber sealant of machine 2 [non-objectionable] (after 10d incubation and stored in glycerin stocks) at time point t = 0 (gray curve) and time point t = 10 (A and B) with the addition of different media (medium 6: A; medium 7: B). The various colours (orange, red, green and blue) show the four independent replicates. n = 4**

### 4.2.3 Effect of media temperature on the melting behavior of gDNA of biofilms

One factor that can affect the stability of gDNA is temperature. Temperature can affect the structure and stability of DNA molecules, which in turn can affect their melting behaviour. Understanding the effect of temperature on the melting behaviour of gDNA in biofilms can have practical implications for a wide range of applications.

In Figure 27, the gDNA melting curve of the biofilm sample of machine 2 rubber sealant (after 10 d incubation and storage in glycerol storage) is presented. The grey curve represents the beginning of the experiment, while the purple curve represents a sample incubated at room temperature. Multiple determinations showed a very similar curve evolution, but with clear deviations from the original curve at t=0. The initial curve had a main peak at 86.5 °C and two smaller peaks at 81.5 °C and 82.5 °C. The treatment at 40°C temperature resulted in a main peak of about 86.5 °C and two smaller peaks of 83 °C and 89.5 °C, which were observed in all repetitions. When treated at 50 °C, the main peak shifted to 87°C and additional peak values were observed at 81.5 °C and 89.5 °C. At 60 °C, the largest separations within the repetitions were visible and the main peaks were between 85.5 °C and 87.5 °C, with higher peaks at 81.5 °C, 83 °C, and 89.5 °C in different curves.

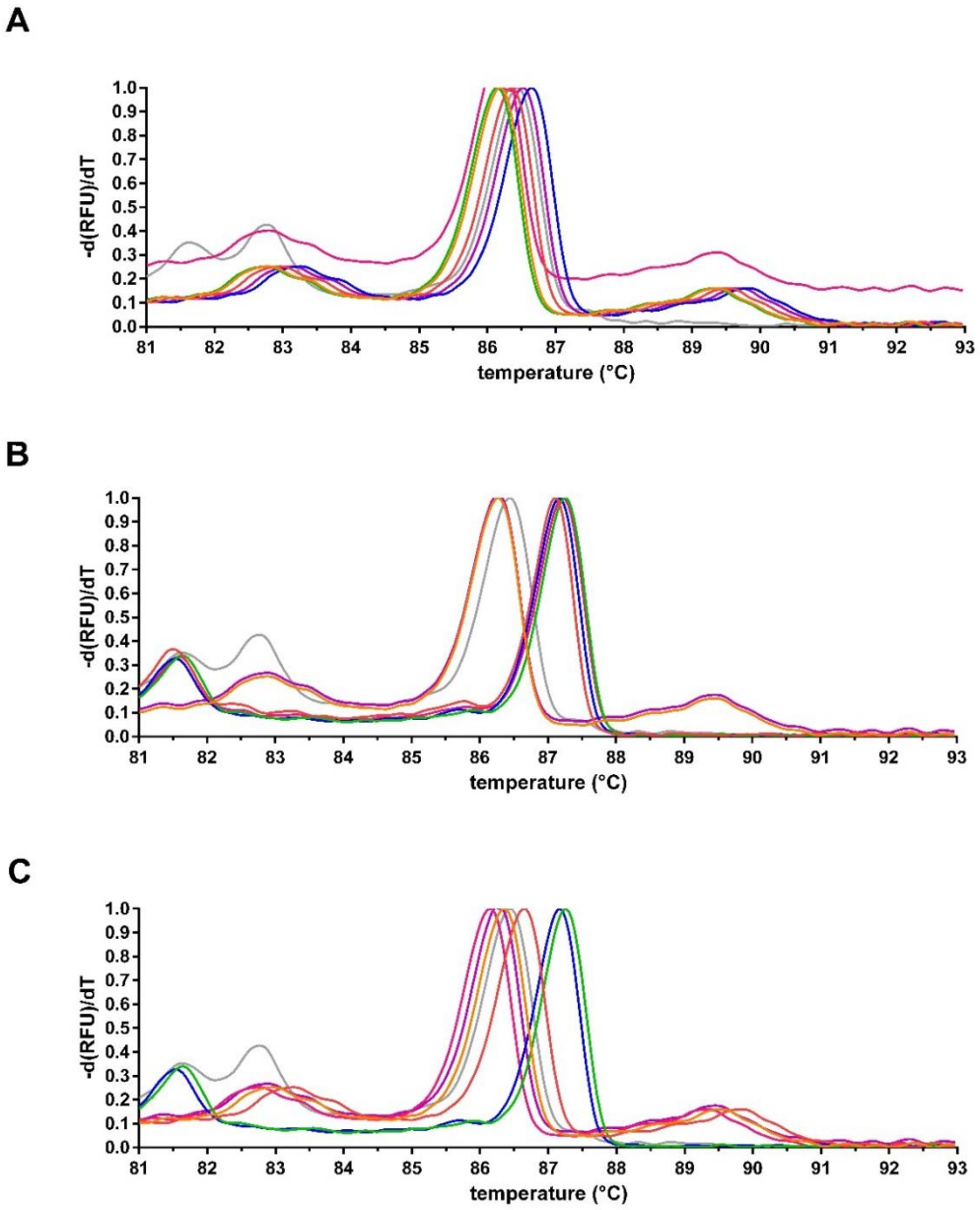



**Figure 27 Normalized melting curve of the active melting region of gDNA of the biofilm sample from the rubber sealant of machine 2 [non-objectionable ] (after 10d incubation and stored in glycerin stocks) at time point t = 0 (gray curve) and time point t = 10 (A-C) with the addition of medium 10 on different media temperatures (A: 40 °C,B: 50 °C and C: 60 °C) of medium 4. The various colours (orange, red, green, pink and blue) show the five independent replicates. The purple curve was incubated at room temperature. n = 5**

### 4.2.4 Changes in the microbial community of biofilms after (re)cultivation

Determining the change in microbial composition in re-cultivation experiments is important for several reasons. First, it can provide insight into the effectiveness of the re-cultivation strategy in restoring microbial diversity and function. Second, changes in microbial composition can provide information on the resilience of the ecosystem to environmental stressors and disturbance. Finally, understanding the microbial community dynamics in

reclamation experiments can provide valuable information for developing more effective and sustainable strategies for household ecosystem re-cultivation.

16S sequencing was used to validate the results of the melting curve analysis. The original biofilms as well as the biofilms after a cultivation of 10 d were sequenced. Figures 28 to 33 show the changes in the composition of the biofilms of the re-cultivation experiments at family level.

As depicted in Figure 28 the number of families present with more than 0.01% relative proportions in the DNA sample decreased from 10 in the initial sample (A-M1d) to 3 in the course of the recultivation experiments (C-M1d and D-M1d). In one case (B-M1d), the number of families increased to 30. Thus, a certain complexity remains, which did not correspond to the original biofilm. The proportion of undefined families was 98.5% in the initial sample (A-M1d) and between 84.8% and 50% in the recultivation experiments (B-M1d to D-M1d). The initial sample contained mainly *Enterobacteriaceae* with 0.4%. All other families were also present with less than 1%. In the recultivated biofilms, *Enterobacteriaceae* were also the most frequently represented family with 3.3% and 4.3%, respectively (C-M1d and D-M1d). In one replicate (B-M1d), on the other hand, *Corynebacteriaceae* was most frequently represented with 25.9%. Further, *Sphingomonadaceae, Pseudomonadaceae, Propionibacteriaceae, Microbacteriaceae, Staphylococcaceae, Beijerinckiaceae, Rhizobiaceae* and *Enterobacteriaceae* followed with prevalences above 1%. 3 out of 10 originally present families (*Enterobacteriaceae, Staphylococcaceae* and *Rhodobacteriaceae*) were also present in all recultivated biofilms.

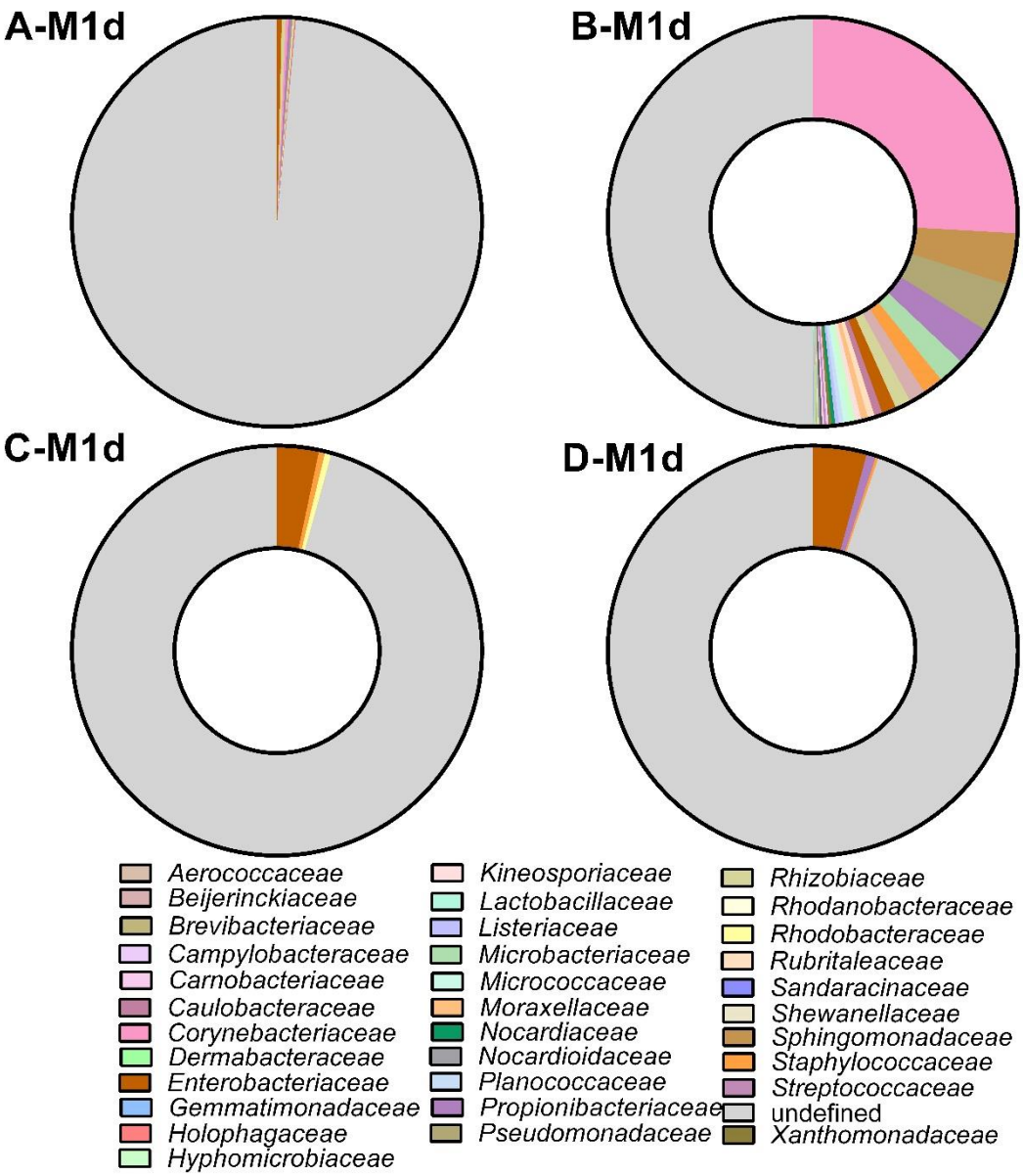



**Figure 28 Microbial community of initial biofilm sample from the detergent drawer of machine 1 [malodor] (after incubation of 10d with medium 4 and storage in glycerol stocks) at family level as selected recultivation experiments (3 of 5 replicates (B-M1d to D-M1d), remaining experiments are shown in the appendix; A-M1d shows the original biofilm). The relative prevalences of the respective families are illustrated. Only families with a prevalence of at least 0.01% were considered in the evaluation. n = 5**

In addition to the family level, the genus level of the sequential data was also analysed. Table 11 indicates that low concentrations of genera were identified in all samples. The diversity of the genera with a total share in the sample of more than 0.1% remained the same after reclamation (C-M1d and D-M1d). In the case of sample B-M1d, the diversity increased even more and genera with a total share of 4.04% could be detected. Furthermore, it could be

seen that smaller proportions were present in the initial sample, while larger proportions of the genera in the total sample could be seen in the reclamation samples.

**Table 11 Overview of genera with the highest relative prevalences (> 0.1 %) in biofilm samples A-M1d (original biofilm from a malodor detergent drawer of machine 1) and B-M1d to D-M1d (multiple determinations of recultivation experiments cultivated with medium 4), sorted by their proportions.**

| A-M1d | | B-M1d | | C-M1d | | D-M1d | |
|---|---|---|---|---|---|---|---|
| amount (%) | genus | amount (%) | genus | amount (%) | genus | amount (%) | genus |
| 0.22 | *Rhizobium* | 4.04 | *Sphingomonas* | 0.49 | *Staphylococcus* | 0.73 | *Cutibacterium* |
| 0.21 | *Cutibacterium* | 3.96 | *Pseudomonas* | 0.49 | *Paracoccus* | 0.22 | *Staphylococcus* |
| 0.13 | *Pseudomonas* | 3.11 | *Cutibacterium* | | | | |
| | | 1.24 | *Staphylococcus* | | | | |
| | | 0.64 | *Brevundimonas* | | | | |
| | | 0.50 | *Acinetobacter* | | | | |
| | | 0.48 | *Devosia* | | | | |
| | | 0.38 | *Macrococcus* | | | | |
| | | 0.37 | *Kurthia* | | | | |
| | | 0.34 | *Kineococcus* | | | | |
| | | 0.33 | *Glutamicibacter* | | | | |
| | | 0.30 | *Brochothrix* | | | | |
| | | 0.29 | *Clavibacter* | | | | |
| | | 0.28 | *Rhodococcus* | | | | |
| | | 0.24 | *Neorhizobium* | | | | |
| | | 0.23 | *Microbacterium* | | | | |
| | | 0.21 | *Luteimonas* | | | | |
| | | 0.18 | *Campylobacter* | | | | |
| | | 0.16 | *Carnobacterium* | | | | |
| | | 0.13 | *Aeromicrobium* | | | | |
| | | 0.12 | *Frondihabitans* | | | | |
| | | 0.12 | *Streptococcus* | | | | |
| | | 0.12 | *Shewanella* | | | | |
| | | 0.11 | *Kocuria* | | | | |

In addition to the graphical evaluation, the Bray-Curtis index was determined to assess the difference between two biofilms based on the sequencing data. The family level was taken into account. The results of the comparison of relevant sample pairings are shown in Table 12. The table displays that the Bray-Curtis index at the family level assumes very high values between 0.81 and 0.95 between the initial sample (A-M1d) and the reclamation samples (B-M1d to D-M1d), thus indicating a dissimilarity of the community, as well as between the samples of the reclamation trials (B-M1d to D-M1d) with values between 0.92 and 0.94. Only the two samples C-M1d and D-M1d indicated a certain similarity with an index of 0.26.

**Table 12 Dissimilarity of the sequenced recultivated samples (B-M1d to D-M1d, cultivated with medium 4) based on the original malodor detergent drawer samples of machine 1 (A-M1d) on family level measured by the Bray-Curtis index. Communities with a value of 0 are identical and those with a value of 1 are maximally dissimilar (Wong *et al.*, 2016).**

| Sample 1 | Sample 2 | Bray-Curtis Index (family) |
|---|---|---|
| A-M1d | B-M1d | 0.95 |
| | C-M1d | 0.85 |
| | D-M1d | 0.81 |
| B-M1d | C-M1d | 0.94 |
| | D-M1d | 0.92 |
| C-M1d | D-M1d | 0.26 |

Figure 29 demonstrates that during the recultivation studies, the number of families with more than 0.01% relative shares in the DNA sample dropped from 14 in the initial sample (A-M1r) to between 8 and 7. (B-M1r to D-M1r). As a result, a level of complexity remained that did not correspond to the original biofilm. In the initial sample (A-M1r), the percentage of undefined families was 32.2%, and in the recultivation trials, it ranged from 32.5% to 46.3%. (B-M1r to D-M1r). *Rhizobiaceae* and *Sphingomonadaceae* made up the majority of the first sample, with 20.2% and 16.5%, respectively. The following families are also well-represented: *Xanthomonadaceae*, *Moraxellaceae* (more than 10%), *Enterobacteriaceae*, and *Beijerinckiaceae* (more than 1% each). Less than 1% of all other families were present. Over 20% of the recultivated biofilms were made up of of *Xanthomonadaceae*. Each of the two families, *Rhizobiaceae* and *Enterobacteriaceae*, accounted for over 10% of the total biofilm. All recultivated biofilms contained members of seven of the 14 original extant families (*Rhizobiaceae, Sphingomonadaceae, Xanthomonadaceae, Moraxellaceae, Enterobacteriaceae, Caulobacteraceae*, and *Acetobacteraceae*).

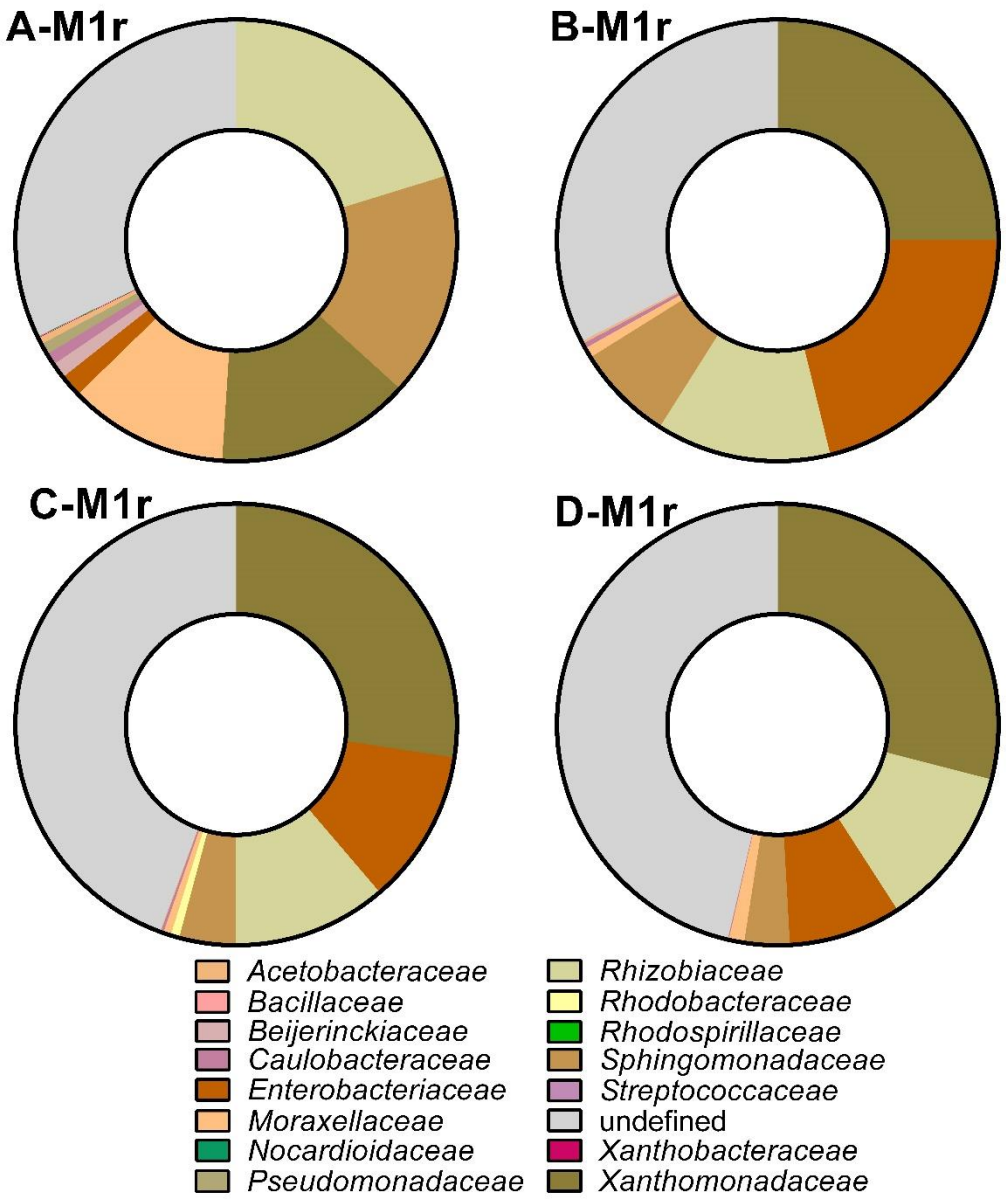



**Figure 29 Microbial community of initial biofilm sample from the rubber sealant of machine 1 [malodor] (after incubation of 10d with medium 4 and storage in glycerol stocks) at family level as selected recultivation experiments (3 of 5 replicates (B-M1r to D-M1r), remaining experiments are shown in the appendix; A-M1r shows the original biofilm). The relative prevalences of the respective families are illustrated. Only families with a prevalence of at least 0.01% were considered in the evaluation. n = 5**

The genus level of the sequential data was also examined in addition to the family level. Results related to recultivation are reported in Table 13 for relevant taxa with a prevalence of greater than 0.1%.

The table's findings indicate that the initial sample contained members of the three genera *Sphingobium* (16.47%), *Stenotrophomonas* (14.28%), and *Acinetobacter* (11.58%) (A-M1r). *Stenotrophomonas* in particular seems to have established itself in the recultivation experiments (B–M1r to D-M1r), with prevalences ranging from 24.95% (D-M1r)

to 29.00% (B-M1r). *Sphingobium* and *Enterobacter* also appeared to be well-established. After reclamation, the diversity of the genera with a cumulative share in the sample of more than 0.1% remained unchanged (B-M1r to D-M1r). Furthermore, it was evident that the initial sample contained less of the genera, whereas the reclamation samples contained more of the genera overall.

**Table 13 Overview of genera with the highest relative prevalences (> 0.1 %) in biofilm samples A-M1r (original biofilm of the malodor rubber sealant of machine 1) and B-M1r to D-M1r (multiple determinations of recultivation experiments cultivated with medium 4), sorted by their proportions.**

| A-M1r | | B-M1r | | C-M1r | | D-M1r | |
|---|---|---|---|---|---|---|---|
| amount (%) | genus | amount (%) | genus | amount (%) | genus | amount (%) | genus |
| 16.47 | *Sphingobium* | 24.95 | *Stenotrophomonas* | 27.39 | *Stenotrophomonas* | 29.00 | *Stenotrophomonas* |
| 14.28 | *Stenotrophomonas* | 20.51 | *Enterobacter* | 10.48 | *Enterobacter* | 7.12 | *Enterobacter* |
| 11.58 | *Acinetobacter* | 7.13 | *Sphingobium* | 3.72 | *Sphingobium* | 3.32 | *Sphingobium* |
| 1.13 | *Enterobacter* | 0.76 | *Acinetobacter* | 0.64 | *Rhodobacter* | 1.13 | *Acinetobacter* |
| 0.85 | *Brevundimonas* | 0.37 | *Brevundimonas* | 0.57 | *Acinetobacter* | | |
| 0.84 | *Pseudomonas* | 0.16 | *Roseomonas* | 0.41 | *Sphingopyxis* | | |
| 0.50 | *Roseomonas* | | | 0.19 | *Brevundimonas* | | |
| | | | | 0.13 | *Klebsiella* | | |

The Bray-Curtis index was established to evaluate the distinction between two biofilms based on the sequencing data in addition to the graphical evaluation. The family unit was taken into consideration. Table 14 displays the findings of the comparison of pertinent sample pairings.

The table reveals that the Bray-Curtis index between the initial sample (A-M1r) and the reclamation samples (B-M1r to D-M1r) at the family level assumed high values between 0.45 and 0.48, showing community dissimilarity. The index values in the reclamation samples were extremely low, ranging from 0.07 to 0.19, and so indicated a comparable community.

**Table 14 Dissimilarity of the sequenced recultivated samples (B-M1r to D-M1r, cultivated with medium 4) based on the original malodor rubber sealant samples of machine 1(A-M1r) on family level measured by the Bray-Curtis index. Communities with a value of 0 are identical and those with a value of 1 are maximally dissimilar (Wong *et al.*, 2016).**

| Sample 1 | Sample 2 | Bray-Curtis Index (family) |
|---|---|---|
| A-M1r | B-M1r | 0.45 |
| | C-M1r | 0.48 |
| | D-M1r | 0.47 |
| B-M1r | C-M1r | 0.15 |
| | D-M1r | 0.19 |
| C-M1r | D-M1r | 0.07 |

Figure 30 shows that the number of families with a relative proportion greater than 0.01% in DNA samples decreased from 13 in the initial sample (A-NO1d) to 7 over the course of regeneration trials (B–NO1d to C-NO1d). In one case (D-NO1d), the number of family members increased to 28. A certain complexity remained, which did not correspond to the original biofilm. The proportion of undefined families was 44.3% in the original sample (A-NO1d) and 11.7% and 59.3% in the recovery experiments (B–NO1d to D-NO1d), respectively. The first sample contained mainly *Rhodobacteriaceae* at 33.1%, followed by *Xanthomonadaceae* (12.2%), *Sphingomonadaceae* (7.6%) and *Caulobacteriaceae* (1.8%). All other families were also less than 1%. In recultured biofilms, *Xanthomonadaceae* was the most abundant family, at 39.9% and 45.1% (B-NO1d and C-NO1d), respectively. However, in replicate D-NO1d, *Enterobacteriaceae* were most frequently represented at 71.0%. *Sphingomonadaceae*, *Xanthomonadaceae*, *Staphylococcaceae*, *Propionibacteriaceae* and *Planococcaceae* followed with prevalence >1%. Five of the 13 originally present families (*Xanthomonadaceae, Sphingomonadaceae, Rhodobacteraceae, Beijerinckiaceae* and *Enterobacteriaceae*) were also present in all recultured biofilms.

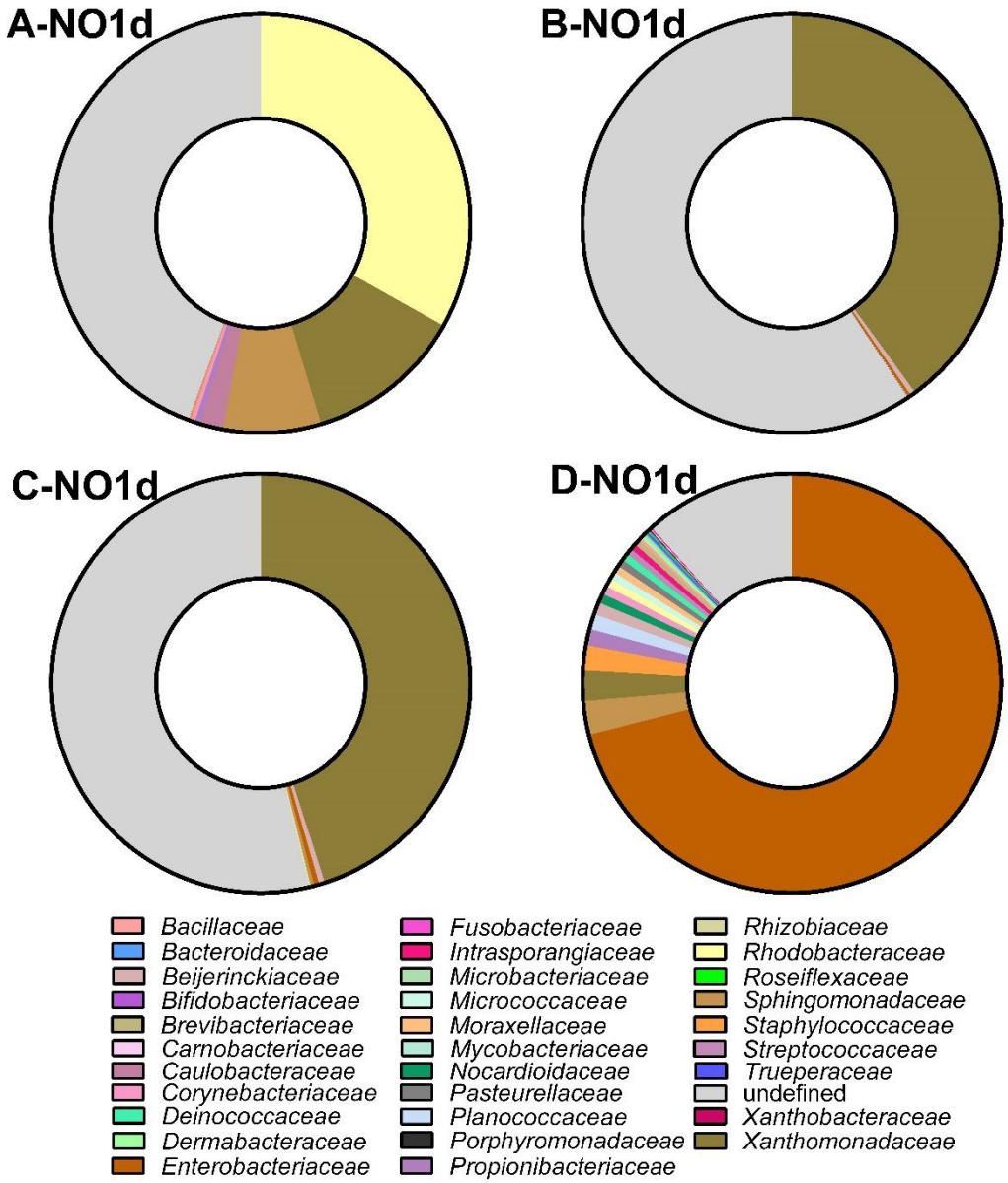



**Figure 30 Microbial community of initial biofilm sample from the detergent drawer of machine 2 [non-objectionable] (after incubation of 10d with medium 4 and storage in glycerol stocks) at family level as selected recultivation experiments (3 of 5 replicates (B-NO1d to D-NO1d), remaining experiments are shown in the appendix; A-NO1d shows the original biofilm). The relative prevalences of the respective families are illustrated. Only families with a prevalence of at least 0.01% were considered in the evaluation. n = 5**

In addition to the family level, the genus level of continuous data was also analyzed. Table 15 shows that mainly *Rhodobacter* (33.13%) and *Stenotrophomonas* (12.21%) were found in the original samples. Genus diversity with a total percentage >0.1% in the sample decreased after recultivation (B-NO1d and C-NO1d), with *Stenotrophomonas* being predominant (39.97% and 45.05%, respectively). In sample D-NO1d, the diversity increased further and the respective overall percentage of *Stenotrophomonas* decreased to a maximum of 2.05%. In addition, we found that the original sample contained a smaller proportion of genera, whereas the recultured samples contained a larger proportion of genera in the overall sample.

**Table 15 Overview of genera with the highest relative prevalences (> 0.1 %) in biofilm samples A-NO1d (original biofilm of the detergent drawer of machine 2) and B-NO1d to D-NO1d (multiple determinations of recultivation experiments cultivated with medium 4), sorted by their proportions.**

| A-NO1d | | B-NO1d | | C-NO1d | | D-NO1d | |
|---|---|---|---|---|---|---|---|
| amount (%) | genus | amount (%) | genus | amount (%) | genus | amount (%) | genus |
| 33.13 | *Rhodobacter* | 39.97 | *Stenotrophomonas* | 45.05 | *Stenotrophomonas* | 2.05 | *Stenotrophomonas* |
| 12.21 | *Stenotrophomonas* | | | 0.26 | *Novosphingobium* | 2.04 | *Sphingomonas* |
| 7.52 | *Sphingopyxis* | | | | | 1.26 | *Staphylococcus* |
| 1.78 | *Brevundimonas* | | | | | 1.10 | *Cutibacterium* |
| 0.43 | *Cutibacterium* | | | | | 0.88 | *Sporosarcina* |
| 0.13 | *Staphylococcus* | | | | | 0.69 | *Jeotgalicoccus* |
| | | | | | | 0.61 | *Paracoccus* |
| | | | | | | 0.57 | *Haemophilus* |
| | | | | | | 0.56 | *Sphingobium* |
| | | | | | | 0.56 | *Deinococcus* |
| | | | | | | 0.49 | *Streptococcus* |
| | | | | | | 0.45 | *Glutamicibacter* |
| | | | | | | 0.43 | *Brevibacterium* |
| | | | | | | 0.37 | *Nocardioides* |
| | | | | | | 0.30 | *Acinetobacter* |
| | | | | | | 0.28 | *Psychrobacter* |
| | | | | | | 0.28 | *Luteimonas* |
| | | | | | | 0.25 | *Bacillus* |
| | | | | | | 0.24 | *Brachybacterium* |
| | | | | | | 0.14 | *Arthrobacter* |
| | | | | | | 0.12 | *Bacteroides* |
| | | | | | | 0.12 | *Truepera* |

Another method was the determination of the Bray-Curtis index to assess the differences between he two biofilms based on sequencing data. Family level was considered. Table 16 shows that the Bray-Curtis index at the family level between the original sample (A-NO1d) and the regenerated samples (B–NO1d to D-NO1d) assumes very high values between 0.74 and 0.89, thus , the community has differences. Because the index values within the recultured samples showed both very low values (0.07 for B-NO1d and C-NO1d) and similar communities, and very high values(0.95 for B-NO1d and D-NO1d or C-NO1d and D-NO1d) , a high level of dissimilarity.

**Table 16 Dissimilarity of the sequenced recultivated samples (B-NO1d to D-NO1d, cultivated with medium 4) based on the original non-objectionable detergent drawer samples of machine 2 (A-NO1d) on family level measured by the Bray-Curtis index. Communities with a value of 0 are identical and those with a value of 1 are maximally dissimilar (Wong *et al.*, 2016).**

| Sample 1 | Sample 2 | Bray-Curtis Index (family) |
|---|---|---|
| A-NO1d | B-NO1d | 0.74 |
| | C-NO1d | 0.75 |
| | D-NO1d | 0.89 |
| B-NO1d | C-NO1d | 0.07 |
| | D-NO1d | 0.95 |
| C-NO1d | D-NO1d | 0.95 |

Figure 31 shows that the number of families present in the relative fraction greater than 0.01% in the DNA samples decreased from 9 in the initial sample (A-NO1r) to between 5 and 4 during the regeneration attempts (B-NO1r to D-NO1r). is shown. Although some complexity was retained, this did not correspond to the original biofilm. The proportion of undefined families was 48.6% in the initial sample (A-NO1r) and 61.1% to 68.8% in the reculture attempts (B–NO1r to D-NO1r). The original sample contained mainly *Rhodobacteriaceae* and *Sphingomonadaceae* at 25.5% and 24.6%, respectively. All other families were present in less than 1%. More than 30% of the recultured biofilms were predominantly *Xanthomonadaceae*. One of the nine originally present families of his (*Xanthomonadaceae*) was also present in all recultured biofilms.

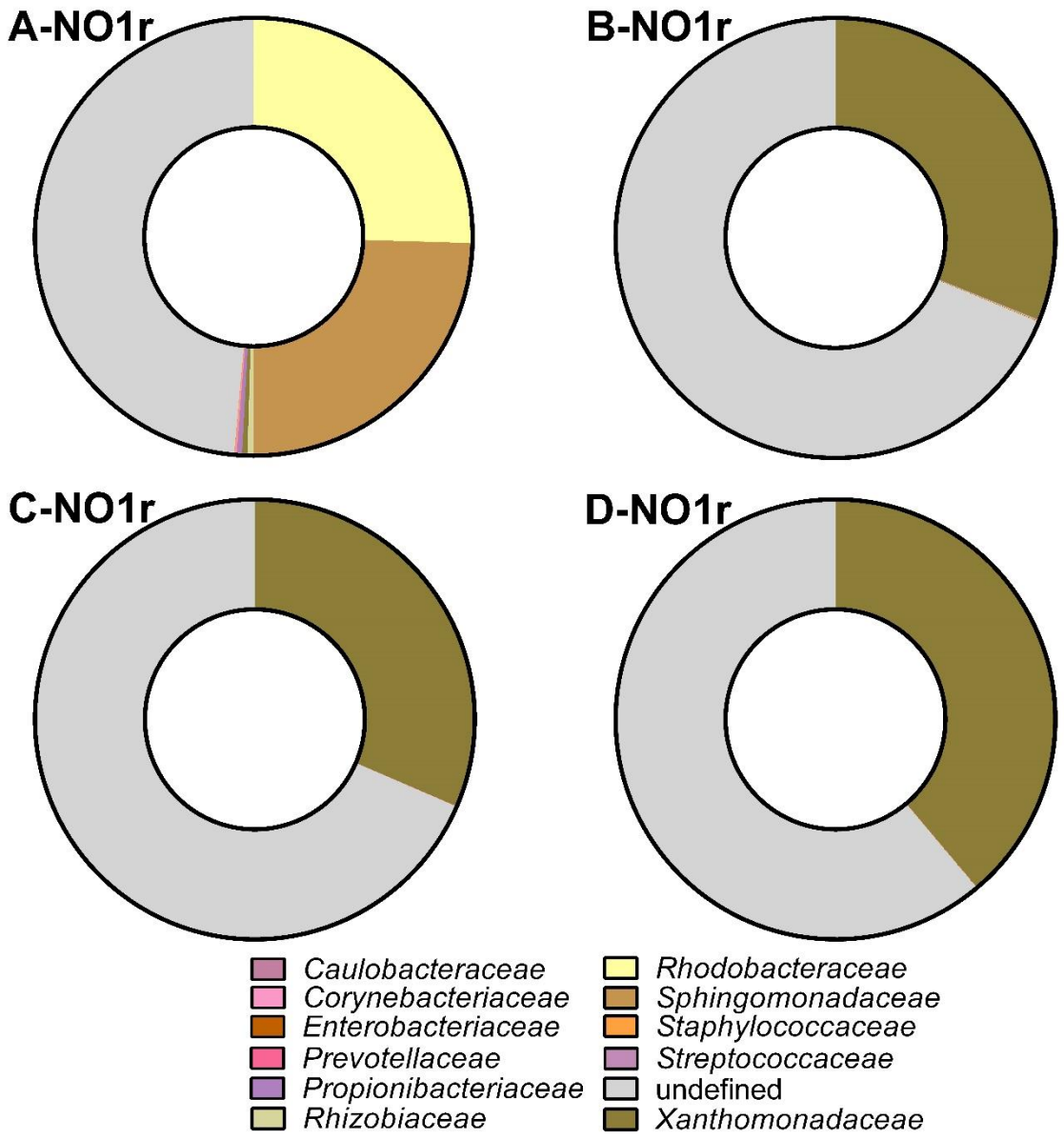



**Figure 31 Microbial community of initial biofilm sample from the rubber sealant of machine 2 [non-objectionable] (after incubation of 10d with medium 4 and storage in glycerol stocks) at family level as selected recultivation experiments (3 of 5 replicates (B-NO1r to D-NO1r), remaining experiments are shown in the appendix; A-NO1r shows the original biofilm). The relative prevalences of the respective families are illustrated. Only families with a prevalence of at least 0.01% were considered in the evaluation. n = 5**

In addition to the family level, the genus level of the segregation data was also analyzed. Table 17 shows results related to reuse and lists related genera with prevalence >0.1%.

This table shows that in the original sample (A-NO1r), two main genera were identified: *Rhodobacter* (25.46%) and *Sphingopyxis* (24.44%). In reculture experiments (B–NO1r to D-NO1r), *Stenotrophomonas* predominated with prevalence ranging from 31.07% (B-NO1r) to 38.85% (D-NO1r). Genus diversity with a total percentage >0.1 in the sample increased after

reuse (B–NO1r to D-NO1r). In addition, it could be found that the original sample contained a smaller proportion of genera, whereas the recultured samples contained a larger proportion of genera in the overall sample.

**Table 17 Overview of genera with the highest relative prevalences (> 0.1 %) in biofilm samples A-NO1r (original biofilm of the non-objectionable rubber sealant of machine 2) and B-NO1r to D-NO1r (multiple determinations of recultivation experiments cultivated with medium 4), sorted by their proportions.**

| A-NO1r | | B-NO1r | | C-NO1r | | D-NO1r | |
|---|---|---|---|---|---|---|---|
| amount (%) | genus | amount (%) | genus | amount (%) | genus | amount (%) | genus |
| 25.46 | *Rhodobacter* | 31.07 | *Stenotrophomonas* | 31.44 | *Stenotrophomonas* | 38.85 | *Stenotrophomonas* |
| 24.44 | *Sphingopyxis* | | | | | | |
| 0.39 | *Stenotrophomonas* | | | | | | |
| 0.25 | *Cutibacterium* | | | | | | |
| 0.15 | *Brevundimonas* | | | | | | |
| 0.10 | *Novosphingobium* | | | | | | |

As well as the graphical evaluation, the Bray-Curtis index was determined to assess the difference between two biofilms on the basis of the sequencing data. The family level was taken into account. Table 18 shows that the Bray-Curtis index at the family level between the initial sample (A-NO1r) and the reclamation samples (B-NO1r to D-NO1r) had very high values of 0.99 and thus there was a dissimilarity of the community. The index values within the reclamation samples showed very low values between 0.01 and 0.11 and thus a similar community.

**Table 18 Dissimilarity of the sequenced recultivated samples (B-NO1r to D-NO1r, cultivated with medium 4) based on the original non-objectionable rubber sealant samples of machine 2 (A-NO1r) on family level measured by the Bray-Curtis index. Communities with a value of 0 are identical and those with a value of 1 are maximally dissimilar (Wong *et al.*, 2016).**

| Sample 1 | Sample 2 | Bray-Curtis Index (family) |
|---|---|---|
| A-NO1r | B-NO1r | 0.99 |
| | C-NO1r | 0.99 |
| | D-NO1r | 0.99 |
| B-NO1r | C-NO1r | 0.01 |
| | D-NO1r | 0.11 |
| C-NO1r | D-NO1r | 0.11 |

Figure 32 shows that the number of families present in the relative fraction greater than 0.01% in DNA samples decreased from 12 in the initial sample (A-NO2d) to between 5 and 4 over the course of regeneration attempts (B-NO2d to D-NO2d). is shown. Although some complexity was retained, this did not correspond to the original biofilm. The proportion of undefined families was 45.6% in the first sample (A-NO2d) and 61.7% to 81.1% in the reculture attempts (B–NO2d to D-NO2d). The original sample contained mainly *Sphingomonadaceae*

and *Rhizobiaceae* at 20.7% and 15.3%, respectively. *Rhodobacteriaceae* and *Caulobacteriaceae* are still well represented at >2%. All other families were present in less than 1%. In recultured biofilms, *Rhizobiaceae* (>15%) and *Xanthomonadaceae* (>3%) were predominantly present. Two of the 12 originally present families (*Rhizobiaceae* and *Enterobacteriaceae*) were also present in all recultured biofilms.

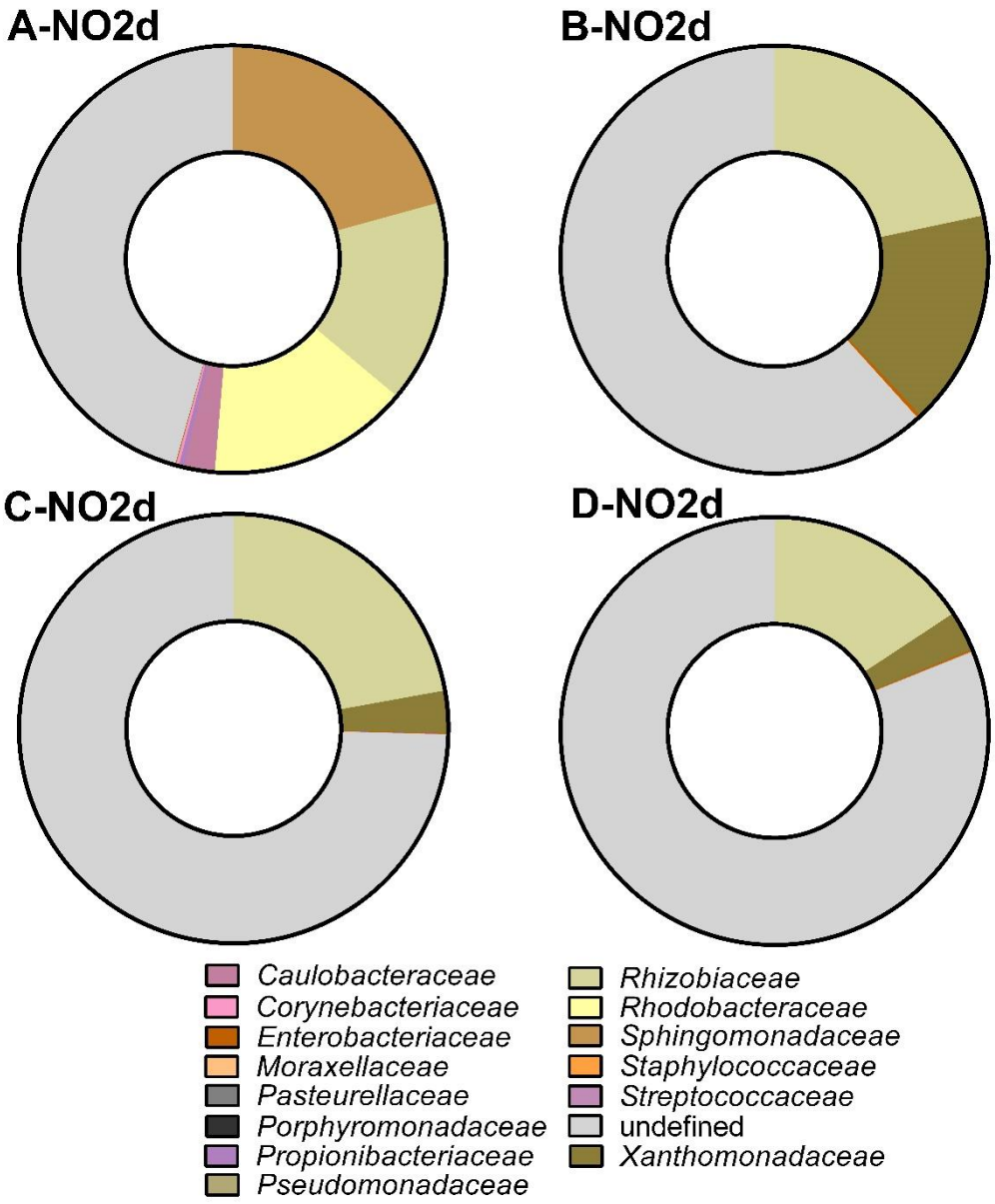



**Figure 32 Microbial community of initial biofilm sample from the detergent drawer of machine 3 [non-objectionable] (after incubation of 10d with medium 4 and storage in glycerol stocks) at family level as selected recultivation experiments (3 of 5 replicates (B-NO2d to D-NO2d), remaining experiments are shown in the appendix; A-NO2d shows the original biofilm). The relative prevalences of the respective families are illustrated. Only families with a prevalence of at least 0.01% were considered in the evaluation. n = 5**

In addition to the family level, the genus level of the segregation data was also analyzed. Table 19 shows the results related to reuse and lists relevant genera with prevalence >0.1%.

The table illustrates that in the original sample (A-NO2d), two main genera were identified: *Sphingopyxis* (20.40%) and *Rhodobacter* (15.28%). In reculture trials (B-NO2d to D-NO2d), *Stenotrophomonas* appeared to predominate with prevalence ranging from 2.99% (D-NO2d) to 16.34% (B-NO2d). Genus diversity with a total percentage >0.1 in the sample increased after reuse (B–NO2d to D-NO2d). In addition, the study found that the recultured samples contained a higher proportion of genera in the overall sample, whereas the initial sample contained a smaller proportion of genera.

**Table 19 Overview of genera with the highest relative prevalences (> 0.1 %) in biofilm samples A–NO2d (original biofilm of the non-objectionable detergent drawer of machine 3) and B-NO2d to D-NO2d (multiple determinations of recultivation experiments cultivated with medium 4), sorted by their proportions.**

| A-NO2d | | B-NO2d | | C-NO2d | | D-NO2d | |
|---|---|---|---|---|---|---|---|
| amount (%) | genus | amount (%) | genus | amount (%) | genus | amount (%) | genus |
| 20.40 | *Sphingopyxis* | 16.34 | *Stenotrophomonas* | 3.18 | *Stenotrophomonas* | 2.99 | *Stenotrophomonas* |
| 15.28 | *Rhodobacter* | | | | | | |
| 2.34 | *Brevundimonas* | | | | | | |
| 0.37 | *Cutibacterium* | | | | | | |
| 0.33 | *Novosphingobium* | | | | | | |

In addition to the graphical evaluation, the Bray-Curtis index was determined to assess the difference between two biofilms based on the sequencing data. The family level was taken into account. The results of the comparison of relevant sample pairings are shown in Table 20.

The table shows that the Bray-Curtis index at the family level between the initial sample (A-NO2d) and the reclamation samples (B-NO2d to D-NO2d) had high values between 0.58 and 0.67 and thus there was a dissimilarity of the community. The index values within the reclamation samples showed very low values between 0.15 and 0.34 and thus a similar community.

**Table 20 Dissimilarity of the sequenced recultivated samples (B-NO2d to D-NO2d, cultivated with medium 4) based on the original non-objectionable detergent drawer samples of machine 3 (A-NO2d) on family level measured by the Bray-Curtis index. Communities with a value of 0 are identical and those with a value of 1 are maximally dissimilar (Wong *et al.*, 2016).**

| Sample 1 | Sample 2 | Bray-Curtis Index (family) |
|---|---|---|
| A-NO2d | B-NO2d | 0.67 |
| | C-NO2d | 0.61 |
| | D-NO2d | 0.58 |
| B-NO2d | C-NO2d | 0.22 |
| | D-NO2d | 0.34 |
| C-NO2d | D-NO2d | 0.15 |

Figure 33 shows that the number of families with a relative share of more than 0.01% of DNA samples in the first sample (A-NO2r) decreased from 12 to 5 to 4 families in the covery experiment (B-NO2r to D-NO2r). Consequently, some complexity remained that did not correspond to the original biofilm. The proportion of non-defined families was 30.9% in the initial sample (A-NO2r) and between 62.33% and 70.1% in the replication study (B-NO2r to D-NO2r). The first sample contained *Xanthomonadaceae* and *Moraxellaceae*, 20.8% and 16.3%, respectively. *Sphingomonadaceae, Acetobacteraceae, Caulobacteriaceae, Rhodobacteriaceae* and *Beijerinckiaceae* were represented by more than 2% of the population. All other families had less than 1%. In the biofilms cultivated, *Xanthomonadaceae* (over 27%) and *Beijerinckiaceae* (over 1%) were mainly present. Of the 12 species that were originally present three also appeared in all re-cultured biofilms (*Xanthomonadaceae, Beijerinckiaceae* and *Enterobacteriaceae*).

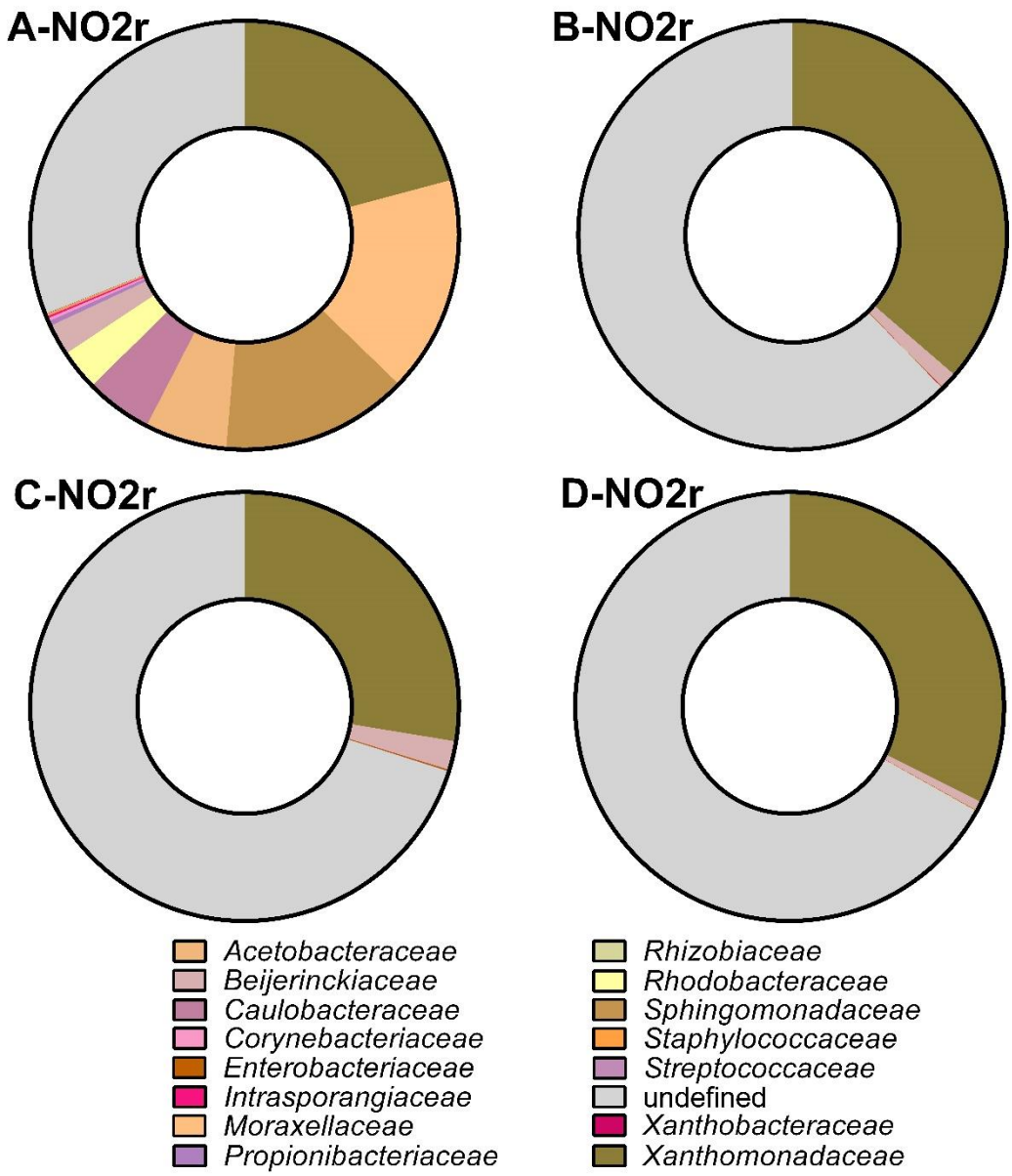



**Figure 33 Microbial community of initial biofilm sample from the rubber sealant of machine 3 [non-objectionable] (after incubation of 10d with medium 4 and storage in glycerol stocks) at family level as selected recultivation experiments (3 of 5 replicates (B-NO2r to D-NO2r), remaining experiments are shown in the appendix; A-NO2r shows the original biofilm). The relative prevalences of the respective families are illustrated. Only families with a prevalence of at least 0.01% were considered in the evaluation. n = 5**

In addition to family levels, the genus levels of sequential data were also analyzed. Table 21 shows the results associated with recultivation, with relevant species with more than 0.1% incidence.

The table shows that in the first sample (A-NO2r), the two species of *Stenotrophomonas* (20.84%) and *Sphingomonas* (10.82%) were mainly identified. In the cultivation studies (B-NO2r to D-NO2r), *Stenotrophomonas* seemed to dominate with prevalences of 27.60% (C-NO2r) to 36.32% (B-NO2r). The diversity of the generation of more than 0.1% of the sample decreased after reclamation (B-NO2r to D-NO2r). In addition, it was observed that lower proportions were present in the initial sample, while higher proportions of generations in the total sample could be observed in the reclamation samples.

**Table 21 Overview of genera with the highest relative prevalences (> 0.1 %) in biofilm samples A-NO2r (original biofilm of the non-objectionable rubber sealant of machine 3) and B-NO2r to D-NO2r (multiple determinations of recultivation experiments cultivated with medium 4), sorted by their proportions.**

| A-NO2r | | B-NO2r | | C-NO2r | | D-NO2r | |
|---|---|---|---|---|---|---|---|
| amount (%) | genus | amount (%) | genus | amount (%) | genus | amount (%) | genus |
| 20.84 | *Stenotrophomonas* | 36.32 | *Stenotrophomonas* | 27.60 | *Stenotrophomonas* | 32.35 | *Stenotrophomonas* |
| 10.82 | *Sphingomonas* | | | | | | |
| 6.19 | *Roseomonas* | | | | | | |
| 5.03 | *Brevundimonas* | | | | | | |
| 3.09 | *Paracoccus* | | | | | | |
| 3.09 | *Sphingopyxis* | | | | | | |
| 1.55 | *Acinetobacter* | | | | | | |
| 0.36 | *Cutibacterium* | | | | | | |
| 0.24 | *Novosphingobium* | | | | | | |
| 0.14 | *Janibacter* | | | | | | |
| 0.13 | *Staphylococcus* | | | | | | |

In addition to the graphical evaluation, the Bray-Curtis index was determined to assess the difference between two biofilms based on the sequencing data. The family level was taken into account. The results of the comparison of relevant sample pairings are shown in Table 22.

The table shows that the Bray-Curtis index at the family level between the initial sample (A-NO2r) and the reclamation samples (B-NO2r to D-NO2r) had high values between 0.53 and 0.58 and thus there was a dissimilarity of the community. The index values within the reclamation samples showed very low values between 0.07 and 0.14 and thus a similar community.

**Table 22 Dissimilarity of the sequenced recultivated samples (B-NO2r to D-NO2r, cultivated with medium 4) based on the original non-objectionable rubber sealant samples of machine 3 (A-NO2r) on family level measured by the Bray-Curtis index. Communities with a value of 0 are identical and those with a value of 1 are maximally dissimilar (Wong *et al.*, 2016).**

| Sample 1 | Sample 2 | Bray-Curtis Index (family) |
|---|---|---|
| A-NO2r | B-NO2r | 0.58 |
| | C-NO2r | 0.53 |
| | D-NO2r | 0.58 |
| B-NO2r | C-NO2r | 0.14 |
| | D-NO2r | 0.07 |
| C-NO2r | D-NO2r | 0.10 |

## 4.3 Malodor model for investigating effects on bad textile odor

The influence of rhizobia on malodor was investigated by carrying out experiments based on the established model for the reproducible production of malodor by Zinn *et al.* (2021). Different rhizobial strains, which were identified by 16S sequencing, were added and the subsequent change in odor was evaluated using a sniffer panel (Figure 34). Furthermore, the model was adapted using one biofilm from a non-objectonable household and one from a malodor household as the basis for the experiment. In this case, known "malodor bacteria" (non-objectonable household, Figure 35 A) and various rhizobia strains (malodor household, Figure 35 B) were applied.

The evaluation of the malodor model (Figure 34) shows that especially the two *Rhizobium* species found in the washing machines (*Rhizobium flavum* (*R. flavum)* and *Rhizobium leguminosarum* (*R. leguminosarum*)) led to a reduction of >50% of the malodor in general and the cheesy and pungent odors. Furthermore, *Rhizobium pisi*, was found to show equally good reduction against the three odor attributes. *Bradyrhizobium japonicum*, which was tested as a control, only showed minor reductions against general malodor (- 11.3%) and the cheesy (-22%) and pungent odors (-60%).

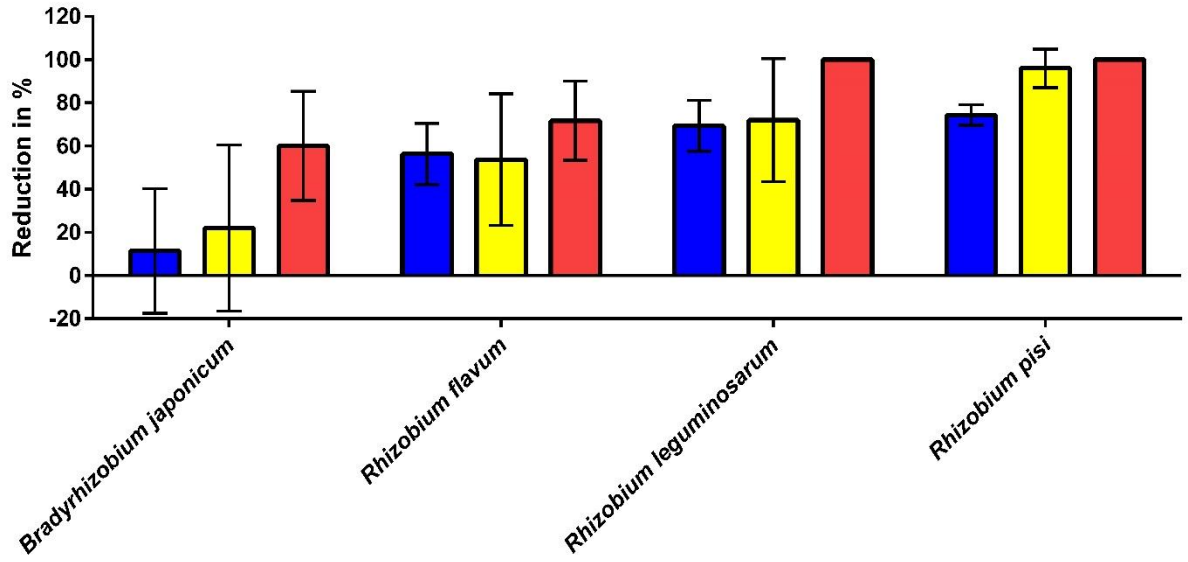



**Figure 34 malodor reduction by *Bradyrhizobium* and *Rhizobium* strains for general malodor (blue), cheesy odor (yellow) and pungent odor (red) using the malodor-model described in (Zinn *et al.*, 2021) (n = 5). All reductions shown are in comparison to the positive control (100% odor intensity of the three odor attributes). The samples were evaluated by a trained sniffer panel.**

The evaluation of the adapted malodor model (Figure 35) shows that the addition of *M. osloensis* to a biofilm that did not have an odor led to the formation of malodor (A). The same effects in a weakened form could be observed for the bacteria from the malodor model of Zinn *et al.* (2021) (*S. hominis, C. jeikeium* and *M. luteus*). Sub-figure B shows a malodor household as positive control and a corresponding treatment with different rhizobial strains. Especially *R. pisi* showed good properties and a reduction of malodor of up to 80%. *R. flavum* and *R. leguminosarum* also showed malodor reduction of 60% and 70% respectively. The lowest effect in the trials was shown by *B. japonicum* with a malodor reduction of approx. 40%.

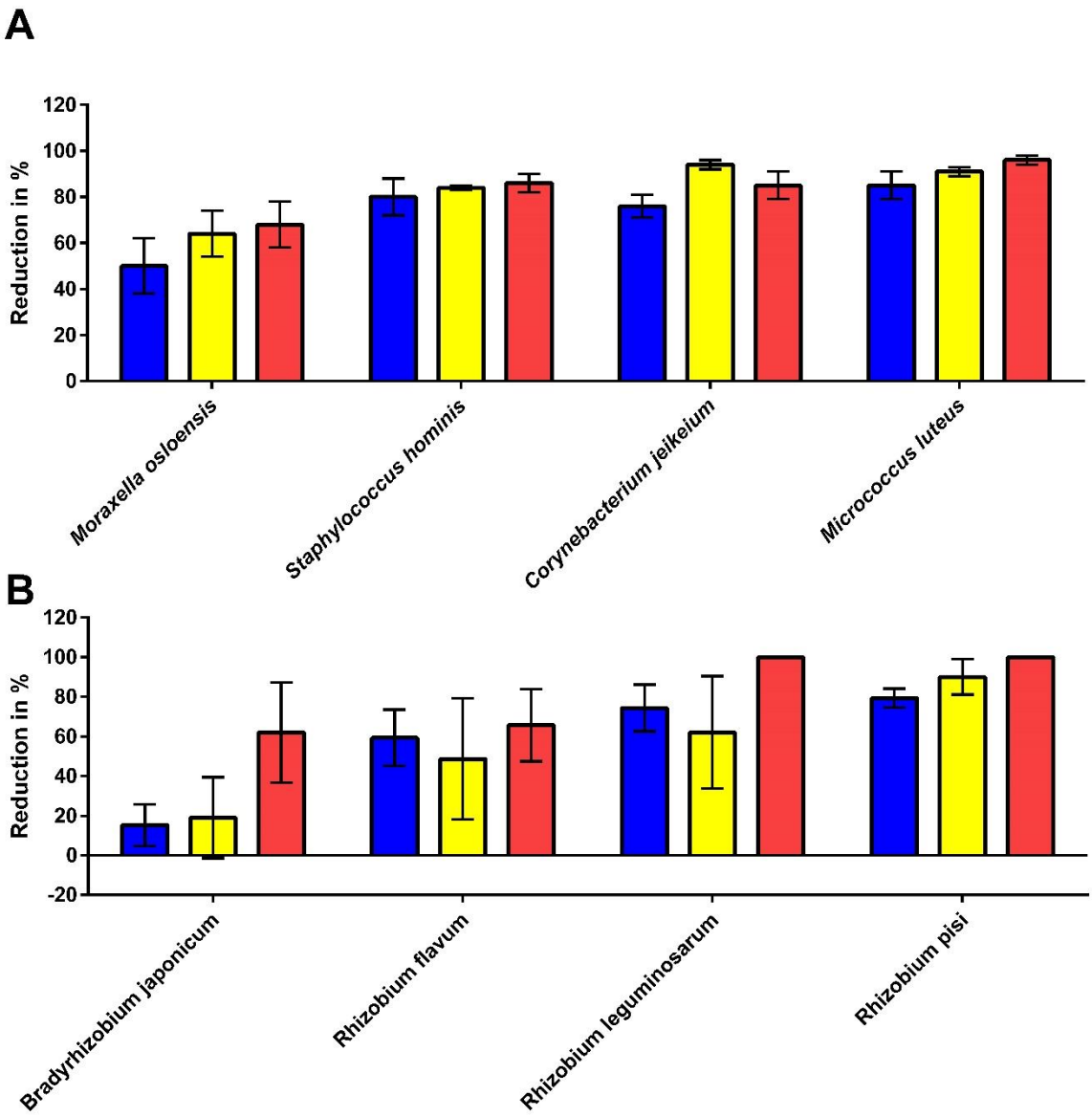



**Figure 35 malodor reduction by *Bradyrhizobium* and *Rhizobium* strains for general malodor (blue), cheesy odor (yellow) and pungent odor (red) using an adapted malodor-model described in (Zinn *et al.*, 2021) (n = 5). (A: non-objectonable household biofilm as positive control, potential malodor causative bacteria were tested; B: a malodor household biofilm was used as positive control, potentially protective bacteria were tested). All reductions shown are in comparison to the positive control (100% odor intensity of the three odor attributes). The samples were evaluated by a trained sniffer panel.**

# 5. Discussion

## 5.1 Analysis of the microbial communities in the laundering cycle

Microorganisms are an important factor in the wash-and-use cycle of laundry and can cause undesirable aesthetic effects, such as malodor formation, biofilm formation as well as potential health risks. Microorganisms such as *S. aureus*, *P. aeruginosa*, *E. hirae* or *C. albicans* can survive a wash cycle at 40 °C and a temperature holding time of 45 min (Honisch, Stamminger, *et al.*, 2016). As the European Union has been suffering from an energy crisis since the beginning of 2022 (McWilliams *et al.*, 2022) and energy prices have risen significantly for consumers in this context, it is a strategy to save electricity by using washing machine programmes with lower temperatures. The current climate problem is also leading to a general effort to consume increasingly less energy (Hoque *et al.*, 2022). The survey of households (Figure 5) showed that in 2020, before the current energy crisis, more than half of households prefer to wash at 30 °C or 40 °C. These households also indicated that they use washing machines with lower temperatures. These households indicated that a 60 °C washing programme is used at least once a month. This interval is recommended by Forum Waschen to consumers for hygienic washing (Anonymous, 2022a). In addition to 60 °C, a AOB-containing heavy-duty detergent should be used.

The household survey showed that the second half of the respondents rarely or only very irregularly use washing programmes above 40 °C. This leads to a situation where microorganisms are no longer killed by the temperature (Honisch, Brands, *et al.*, 2016). The use of a full detergent containing bleach would compensate for the low temperatures and lead to almost complete reductions in the most common microorganisms (Honisch *et al.*, 2014; Honisch, Brands, *et al.*, 2016; Lichtenberg *et al.*, 2006; Linke *et al.*, 2011). Honisch *et al.*(2014), showed that the addition of a bleach-containing heavy-duty detergent completely killed the test germs *S. aureus*, *E. hirae*, *P. aeruginosa* and *T. mentagrophytes* at a washing temperature of 30 °C and a holding time of 15 min (Honisch *et al.*, 2014). *C. albicans* as a representative of the yeasts must be treated at a washing temperature of 52 °C and a holding time of 15 min to show a complete reduction. It was shown that the use of a bleach-containing heavy-duty detergent at low washing temperatures (30 °C) shows significantly better reductions of microorganisms than the use of a bleach-free detergent (Schages *et al.*, 2020).

Since the reduction of the washing temperature has a considerable influence on electricity consumption, it can be assumed that consumers will increasingly use low-temperature programmes due to the current situation. An average wash cycle in Western Europe consumes about 0.95 kWh, which translates into an annual consumption of 156.2 kWh

per household for a washing machine (Pakula & Stamminger, 2010). Although the 3.8% share of total electricity consumption can be considered low, it is an easy way to save energy, because a wash cycle at 60 °C consumes about 1 kWh, whereas a wash cycle at 30 °C consumes only about 0.3 kWh (Anonymous, 2017; Zinn & Bockmühl, 2022) . In addition to the high electricity prices, there is also high inflation (Coibion *et al.*, 2021), which also drives households to save more energy costs than before.

The evaluation of the questionnaire (Figure 4) also showed that many households now own a washing machine that is less than 8 years old. This also leads to consumers washing at lower temperatures, as new washing machines no longer have high-temperature programmes (e.g. 90 °C). In addition, there is information from the detergent manufacturers, who in their advertisements call for washing at low temperatures (30 °C) for energy and sustainability reasons (Anonymous, 2022b). From a hygienic point of view, consumer education with reference to the recommendation of *Forum Waschen* should be an important step in the future (Anonymous, 2022a).

Since the current trend of machine and detergent manufacturers is towards low temperature washing programmes, and the present study showed that already in 2020 more than 74% of consumers have already come into contact with the issue of malodor, albeit in various ways (Figure 6), it can be assumed that the problem of textile malodor will increase in importance in the near future. The survey showed that 74% of households that had already have to deal with the issue of laundry malodor, about 50% have perceived the typical malodor. Some households stated that there was a problem with laundry malodor, but described the odor with other attributes (e.g. sweaty) than typical malodor ("musty"). This occurs mainly with laundry that has been dried indoors or with textiles that have already been dried and stored in humid conditions (Kubota *et al.*, 2012; Munk *et al.*, 2001; Nagoh *et al.*, 2005). Furthermore, this smell is described in the literature as "wet-and-dirty-dustcloth-like" or "wet fabric" malodor and is associated with the attribute "musty" (Kubota *et al.*, 2012; Nagoh *et al.*, 2005; Takeuchi *et al.*, 2012; Zinn *et al.*, 2021). This description was also used by 60% of the households surveyed (Figure 6).

According to the present study, liquid detergents are favored by nearly 50% of homes (see Figure 5). Del Rosario Augustin *et al.* (2023) investigated the formation of *P. aeruginosa* biofilms on cellulose surfaces in a recent study. The combination of liquid detergent and enzyme (kind of enzyme not specified) resulted in a substantial decrease ($p < 0.001$) in viable bacteria within biofilms. This therapy disturbed and largely eliminated the biofilms (del Rosario Augustin *et al.,* 2023).

Bridier *et al.* advocated including enzymes such as proteases, cellulases, polysaccharide depolymerases, alginate lyases, dispersion B, and DNases into detergent formulations to destabilize EPS (Bridier *et al.*, 2010). Prior research has demonstrated that

enzymes are efficient in degrading the physical structure of EPS in biofilms (Augustin *et al.*, 2004; De Bivar Xavier *et al.*, 2005; Lequette *et al.*, 2010). Nevertheless, enzymes such as amylases, mannanases, proteases, and cellulases are already present in liquid detergents (Henkel AG & Co. KGaA, 2023; The Procter & Gamble Company, 2023). Yet, in normal norm testing, these enzymes showed insufficient logarithmic decrease (> 5-log levels) (Anonymous, 2015b; Working Group Hygiene and Microbiology of Rhine-Waal University of Applied Sciences, o. J.). As a result, future research may need to concentrate on strengthening the enzyme composition of liquid detergents in order to match the efficacy of powder detergents.

This research reports the first time the determination of microbial burden in the detergent drawer and the rubber sealant of washing machines as well as on towels used in the household has been carried out. The focus so far has been primarily on the microbiome of these sample locations. The results showed above all that no significant difference can be perceived between the single-split chamber and the rubber sealant of washing machines (Figure 7). Both sample locations show total bacterial counts of 1 x $10^5$ cfu/cm² which is significantly less than, for example, kitchen sponges with a bacterial count of 1 x $10^7$ to 1 x $10^9$ cfu/sponge (Jacksch *et al.*, 2020), but is significantly more than, for example, on the toilet seat (approx. $10^2$/cm²; (Ojima *et al.*, 2002). The main difference between a toilet seat and a washing machine rubber sealant is, *inter alia*, the material used. On the one hand, thermoset, which has antimicrobial properties, is often used for toilet seats, whereas the washing machine rubber sealant is preferably made of ethylene propylene diene (monomer) rubber (EPDM) (Hutchins *et al.*, 2020; Jaglarz, 2020; Moritz *et al.*, 2010). This has already been identified in previous studies as a material that promotes biofilms. This is due to additives such as plasticisers, fatty acids, solvents and paraffin oils (Kilb *et al.*, 2003; Schmeisser *et al* 2003).

The comparison of the microbial counts of households with young (under 35) or old people (over 60), with or without children and households with or without pets showed no significant differences (Figure 8 - Figure 10). Only total viable counts (TVC) and colony forming units (cfu) were examined in this study. Typical biofilm staining methods such as DAPI or live/dead staining were not used because previous studies by the Hygiene and Microbiology Working Group at the Rhein-Waal University of Applied Sciences show that this is difficult and unsatisfactory on textiles due to the high background fluorescence present (unpublished data). With regard to live/dead staining, the presence of optical brighteners in detergents is another obstacle that has prevented this type of study. In addition, the determination of TVC is the standard method in the field of (household) hygiene and was therefore used for classification in the literature data.

Based on the questionnaire, the households could be divided into those with malodor problems and those without odor problems as well as the bacterial counts compared. With the exception of the staphylococci in the rubber sealant, which showed slightly significantly higher bacterial counts in malodor households, the comparison showed that there were no significant differences between households with or without malodor problems (Figure 13). This result suggests that it is not the number of microorganisms but the composition that is decisive in the formation of malodor.

The evaluation of the metagenome analysis showed that mainly the two species *Pseudomonas* and *Acinetobacter* were found in the washing machine (Figure 11). It was also possible to identify gram-positive genera with *Microbacterium* and *Paracoccus* and soil bacteria like *Rhizobium*, *Agrobacterium* and *Bosea*, which have already been found in washing machines (Bockmühl *et al.*, 2019; Jacksch *et al.*, 2020; Nix *et al.*, 2015). The Shannon diversity of bacterial communities was significantly lower ($p < 0.05$) in towels (exp (H') =19.97 ± 5.32) and rubber sealants (exp (H') =28.96 ± 6.61) compared to the detergent drawer (exp (H') =33.06 ± 8.66). In contrast, there were no significant differences in species richness (towels: n = 591 ± 357.9; detergent drawer: n = 742 ± 257.1; rubber sealant: n = 645 ± 335.6). Beta diversity showed a high degree of variation between the different sampling sites. Specifically, detergent drawer samples were found to have more species in common with rubber sealant samples (Bray-Curtis index = 0.36) than towel samples compared to detergent drawer (Bray-Curtis index = 0.68) or rubber sealant (Bray-Curtis index = 0.52).

The genus *Paracoccus* is associated with the formation of sweat odor (Teufel *et al.*, 2010). They have been found mainly in the axillary region of humans and are referred to as nitrogen respiration organisms (Callewaert *et al.*, 2013; Ross *et al.*, 2018). Like *Paracoccus*, *Pseudomonas* is a group of bacteria associated with nitrogen respiration. *Pseudomonas* has already been identified as the main coloniser of the washing machine in previous studies (Bockmühl *et al.*, 2019; Jacksch *et al.*, 2020; Nix *et al.*, 2015; Schages *et al.*, 2020). For aquatic bacteria (Mena & Gerba, 2009), a washing machine provides an optimal habitat to survive and establish itself. The same applies to *Acinetobacter*, which are known water germs and have also been found in food or drinking water (Carvalheira *et al.*, 2021). Basically, the drinking water used to operate a washing machine is a major input factor of microorganisms into the machine. Since the washing machine does not need water continuously, rest periods occur and the water stagnates. During this time, existing microorganisms can multiply and thus get into the washing machine and onto the laundry. A study by Chen and Li Li (2013) showed the effects of bacteria adhering to cast-iron pipes on tap water in a distribution system where water stagnation occurs repeatedly. The results showed that mainly *Rhizobium*, *Pseudomonas*, *Brevundimonas* and *Lactococcus* were identified as genera in the tap water (Chen *et al.*, 2013). The genus *Bosea* was originally found

in root nodules of *Vavilovia formosa* (Safronova *et al.*, 2015). Since 2016, an inclusion in the genera *Rhizobium* and *Agrobacterium* has been discussed but not finalised due to bureaucratic reasons (de Lajudie & Young, 2020; Young & Young, 2017). *Bosea* is very closely related to *Rhizobium* and *Agrobacterium*, which is why there are always shifts of individual species between the genera (Young *et al.*, 2001).

Furthermore, with *Corynebacterium*, *Micrococcus*, *Brevibacterium* or *Mycobacterium*, various strains were found that are associated with human skin (Finley *et al.*, 2013; Yang *et al.*, 2022).

In summary, three major sources of microorganisms entering a washing machine have been identified: drinking water, people wearing clothes and the washing machine itself. A similar connection was already established by van Herreweghen *et al.* (2020) in connection with the formation of malodor. In addition to wearing and washing the clothes, the authors identified storage before washing, the dryer and wearing the clothes again as other major influencing factors (Van Herreweghen *et al.*, 2020).

The metagenome analysis could also be divided into malodor-affected and non-affected households with the help of the questionnaire (Figure 14 - Figure 16). Different soil bacteria were identified on the non-objectonable towels: *Rhodococcus*, *Blastococcus* and *Phenylobacterium*, whereas on the malodor towels, the genera *Moraxella*, *Staphylococcus*, *Corynebacterium* and *Micrococcus* were present which have previously been associated with malodor (Kubota *et al.*, 2012; Zinn *et al.*, 2021).

*Moraxella* was identified only in malodor households, confirming the data of Kubota *et al.* (2012) (Kubota *et al.*, 2012). *M. osloensis* is known to cause opprtunistic infections such as meningitis (Roh *et al.*, 2010) or bacteraemia (Han & Tarrand, 2004), but has also been identified in household air in the UK (Yuan *et al.*, 2007). *Moraxella sp.* and its biodegradation have been linked to the formation of various VOCs such as p-nitrophenol (Spain & Gibson, 1991) or naphthalene-1,6-disulfonic acid (Wittich *et al.*, 1988), which, however, are not known to be components of malodor. It is conceivable that *Moraxella sp.* forms precursor components, which are further processed by other microorganisms and finally form components that are typical in malodor. Furthermore, *M. osloensis* in particular shows high tolerance to desiccation (Kubota *et al.*, 2012), which is probably related to its special fatty acid composition in the cell membrane (Sugimoto *et al.*, 1983), which plays an important role in desiccation tolerance (Beney & Gervais, 2001; Potts, 1994; Singh *et al.*, 2002).

Another possibility in the formation of malodor can be hydrophobic substances in the washing machine. These occur, for example, due to an incorrect dosage of detergent (Anonymous, 2022c). They seem to play a role for some consumers. In conversations during sampling, many households described recalcirtant dirt residues, which seem to consist primarily of skin grease, creams or ointments, as an aesthetic blemish on the

washing machine. Consumers reported a grey, greasy coating that settled not only on the laundry but also in the washing machine. Breaking down this residue could lead to the formation of odor in the washing machine. The formation of sweat works in a similar way, with microorganisms breaking down long-chain fatty acids into shorter chains such as butyric acid or formic acid, thus providing the typical smell of sweat (Kippenberger *et al.*, 2012; Leyden *et al.*, 1981). The same applies to the dishwasher, where malodorous VOCs were also found in the headspace (Howard-Reed *et al.*, 1999). This odor is described as "garlic, metallic, unpleasant" and is formed by dimethyl trisulphide (Ontañón *et al.*, 2019).

To understand a possible risk of infection and how adverse microbial effects like malodor develop, it is necessary to understand the interplay between microbial communities in the washing machine and on laundered items, since it has already been shown that laundering creates a complex microbial exchange pattern (Callewaert *et al.*, 2015). Interestingly, most of the studies related to the microbiological effects of laundering do not consider the actual microbial burden *in vivo* and in general; the “natural” bacterial counts on household textiles or in washing machines have only been investigated in a few studies (Munk *et al.*, 2001; Stapleton *et al.*, 2013). Unfortunately, some of the following studies did not provide bacterial counts for a defined surface, so that some numbers are only approximated. Stapleton *et al.* (2013) detected up to $10^4$ cfu /cm² in the detergent drawer and up to $10^5$ cfu/cm² in the rubber sealant of washing machines (Stapleton *et al.*, 2013). With regard to textiles laundered in household washing machines, Munk *et al.* (2001) showed how the bacterial counts on cotton and polycotton may develop after washing and found 1 x $10^5$ cfu/cm² and 1 x $10^4$ cfu/cm² one day after laundering, respectively (Munk *et al.*, 2001). Lucassen *et al.* (2014) found a mean total viable count (TVC) of approx. $10^2$ cfu/cm$^2$ on hand towels that had been normally used for one week (Lucassen *et al.*, 2014). In addition to the household-related publications there have been some studies investigating the bacterial burden on textiles in health care facilities (Bloomfield *et al.*, 2011; Howe *et al.*, 1961; Smith *et al.*, 1987). In this regards, Blaser *et al.* (1984) found total bacterial counts on objects such as soiled bed sheets and terry towels of $10^4$ – $10^6$ cfu/cm$^2$ (Blaser *et al.*, 1984).

The present study is the first to provide a quantitative and qualitative comparison of bacterial communities in washing machines and on normally used hand towels that have been laundered in these machines. Our results suggest a TVC of aerobic, mesophilic bacteria in the washing machine of approx. $10^5$ cfu/cm$^2$, while the TVC on normally used items was $10^2$ cfu/cm$^2$ after use. These findings are generally consistent with the studies mentioned above and prove a strong bacterial colonization of the detergent drawers and the rubber sealants in household washing machine. However, we could also show that the bacterial burden on used and unwashed textiles in standard households can be considered rather low, compared to microbial counts on textiles in clinical settings, confirming the data of

Lucassen *et al.* (2014) (Lucassen *et al.*, 2014). We refrained from analyzing the microbial reduction on the used textiles that can be achieved by laundering, since the reduction factors that are typical for domestic laundering procedures are well known from other studies (Bockmühl, 2011; Bockmühl, 2017; Callewaert *et al.*, 2015; Honisch, Brands, *et al.*, 2016; Linke *et al.*, 2011; Lucassen *et al.*, 2013). The cross contamination by washing machine biofilms must be considered and has not yet been investigated comprehensively, except from in a few studies suggesting a considerable input of machine-borne microorganisms (Callewaert *et al.*, 2015; Lucassen *et al.*, 2014).

When evaluating disinfecting procedures or products, textile test carriers are currently still artificially contaminated with a bacterial count of $10^8$ cfu/cm² according to the normative procedures (Anonymous, 2015b; Gebel *et al.*, 2001). Given the results of this study, this bacterial count must be considered more relevant to a situation in the health care sector than to the household. A new standard (prEN 17658) explicitly focuses on "chemical textile disinfection for the domestic area" (European Committee For Standardization, 2021) and uses initial counts of > $10^6$ cfu/cm² for bacteria and of > $10^5$ cfu/cm² for yeasts. According to this standard, a reduction of 4 log levels in the cfu of the bacteria is required and of 3 log levels for fungi. Based on the current results, these requirements reflect a consumer-related situation slightly better than the demands of EN 16616 (Anonymous, 2015b), although it is difficult to define requirements for antibacterial effects associated with domestic laundering, since the results of the present study do not include situations of higher risks, such as infections.

The dominant species in the washing machine were water-borne bacteria such as *P. aeruginosa*, and *S. maltophilia* (Table 7 - Table 8), which have been found in washing machines before (Schages *et al.*, 2021). While these data generally support the idea of a low infection risk associated with domestic laundry, our findings clearly show the presence of pathogens on used textiles, which may pose a risk under certain circumstances.

When correlating the occurrence of laundry-related malodor with the quantitative bacterial colonization, no significant differences in the bacterial counts with regards to malodor could be found (Figure 13) apart from significantly higher amounts of *Staphylococcus spp.* in the rubber sealant ($p > 0.05$) of malodor machines.

While the amount of bacteria does not seem to be a suitable to explain the development of laundry associated malodor, the qualitative analysis of the samples with and without malodor, yielded interesting results. In general the findings show a “typical” bacterial washing machine colonization, which is consistent with the data of Nix *et al.* (2015) and Jacksch *et al.* (2020) who showed that *Brevundimonas sp.*, *Pseudomonas sp.*, *Methylobacterium sp.*, *Acinetobacter sp.* and *Rhizobium sp.* were dominant species in domestic washing machines (Jacksch *et al.*, 2020; Nix *et al.*, 2015). Nonetheless, these microbial communities have not been related to the formation of malodor yet, except in isolated

findings, linking the presence of *M. osloensis* with musty textile odor (Kubota *et al.*, 2012; Takeuchi *et al.*, 2013). Since in the present study *Moraxella* could not be found in all samples from households with malodor experience, it is questionable if one single species may be responsible for the development of laundry malodor.

Recently, Zinn *et al.* (2021) showed that a combination of *M. luteus*, *S. hominis* and *C. jeikeium* can also lead to the formation of malodor (Zinn *et al.*, 2021). Two of these genera (*Staphylococcus* and *Micrococcus*) were detected on malodor towels in the current investigation as well. Gattlen *et al.* (2010) were also able to isolate staphylococci from washing machines in a culture-dependent approach (Gattlen *et al.*, 2010). Furthermore, Madsen *et al.* (2018) were able to show that *S. hominis* was found in 13 - 25% in air samples of living rooms (Madsen *et al.*, 2018). A similar study by Kooken *et al.* (2012) showed that about two thirds of the environmental samples from indoor air were *Micrococcus* (Kooken *et al.*, 2012). Likewise, Callewaert *et al.* (2015) found *Micrococcus sp.* on worn cotton clothing and suggested that skin-derived staphylococci and corynebacteria are enriched on textiles during washing, while micrococci remain abundant (Callewaert *et al.*, 2015).

However, the Shannon diversity of bacterial communities was significantly lower ($p < 0.05$) in non-objectionable ($\exp(H') = 10.56 \pm 9.38$) compared to malodor detergent drawers ($\exp(H') = 15.03 \pm 7.28$). Likewise, significant differences between the malodor rubber sealants ($\exp(H') = 10.57 \pm 8.02$) and the non-objectionable rubber sealants ($\exp(H') = 5.03 \pm 4.09$) could be observed (Table 9).

Biofilms in washing machines behave like all biofilms. For example, *Pseudomonas sp*., which was found to be the main coloniser in the washing machine, is a typical biofilm former with a quorum sensing network. *Pseudomonas* has at least three different quorum sensing pathways, two of which follow the AHL-based network (Bramhachari, 2018) and one of which is based on so-called *Pseudomonas* quinolone signals (Galloway *et al.*, 2012). Rhizobia also communicate with each other in biofilms via quorum sensing, controlling nodulation, biofilm formation, symbiosis with legumes and nitrogen fixation (Amrutha *et al.*, 2018). *Rhizobium sp*., on the other hand, has AHL molecules composed of long-chain fatty acids, which play a dual role in swarming (Daniels *et al.*, 2006). The cinIR operon is a QS system involved in the synthesis of AHL ligands and is necessary for swarming behaviour. The cinIR operon is autoregulated, leading to increased expression and AHL production.

In the natural habitat, EPS glucomannan produced by *Rhizobium sp.* interacts with cell surface lectin glycoproteins in various legume hosts (Laus *et al.*, 2006). The sugars required for EPS synthesis may be derived from animal polysaccharides, and these sugars may also serve as signals for biofilm formation, as observed in *Bacillus sp.* (Beauregard *et al.*, 2013). Surface proteins were also found to be essential for root adherence and/or biofilm formation in *Rhizobium* and *Pseudomonas spp.* (Ausmees *et al.*, 2001; Hinsa *et al.*, 2003).

The same could also be responsible for adhesion in the washing machine and should be further investigated in future work.

Since washing machines form a different habitat than soil, biofilm formation is necessary even under unfavourable conditions. Nutrient limitation is a decisive factor for the formation of a biofilm of *R. leguminosarum* on inert surfaces (Flemming *et al.*, 2016; Fujishige *et al.*, 2006; Rinaudi *et al.*, 2006; Russo *et al.*, 2006). Thus, based on the literature, an establishment of rhizobia in washing machines may also possible for future applications.

Although PCA (Figure 17) suggests a role of microbial colonizers of the washing machine and microorganisms present on the used textile in malodor formation, it cannot be excluded that microbial contaminations that are introduced after laundering (e. g., during drying) might influence malodor formation as well. To prove the idea of malodor-protecting bacteria, we used a model to generate laundry-associated malodor *in vitro* published by Zinn *et al.* (2021) (M. Zinn *et al.*, 2021). In this study, it could be shown that, amongst other substances, dimethyl disulfide, dimethyl trisulfide and indole may in particular account for the wet-fabric-like malodor. Apart from being volatile, malodorous substances, Weisskopf and Coworkers (2021) recently showed that dimethyl disulfide and dimethyl trisulfide can inhibit the growth of soil bacteria and promote the growth of *Pseudomonas sp.* (Weisskopf *et al.*, 2021), which is in line with the identified microbial community patterns of the malodor and non-objectionable detergent drawers in the present study.

## 5.2 *Ex situ* model for investigating microbial communities isolated from household washing machines

### 5.2.1 Relationship of the melting behaviour and the composition of the microbial community

With the methodology presented in this work, it was possible to cultivate a biofilm over 10 days, which had similar melting curve behaviour to the original biofilm. As a cultivation medium, the composition of 0.001% potato starch and 0.001% meat extract solution in type 1 water (medium 4) proved to be the best cultivation medium for washing machine biofilms (Table 10).

The multiple determinations confirm the experimental procedure and show a certain reproducibility of the results. The occurrence of altered and shifted melting curves, such as in Figure 19, suggest that not all biofilms have the same composition and nutrient requirements. The diversity of biofilms in the household is influenced by various human factors such as frequency of use, age of the machine and water hardness (Abeliotis *et al.*, 2015; Bockmühl *et al.*, 2019; Raghupathi *et al.*, 2018).

In addition to the laundry, other influencing factors such as water, detergent and the washing machine itself are considered to be sources of entry for microorganisms in the washing machine (Lucassen *et al.*, 2014), therefore a wide range of nutrient compositions (Table 1) were tested. Since there are various sources, the nutrient requirements were also adapted to the corresponding milieu, so that not all biofilms could be equally cultivated with a fixed media composition in terms of their initial composition.

This explains why the melting behavior of some biofilms after 10 d was similar to the initial sample, while other biofilms under the same conditions showed melting curves that were sometimes significantly different (Figure 19 - Figure 24). The similarities and differences of the biofilm samples are supported by the Bray-Curtis dissimilarity. While the family-level value for the detergent drawer of machine 2 and medium 4 compared to the initial sample was 0.24, indicating a high degree of similarity, the values for corresponding samples from the rubber sealant of machine 2 with medium 4 were 0.99. These high values indicate a significantly different microbial composition (Wong *et al.*, 2016). These data are supported by the calculation of the Euclidean distance, which for the active melting range for the detergent drawer of machine 2 and medium 4 showed a significantly smaller value of 0.23 than the rubber sealant of machine 2 with medium 4 (0.80). A correlation between these two measured variables could be recorded (Figure 37, see section 5.2.3).

For further evaluation of the *ex situ* biofilms, a Principal Component Analysis (PCA) was conducted. Each data point obtained from the melting curve analysis was considered and plotted on the first two principal components, as shown in Figure 36. The PCA yielded distinct clusters, particularly for malodorous households, which were easily discernible for both the rinsing chamber (red cluster) and the rubber seal (blue cluster). However, it should be noted that there were some outliers lying outside the clusters. The results could be further validated with a larger dataset.

In the case of no-odor households, clear patterns or clusters were not discernible. Although a large data cluster of no-odor households was present, there was no clear differentiation between the sampling locations. This observation was also confirmed by the microbial composition of the individual sampling sites in no-odor households, as evidenced by the Bray-Curtis Index of 0.35, particularly in machine 3 (no odor). This indicated the presence of a similarity in the microbial composition. These values were particularly apparent in the PCA on the second principal component above the value of 0 (blue and red squares). The Bray-Curtis index of the sampling sites of machine 2 (no odor) was 0.99, indicating complete dissimilarity between the two sites. In the present PCA, these values were found on the second principal component both above the value of 0, along with the values of machine 3, and below 0 (red squares).

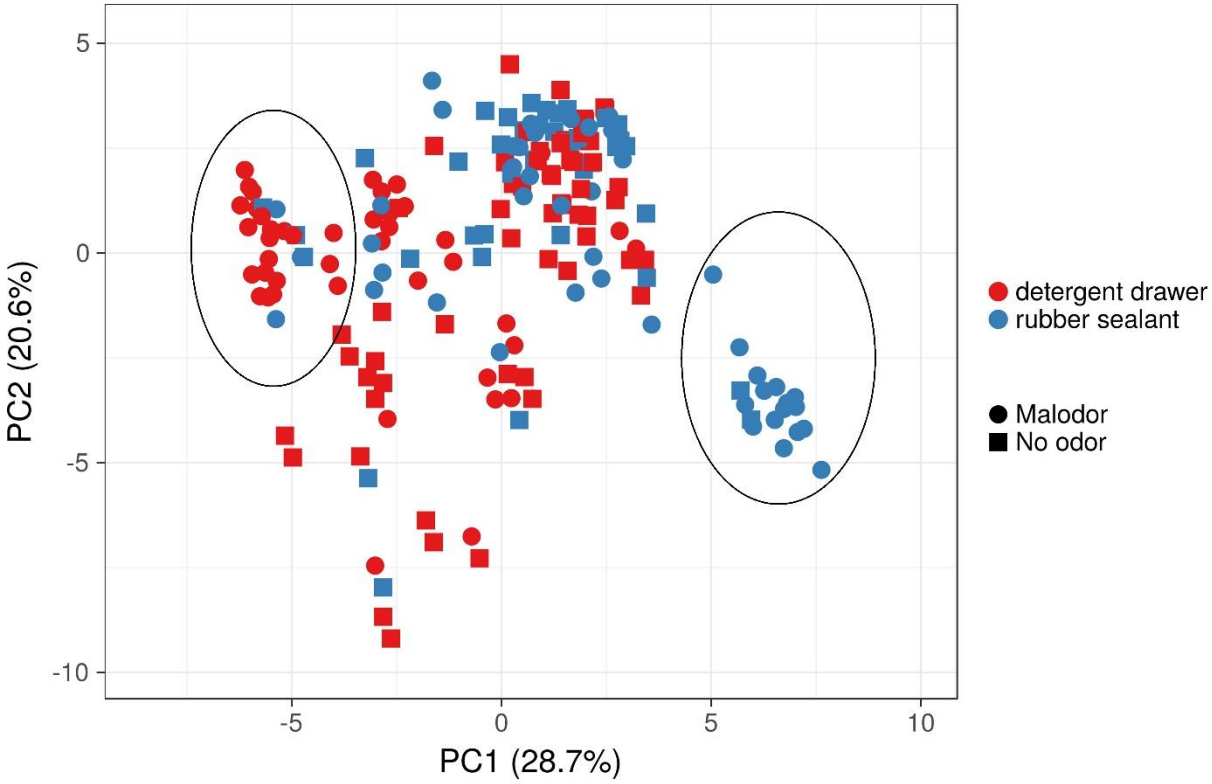



**Figure 36 Principal component analysis (first two principal components) of standardised melting curves of *ex situ* biofilms. A distinction is made between detergent drawer (red) and rubber sealant (blue), as well as between malodor (circle) and no odor (square) households. Black ellipses indicate clusters that have formed.**

The differences in biofilms demonstrate their complexity, dynamics and diversity, which complicates their analysis and the development of suitable models to study them (Hansen *et al.*, 2019). The goal, which is more realistic for the time being, should therefore be the preservation of an appropriate diversity of a biofilm and not its identical reproduction after cultivation in the laboratory. The combination of parameters that are important (Sinner, 1960) cannot all be included in a simplified *ex situ* model. In the current experiments, for example, the influence of mechanics in terms of shear forces was not taken into account. In further experiments, the use of a pump or a type of drum could better incorporate this aspect as realistically as possible based on the processes of washing machine programmes. A comparable methodology taking flow rates into account has already been established by Ledwoch *et al.* (2020) for sink pipes (Ledwoch *et al.*, 2020). Transferring the idea to washing machine biofilms makes sense, as the influence of flow rate on biofilms from rivers has already been identified as an important factor in their composition (Romero *et al.*, 2020). Furthermore, changes in biofilm formation could be identified in fast-flowing aquatic environments (P. Stoodley *et al.*, 2002). Therefore, it cannot be ruled out that the same applies to biofilms in the household that are formed in an aquatic environment such as in washing machines,

dishwashers or coffee machines). Supporting this hypothesis, Raghupathi *et al.* (2018) referred to mechanics as a factor defining the microbiome in the dishwasher (Raghupathi *et al.*, 2018).

Furthermore, the volume of water and the liquor ratio used in the washing process are among the most important factors of microbial reduction in household washing machines (Bockmühl *et al.*, 2019; Heinzel *et al.*, 2010). Similarly, long-term incubation at room temperature may have an influence on biofilm composition. Since the washing temperature has a significant influence on the reduction of microorganisms in the washing machine (Bockmühl *et al.*, 2019; Honisch *et al.*, 2014; Honisch, Brands, *et al.*, 2016; Schages *et al.*, 2020), a selection of certain microorganisms is possible. In the present experiments, the possibility cannot be ruled out that species accumulated that would not be present in the biofilm in the long term in the scenario of household use or that would be displaced by other species.

The visual evaluation of the melting curves of the recultivation of biofilms from glycerol stocks partly suggests that a similar microbial community is present in the samples. The differences that occurred in the melting curves could have been caused by the storage time of the glycerol stocks, which has already been identified as an influencing factor on the cells (Hubálek, 2003). This influence could be more pronounced in biofilms than in a single species. Thus, the complexity and diversity of biofilms also complicate the analysis (Hansen *et al.*, 2019) in relation to recultivation.

Long-term frozen storage of bacterial strains mixed with glycerol is a widely used method for preserving microorganisms. The addition of glycerol protects the cells from freezing damage by penetrating the bacterial cell wall and cytoplasmic membrane, thereby reducing osmotic stress. However, there are several influencing factors such as bacterial species, pH, cell composition or medium that determine the effectiveness of glycerol as cryoprotection. Similarly, the medium used for re-growth from the frozen stock is decisive for viability after freezing. For some species, glycerol has no protective function or is even toxic to the cell (Hubálek, 2003). Since the composition of the biofilms used for this work is unknown and the media composition with low concentrations of meat extract and potato starch is rather atypical, there is a possibility that not all originally contained bacterial strains regenerate after freezing. Due to slight changes in the community with each further recultivation, the effects of various factors may accumulate until, at a certain point, more significant differences become visible. In conclusion, the development of an *ex situ* model for biofilm recultivation is complex. The previously discussed aspects that may have an influence on the cultivated biofilms also apply to this series of experiments.

Since Bray-Curtis values close to 0 stand for an almost identical community of two samples (Wong *et al.*, 2016), the similarity in recultivation visually assessed by the melting curves is statistically supported by the sequencing data. The trend of the melting curves of the

recultivation experiments is constant within the replicates and partly shows large differences from the initial sample. The Bray-Curtis constant at family level is at a minimum of 0.45 in all tested combinations compared to the initial sample. Also in the case of recultivation of biofilm samples from glycerol stocks, the Euclidean distance between the active melting areas matches the Bray-Curtis indices, so that with a lower Bray-Curtis dissimilarity, the Euclidean distance is also lower (discussion in section 5.2.3).

The high proportion of different genera (*Sphingomonas*, *Cutibacterium*, *Microbacterium*, *Rhodobacter*, *Stenotrophomonas*, *Pseudomonas* and *Enterobacter*) is striking for the biofilms of the recultivation experiments. The occurrence of *Microbacterium* has already been proven in previous studies (Bockmühl, 2017). This genus has been identified as soil bacteria (Shimkets *et al.*, 2006) and possible contaminants in laboratory investigations (Salter *et al.*, 2014). Possible sources of DNA contamination include molecular biology water (Bohus *et al.*, 2011; Kéki *et al.*, 2013; Kulakov *et al.*, 2002; McAlister *et al.*, 2002; McFeters *et al.*, 1993; Nogami *et al.*, 1998; Shen *et al.*, 2006), PCR reagents (Corless *et al.*, 2000; Grahn *et al.*, 2003; Maiwald *et al.*, 1993; Newsome *et al.*, 2004; Rand & Houck, 1990; Tanner *et al.*, 1998) and DNA extraction kits themselves (Mohammadi *et al.*, 2005). To exclude contamination in this study, all media and reagents used were also sequenced as negative controls. For this reason, *Sphingomonas*, *Stenotrophomonas* and *Pseudomonas* (Barton *et al.*, 2006; Bohus *et al.*, 2011; Corless *et al.*, 2000; Grahn *et al.*, 2003; Kéki *et al.*, 2013; Kulakov *et al.*, 2002; Laurence *et al.*, 2014; Maiwald *et al.*, 1993; McAlister *et al.*, 2002; McFeters *et al.*, 1993; Newsome *et al.*, 2004; Nogami *et al.*, 1998; Rand & Houck, 1990; Shen *et al.*, 2006; Tanner *et al.*, 1998) can all be excluded as contaminants. The genus *Sphingomonas* belongs to the common bacteria and is found especially in aqueous and terrestrial environments such as plant root systems. Furthermore, *Sphingomonas* is able to utilise organic compounds and survive under nutrient-poor conditions (Shimkets *et al.*, 2006). In households, *Sphingomonas* has been found in the biofilms of shower curtains (Kelley *et al.*, 2004). The authors attributed the presence to a combination of nutrients from soap residues and humidity. The presence of other genera such as *Pseudomonas*, *Stenotrophomonas* or *Rhodobacter* is attributed to the fact that they are known water germs for which a washing machine creates ideal growth conditions. The aforementioned water germs have also been identified in washing machines in previous studies (Jacksch *et al.*, 2020; Nix *et al.*, 2015).

Assuming that the species with the largest relative proportions are primarily responsible for the largest peaks in melting behaviour, hypotheses regarding the influence of certain species can be made. Since a high GC content leads to an increased melting temperature Tm (Khandelwal & Bhyravabhotla, 2010; Liu *et al.*, 2007), a shift to the right towards higher

temperatures in the melting curve analysis means, among other things, an increased GC content compared to peaks further to the left.

*Enterobacter cloacae* as a type species of the genus *Enterobacter*, was strongly represented in the rubber sealant of machine 1 (Table 13), and close relatives have a GC content of about 55% in their DNA (Mustafa *et al.*, 2020; Ren *et al.*, 2010). Similar values were also shown by the genera *Microbacterium* (GC content: 69.1%; (Leipniz-Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, 2022), *Cutibacterium* (GC content: 54%; (Kim *et al.*, 2022)) or *Sphingomonas* (GC content: 65.7%; (Pan *et al.*, 2016)). In contrast, the type species *Acinetobacter baumannii* (Table 13) as a representative of the genus *Acinetobacter* (GC content: 39%; (National Center for Biotechnology Information, 2022)) and the type species *M. osloensis* (GC content: 43.6%, (Lim *et al.*, 2018)) as a representative of the family *Moraxellaceae* have a lower GC content. *P. aeruginosa* as the type species for the corresponding genus has a GC content of 67.2% (Raychaudhuri & Tipton, 2004). With regard to the data shown, it can thus be assumed that the main peak at 86 °C, which recurs in all samples, is caused, among other things, by the genera with higher GC contents. For this reason, the assignment of the peak with the highest melting temperature makes sense. Accordingly, the assumption arises that the peak at approx. 83 °C is partly caused by the genera *Acinetobacter* and *Moraxella*.

Nevertheless, it should be noted that the GC content of the gDNA is not directly equivalent to that of the ITS amplicons. The selected oligonucleotide pair (ITS1f and ITS2r) for characterization of bacteria and fungi also amplifies the internal transcribed spacer region. The ITS region separates small ribosomal subunit genes from the large subunit in eukaryotic organisms. This region consists of two regions (ITS1 and ITS2) and the 5.8S gene. Another advantage is that the ITS region does not code, making them ideal for identifying organisms (Gardes & Bruns, 1993; White *et al.*, 1990). The ITS region is easily amplified from small, dilute, or highly degraded DNA samples based on the multicopy nature of the rDNA repeat, and various studies found that the ITS region is often widely diverse in morphologically heterogeneous fungal species (Baura *et al.*, 1992; Chen *et al.*, 1992; Gardes *et al.*, 1991; Gardes & Bruns, 1991; Lee & Taylor, 1992), and that, with one noted exception (O'Donnell, 1992), there is little intraspecific variation (Anderson & Stasovski, 2008; Baura *et al.*, 1992; W. Chen *et al.*, 1992; Gardes *et al.*, 1991; Gardes & Bruns, 1991; Lee & Taylor, 1992).

An accurate and direct prediction of the melting temperature for short DNA segments such as oligonucleotide sequences (Khandelwal & Bhyravabhotla, 2010) is not possible due to the unknown DNA mixture. Shoulder formations and broad instead of narrow, clear peaks prevent a clear assignment of these (Caux-Moncoutier *et al.*, 2011). In addition, there is the effect of regions between 40 and 100 base pairs, whose double strands separate

simultaneously at different points in long DNA sequences or entire genomes (Khandelwal & Bhyravabhotla, 2010). Since the ITS primers used generate PCR products of about 600 bp (Andini *et al.*, 2017), this may be the case in the experiments shown and complicate the interpretation of the melting curves. For example, since DNA extracts of *Enterobacter* members can produce multiple signals between 80 °C and 85 °C and the *Pseudomonas* members can produce multiple signals between about 77 °C and 86 °C in a melting curve based on the bacterial ITS region (Andini *et al.*, 2017), the interactions within the diverse DNA mixtures cannot be estimated. In this way, several peaks could be influenced by one genus.

Due to these factors and the limited data available, it is currently not possible to make a prediction about the exact composition (species, genus, family). However, it is possible to roughly compare habitats for similarity in a quick and cost-effective way.

Nevertheless, with a refined procedure adapted to the habitat of an original biofilm, the methodology for the re-cultivation of microbial communities *in vitro*, represents a procedure with potential. With a media composition adapted to the biofilm, it is possible to obtain a similarly composed biofilm recultivated from glycerol stocks. Families or species that are particularly dominant in the biofilm remain present in similar proportions with some variance. Due to the complexity of biofilms, a certain limitation of this method remains.

An additional factor that influences the complexity of the biofilm is the media temperature. In this study, the short-term influence of temperature-controlled media on the melting curves of washing machine biofilms was investigated (Figure 27). The results showed that the melting curves became more diverse with increasing media temperature. This is consistent with the assumption that washing temperature can influence biofilm formation and micorbial community composition (Honisch *et al.*, 2014; Honisch, Brands, *et al.*, 2016; Ponomareva *et al.*, 2018). The melting curves show that the main peak shifts with increasing media temperature. In contrast, the intensity of the minor peak at 83 °C decreases with increasing media temperature and compared to the control. It is possible that a slight change in melting behaviour has occurred due to the depletion of mesophilic bacteria, which are partly responsible for this signal. Thermophilic microorganisms can tolerate heat stress better through different strategies and trigger a population shift. While mesophilic germs with optimal growth conditions of 20 °C - 42 °C tend to grow at room temperature, a media temperature of 50 °C or 60 °C can favour thermophilic germs in their growth (Fuchs, 2007). Thermophilic genera were previously identified in biofilms of washing machines (Jacksch *et al.*, 2020; Nix *et al.*, 2015). These microorganisms can usually tolerate short periods at 70 °C and grow optimally at temperatures between 50 °C and 65 °C as well as in alkaline conditions (Raghupathi *et al.*, 2018).

The DNA of thermophilic microorganisms is stabilised by various mechanisms such as pronounced supercoiling (Charlier & Droogmans, 2005), so a higher melting point of the gDNA of these compared to mesophilic microorganisms is conceivable. This may explain the reduction in intensity of the small peak on the left. At lower temperatures, the genome of less heat-tolerant organisms, possibly with lower GC content, is more likely. However, a slightly varying height of a peak can only be due to different template concentrations (Andini *et al.*, 2017). In the reported experiments, no relevant proportions of typical thermophilic genera could be detected in the source biofilms (Table 11 - Table 21).

The formation of the biofilm matrix, which can buffer extreme temperatures and reduce its effect on the cells, argues for a hardly changed microbial composition of the biofilm (Hall-Stoodley *et al.*, 2004). The increased formation of heat shock proteins or chaperones in biofilms as a stress response to elevated temperatures induce enhanced tolerance to temperature stress (Santos *et al.*, 2019). In addition, the small volume of 2 mL in the experimental set-up ensures that the temperature-controlled medium cools down in a short time. Higher temperatures and/or a different loading rhythm could produce different results. For example, a temperature of 50 °C for 15 min can kill bacteria such as *S. aureus* in the washing machine (Honisch *et al.*, 2014). In dishwashers, a temperature of 60 °C for only 2 min was shown to kill Gram-negative bacteria on surfaces of dishes (Schulze-Struchtrup *et al.*, 2021).

When considering sequencing data of the biofilms studied, an investigation into a decreasing mesophilic population would be useful. Since temperatures in the washing machine fluctuate during a washing programme and can be low or high depending on the programme (Honisch *et al.*, 2014; Honisch, Brands, *et al.*, 2016), both groups, mesophilic and thermotolerant microorganisms, are to be expected.

### 5.2.2 High Resolution Melting Analysis and its potential as a screening tool for changes in microbial communities

The available experimental results show that HRMA can be an effective tool for detecting changes in microbial communities. The analysis of the melting behaviour of the gDNA of biofilms after a temperature treatment has shown that in most cases a differentiation from untreated controls is generated. As a result, it can be assumed that the biofilm community has changed in the course of the treatment. In order to verify these changes and, for example, to be able to investigate the composition at genus or species level in more detail, a comparative method is necessary.

To validate HRMA results, it is useful to use alternative methods to confirm the change and to characterise it. In this context, denaturing gradient gel electrophoresis (DGGE) can be an alternative method to analyse microbial composition (Hjelmsø *et al.*, 2014; Welsh & McLean, 2007). With this approach, the genetic fingerprint of a microbial community is analysed by separating DNA based on its GC content within a gradient gel with increasing concentrations of formamide and urease. In this process, these substances have a denaturating effect and cause the DNA of different species with different GC contents to denature at different times (Welsh & McLean, 2007). The denaturation, in turn, leads to a slower flow of DNA in the gel and eventually ensures that all DNA is characterised by bands localised at different sites in the gel (Levin *et al.*, 2018). Thermal gradient gel electrophoresis follows a principle similar to DGGE. In this case, as with HRMA, DNA is denatured by a temperature gradient (Fuchs, 2007). Thus, the DNA of a sample is separated on the basis of the melting point of the different DNA components by restricting the mobility of the strands through the denaturation of double strands into two single strands (Fuchs, 2007; Zhou & Li, 2015).

Besides these methodically more complex methods, the most accurate method is amplification and sequencing of DNA samples (gold standard test). This allows the composition to be analysed down to the species level, if necessary. Typically, for complex communities such as biofilms, the 16S rRNA of the entire community is used for further processing with different methods (Fuchs, 2007).

Due to the accuracy of the results, selected samples from this work were subjected to sequencing to validate the melting curve results. Additional information on changes in biofilm adhesion, structure and cell number can be collected using microscopic techniques (Azeredo *et al.*, 2017). While scanning electron microscopy is used for structural analysis, fluorescence staining in the context of confocal laser scanning microscopy (CLSM) can be used for studies of the spatial structure of the biofilm (Azeredo *et al.*, 2017; Toyofuku *et al.*, 2016).

The possibility of using HRMA to identify bacteria by their melting behaviour has already been demonstrated in various studies. These have mainly examined clinically relevant human or veterinary pathogenic bacterial species (Andini *et al.*, 2017; Cheng *et al.*, 2006; Morick *et al.*, 2009; Robertson *et al.*, 2009). Using a database, Andini *et al.* (2017) developed an HRMA system to identify commensal as well as pathogenic microorganisms from different phylogenetic families and genera based on their melting behaviour. This achieved an accuracy of 90% at the species level and 95% at the genus level when investigating 89 bacterial species (Andini *et al.*, 2017). Similarly, older studies by Cheng *et al.* (2006) achieved a comparable accuracy of 94 % in the assignment of 54 clinical isolates. In this case, there was also a database of deposited melting curve data used to identify indeterminate colonies. The isolates

that could not be clearly identified belonged to closely related genera or the same genus. For isolates in pure culture, this study showed that meaningful classification using HRMA is potentially possible without DNA extraction using a bacterial suspension (Cheng *et al.*, 2006).

The usefulness of HRMA for differentiating entire microbial communities after exposure to chemical substances was already investigated by Hjelmsø *et al.* (2014). Comparable to the experiments in this thesis, they used HRMA to examine changes in microbial communities from environmental samples. The authors analysed soil samples and contaminated them with two different pesticides (Basamid GR, Tridex GD) with and without the addition of ammonium sulphate as fertiliser (Hjelmsø *et al.*, 2014). Subsequent DNA extraction followed by qPCR and melting curve analysis resulted in statistically significant changes in the melting curves of basamide-treated soils compared to water-treated controls, depending on the duration of the experiment. These results could be confirmed by DGGE. A similarly small difference in melting behaviour in treated samples related to the control was recorded in DGGE. However, the authors noted that even DGGE is not always able to differentiate closely related species and so it is possible that there was a change in the microbiome for which both methods have too low a sensitivity (Hjelmsø *et al.*, 2014).

Inspired by these experiments, Everman and Wang (2017) investigated microbial communities from gastrointestinal tracts and excrement of tadpoles using HRMA. DGGE and sequencing of the corresponding DNA extracts also served as comparative methods here. The microbiomes of five different regions of the gut of the same tadpole could be differentiated on the basis of the melting behaviour. Within an intestinal region, multiple determinations showed similar melting behaviour, suggesting a microbial composition typical for that region. The differences between microbiomes were confirmed by DGGE and 16S sequencing. For all methods, the rectal section and excrement of the animals showed the greatest variance in the microbial community. Validation using sequencing data showed higher significance in some cases for DGGE and in some for HRMA. Compared to Hjelmsø *et al.* (2014), about 50 bp shorter amplicons and more specific primers were chosen in these experiments for better sensitivity (Everman & Wang, 2017).

Furthermore, the investigation of changes in methanogenic communities using HRMA from anaerobic digesters has already been performed and validated using DGGE. In this case, in contrast to the previous analyses, the archaeal rather than the bacterial 16S region was used for PCR and subsequent HRMA. Differences between various microbial communities could also be highlighted in these experiments. Once again, the results of the melting curve analyses agreed with those of the DGGE. In conclusion, the authors concluded that HRMA can be an efficient, low-time and low-cost alternative to DGGE for microbial community analysis (Kim & Lee, 2014). The results showed that not only differences in communities of

bacteria but also archaea can be highlighted by HRMA. This speaks to the potential for diverse applications of HRMA. Variation in use is supported by results from Wang *et al* (2019).

In studies of the oral microbiota of different subjects they used HRMA to show differences in the samples. The authors concluded that the methodology could be used in forensics to identify individuals based on their oral microbiome (Wang *et al.*, 2019).

An important difference between the research shown and the studies discussed is that instead of amplifying the ITS genes, the 16S rRNA genes were usually used as templates for qPCR and subsequent HRMA. The ITS sequence as a phylogenetic marker was chosen based on the results of Andini *et al.* (2016), who showed that ITS regions obtained more complex curves with multiple peaks compared to 16S sequences in a melting curve analysis. This is due to highly conserved regions within a species and greater differences between different species due to polymorphisms in contrast to 16S rRNA. This resulted in improved discrimination between different species (Andini *et al.*, 2017). Thus, it can be assumed that the melting behaviour of ITS amplicons can also reveal changes in a microbial community.

In general, the melting profile of DNA samples in a HRMA is influenced by several factors that limit the method and should be considered when assessing melting curves. These include for example sample volume, amount of DNA and the fluorescent dye, the melting rate and the thermocycler used (Herrmann *et al.*, 2006). When comparing different studies, these factors and their effects should therefore always be taken into account. Some aspects will be discussed below in relation to the method used for the experiments shown.

The template concentration used for qPCR may influence the melting curve by causing the height of the peaks to vary. This occurs especially with samples that do not reach the plateau phase in the qPCR (Andini *et al.*, 2017). The quality of the melting curves obtained can be negatively influenced by this (Life Technologies Corporation, 2009). As in the present experiments very low DNA concentrations were often present, this phenomenon may occur with some samples.

To minimise differences in peak height during a later HRMA, it would be possible to optimise the cell extraction methodology or to use a larger volume of the resulting suspension for DNA extraction. In either case, care should be taken to use similar concentrations of DNA at the outset of the PCR to achieve comparable results (Life Technologies Corporation, 2009). To compensate for a low initial concentration of DNA, the number of PCR cycles can be increased. For example, Cheng *et al.* (2006) used 40 PCR cycles prior to melt curve analysis. However, in this case primer dimers formed, which could be seen in the melting behaviour at a peak of 74 °C (Cheng *et al.*, 2006). In contrast, the formation of primer dimers was minimised in the studies presented in this thesis by using only 33 cycles.

The melting curve also influences the amplicon length, which should be adjusted depending on the application. While short amplicons reduce the fluorescence signal, they allow in parallel a better differentiation of sequence variants. For genotyping, an amplicon length of about 100 base pairs is ideal; for mutation screening, amplicons of 300 bp length are easy to distinguish. The complexity of the melting curve of amplicons increases with their length and complicates the evaluation (Life Technologies Corporation, 2009). Since the differentiation of gDNA from biofilms is not aimed at the detection of differences at the base level, the amplicons used with a length of 600 bp should be sufficient for these purposes, although further investigations could provide clarity in this regard. In addition, with long DNA sequences such as the ITS-PCR product, parallel separations can occur in different regions (Andini *et al.*, 2017; Khandelwal & Bhyravabhotla, 2010). This complicates the interpretation of the melting curves.

Furthermore, the choice of primer has an influence on the results of HRMA. In the experiments shown, primers already established by Andini *et al.* (2017) were used to amplify the ITS regions. In general, it is recommended to investigate several primer sets and to compare the quality of the resulting melting curves. The formation of primer dimers should in particular be minimised (Life Technologies Corporation, 2009). For a general improvement of the quality of the melting curves, the use of degenerated primers would be conceivable in order to maximise the number of possible PCR products. This results in a greater variation with regard to the melting curves, but the sensitivity of the method decreases. For increased sensitivity, e.g. in studies of closely related species, specific primers are useful (Hjelmsø *et al.*, 2014). In this case, a consideration is necessary depending on the proposed use.

In addition, the SYBR-Blue dye used in the experiments is not equally well suited for all applications, as while there is compatibility with most equipment, the accuracy of the melting curves in the case of heteroduplex discrimination needs improvement. Alternatively, the use of saturated fluorescent dyes such as LCGreen is possible (Herrmann *et al.*, 2006; Tong & Giffard, 2012). For other applications, such as in the case of this work for an assessment of the composition of a microbial community, the accuracy should be sufficient (Tong & Giffard, 2012).

Lastly, variance in melting curves is possible and unavoidable when performed repeatedly with the same DNA due to even small variations in performance (Andini *et al.*, 2017). It is therefore to be expected that if the treated biofilms were analysed again, there could be distinguishable differences to the results shown here. By developing an algorithm that accounts for this variation within multiple runs and takes other factors into account, minimising spurious results is potentially possible. Similarly, such an algorithm could account for peak heights varying due to low DNA concentrations (Andini *et al.*, 2017). At what

point a Tm shift of a peak to higher or lower temperatures corresponds to a change in the microbial community is unclear.

The melting temperature increases with increasing amplicon length, independent of the GC content of the sample, but only to a small extent. Tong and Giffard (2012) established a temperature difference of 0.2 °C as the limit for differentiating melting curves of individual bacterial species. Due to the complexity of a biofilm and the interactions within a gDNA of it, this difference is probably not directly applicable to the experiments presented here. However, a rough assessment of whether there is a change in an entire microbial community is already recognisable from the course of the melting profile, according to the present results (Tong & Giffard, 2012).

In general, HRMA is a method with potential as a screening tool (Hjelmsø *et al.*, 2014; J. Kim & Lee, 2014; Tong & Giffard, 2012). Especially with high sample throughput, HRMA can serve as a screening to filter out interesting samples with a clear change in their composition compared to controls (Hjelmsø *et al.*, 2014). This should be confirmed in the future. A similarity in melting behaviour with similar microbial composition according to 16S results was confirmed in this thesis. As is known, the detailed interpretation of peaks was complicated by shoulder formations and broadened peaks (Caux-Moncoutier *et al.*, 2011). The time-saving and rapid methods of qPCR and HRMA can, for example, precede metagenome sequencing of these selected samples and offer temporal and financial relief (Hjelmsø *et al.*, 2014). For this reason, and due to the small sample sizes, it would make sense to subject further interesting DNA extracts from this work to (meta-)genome sequencing and thus highlight the exact differences at different phylogenetic levels.

This is planned and will serve to verify the results obtained and add much more detailed information to them. For other applications, such as for medical purposes or in forensics, HRMA of a microbial community may equally represent a potential use (Cheng *et al.*, 2006; Wang *et al.*, 2019). Given the possibility of direct use of a bacterial suspension of a single isolate for HRMA, research into a method using clinical samples with multiple pathogens, where appropriate, is desirable (Cheng *et al.*, 2006).

### 5.2.3 Statistical correlation of the Euclidean distance and the Bray-Curtis dissimilarity

To investigate a possible existing statistical correlation between the Euclidean distance of the melting curves and the Bray-Curtis dissimilarity, all data from two compared biofilms were used for analysis. First, the values were tested for normal distribution according to D´Agostino and Pearson (D'Agostino & Pearson, 1973). Since the test proved a normal distribution, a linear regression of the data could be carried out. The Bray-Curtis index at family level was plotted as a function of the Euclidean distance as a linear regression and the linear relationship between the two variables was investigated using the coefficient of determination

$R^2$, automatically calculated by the GraphPad PRISM statistical software. The coefficient of determination ($R^2$) can assume values between 0 and 1 or 0% and 100%, whereby high values indicate a good prediction by the model and a low variation away from the determined straight line (Chicco *et al.*, 2021). For example, a value of 0.6 means that 60% of the data fit the regression by the model shown (Corporte Finance Institute, 2022). Values close to 1 correspond to a straight line that fits the linear relationship between two variables.

The results of all data points obtained in this work, including the resulting mathematical functions and coefficients of determination, can be seen in Figure 37. The figure shows that a mathematical function results from the linear regression for the examined correlation, which is described by a slightly positive slope. The straight line had a straight line equation of y = 0.2319x + 0.5286 and a coefficient of determination of 0.4697. The slope of the straight line increased with 0.2319x.

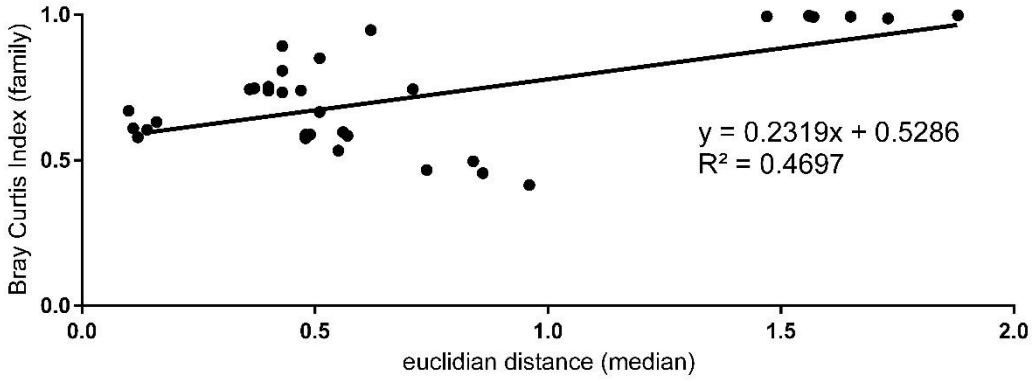



**Figure 37 Linear regression of the family-level Bray-Curtis index variable as a function of Euclidean distance for all sequenced samples (n = 42).**

Figure 37 shows that there is a weak positive correlation between the Euclidean distance of melting curves and the Bray-Curtis dissimilarity of the respective microbial community (Puth *et al.*, 2014). This relationship is assessed using the coefficient of determination $R^2$, which is a statistical measure of how close the data are to the fitted regression line. It is the percentage of dispersion in the response variable that is explained by a linear model. In general, higher values mean that the model is better fitted to the data (Chicco *et al.*, 2021; Freund *et al.*, 2006; Sykes, 1993). The present model explains about 47% of the data with an $R^2$ of 0.4697. In biological systems, which are generally more difficult to predict than, for example, chemical or physical processes, the values of the coefficient of determination are usually below 50% (Welham *et al.*, 2015). Furthermore, it should be noted that a high coefficient of determination is not necessarily a good test, because it can lead to a kind of "bias" of the data, i.e. a systematic false prediction by the regression line.

The results obtained in this work show variance between different washing machine biofilms. Therefore, data related to two different original biofilms could be indicative of whether or not (re-)cultured biofilms differ greatly. The Bray Curtis dissimilarity of two original samples is usually high, with values above 0.80 (example: single wash machine 1 vs single wash machine 2, Bray Curtis dissimilarity: 0.88), indicating a strongly divergent composition (Wong *et al.*, 2016). Similar high values were also obtained, for example, for the cultivation of the biofilm of the induction chamber of machine 1 with medium 4 (Table 12), thus showing a medium similarity in terms of their microbial communities. Some of the recultivations show significantly lower values in different combinations (e.g. cultivation of the biofilm of the rubber sealant of machine 1 with medium 4, Bray-Curtis dissimilarity of 0.45; Table 14). The replicates among themselves show similarities with values sometimes well below 0.20 (e.g. cultivation of the biofilm of the rubber sealant of machine 2 with medium 4, Bray-Curtis dissimilarity of 0.11; Table 18), indicating a high similarity of the respective biofilms (Wong *et al.*, 2016). Similar trends are shown for the Euclidean distance.

In addition to the small amount of data (n = 42), limitations due to the prescribed method must also be taken into account when assessing the significance. For a better comparability, all data of the melting curves were normalised for the temperatures between 81 °C and 91 °C. Thus, all samples could be examined with regard to the same temperature range. For the statistical analysis of the Euclidean distance, an individual adjustment of the observed ranges for each pairing would be useful. Outer areas with background noise and without clear peaks could thus be reduced or eliminated and the significance of the Euclidean distance improved. In addition, differences in the height of the peaks, i.e. varying y-values taken into account in the Euclidean distance, are to a certain extent not necessarily due to differences in the melting behaviour of the DNA, but possibly to different DNA concentrations, for example (Andini *et al.*, 2017). It should also be taken into account that the calculation of the Bray-Curtis dissimilarity does not include the amount of undefined DNA, but may contribute to the melting behaviour of the sample. Therefore, samples with high unknown proportions should be re-examined in order to assess the relationship between Euclidean distance and Bray-Curtis dissimilarity even more precisely.

## 5.3 Malodor model for investigating effects on bad textile odor

With their predominant presence in non-objectionable machines, these findings (Figure 34) strongly suggest that *Rhizobium spp.*, has a positive impact on laundry related malodor. More research is needed, however, to reveal the exact interrelationship of malodorous and (putatively) protective bacterial species For example, although our data indicate *Rhizobium spp.* is a protective means with regards to malodor, we did not find rhizobia in any

case of non-objectionable samples, so the potential role of other bacteria still has to be elucidated.

Recent literature shows that *Rhizobium* interacts with VOCs such as dimethyl disulphide and dimethyl trisulphide, which have been identified as potential substances for the characteristic malodor (Stapleton *et al.*, 2013; Zinn *et al.*, 2021). For example, the presence of dimethyl disulphide and dimethyl trisulphide in *Rhizobium* in the presence of cadmium releases antioxidant mechanisms (Bentley & Chasteen, 2004; Sá *et al.*, 2021). According to the authors, this leads to a reduction in lipid oxidation of up to 80% for dimethyl trisulphide.

The formation of dimethyl disulphide and dimethyl trisulphide can occur via the conversion of amino acids such as methionine or homocysteine but also osmolytes such as dimethylsulphoniopropionates into small volatile sulphur compounds (Kharayat & Singh, 2018; Weisskopf *et al.*, 2021). The S-methyl esters are synthesised via the methanediol, which reacts with acid components such as phenylacetic acid. Rhizobia can intervene in this formation pathway because they contain the gene dddD, which encodes for a class III acyl CoA transferase and enables the bacteria to form dimethyl sulphide, which has a typical smell of the sea, through the precursor dimethyl sulphoniopropionate (Todd *et al.*, 2007). This process prevents the formation of the malodor substances dimethyl disulphide and dimethyl trifulide.

Nonetheless, it seems obvious from our observations, that the question of wether malodor develops in a laundry-related environment must be considered a complex interplay between numerous bacterial groups rather than an effect caused by a distinct species. Moreover, there may be other microorganisms, which have not been in focus here, such as fungi, contributing to this phenomenon.

## 5.4 Future Prospects

In this systematic study, the results showed that a transfer of a washing machine biofilm to the laboratory is possible under certain circumstances. This *ex situ* biofilm shows a high diversity and will enable future experiments with household biofilms. This is particularly interesting for industry, for example to test the behaviour of new detergent formulations under simulated consumer conditions. Furthermore, the laboratory model could be expanded to include the use of a pump or a type of drum substitute in order to better take into account the realistic processes of washing machine programmes. Likewise, the relationship between the Euclidean distance and the Bray-Curtis dissimilarity should be further investigated in the future through more experiments and more repetitions in order to achieve a higher statistical certainty.

Since the present study, with the presence of rhizobia in non-objectionable machines, is indicated for the first time in the prevention of malodor, a suitable rhizobia concentration should first be determined in the laboratory model, with which the washing machines can be treated in future. With the concentrations achieved, it could be ascertained in the *ex situ* laboratory model whether the rhizobia find a niche and establish themselves in the biofilm, or whether they have to be added to the biofilm at regular intervals. Suitable analytical methods for reproducibility and verification of establishment are the qPCR and metagenome analysis methods also used in the present study.

An additional possibility would be to test the rhizobia concentrations obtained in the adapted malodor model, i.e. with an ex-situ biofilm. Besides the malodor reduction capacity, the changes in the main fractions (dimethyl disulphide, dimethyl trisulphide, indole and *p*-cresol) using gas chromatographic analysis would also be informative.

As laboratory models can only represent a small part of reality, as more niches are occupied in a real habitat. Therefore, the medium-term future of this project should lie in trials in a real washing machine, as different concentrations than in the laboratory model may be needed to achieve the same effect (comparison *in vitro* vs. *ex situ*).

Future experiments in washing machines should ideally be carried out under defined conditions in order to keep the influencing factors as low as possible. In addition to defined washing programmes, the detergent should also be subject to defined conditions.

# 6. Conclusion

The formation of biofilms from biofilms, consisting of a diverse microbial community, is widespread in households. Even in the washing machine, numerous microorganisms interact with textiles in a complex way. While microbial cells reach textile surfaces through wear, storage and even the washing machine itself, microbial load reduction takes place, e.g. when washing or drying the garments, resulting in a constant exchange of the microbial community in the textile.

To understand the role of the textile and washing machine microbiota in the putative risk of infection and other adverse effects such as odor nuisance, a microbiological study of the entire washing and use cycle is required. This study provides a comprehensive quantitative and qualitative comparison of bacterial communities in washing machines and on normally used towels washed in these machines, allowing for a better risk assessment of laundry-associated infections and providing evidence for possible mechanisms leading to other problems associated with laundry-associated microorganisms, such as odor. Soil bacteria have been identified as a group of microbial colonisers of washing machines that could act as a kind of protective factor against laundry malodor and should therefore be further investigated. Furthermore, the analysis showed that there is no typical washing machine biofilm, but that a high diversity prevails in the microbial composition of the washing machine.

Moreover, an *ex situ* biofilm model adapted to washing machine biofilms could be developed and established. With the help of this model, biofilms from household washing machines were grown in a laboratory model and showed a high diversity there (up to 29 different families could be detected). Initial comparisons of melting curves and sequencing data showed that a moderately strong, positive correlation between Euclidean distance and Bray-Curtis dissimilarity could be found. Use of HRMA as a rapid test for similarities between biofilms should therefore be pursued in the future.

Likewise, the identified preventively acting bacteria could be tested in the established malodor model. In addition, the model was adapted based on *ex situ* biofilm transfer by establishing a household washing machine biofilm producing malodor as a positive control. These experiments showed a high malodor reduction capacity of different rhizobial species.

In the future, modifying the microbial communities associated with laundry could be a promising way to sustainably combat microbial risks and undesirable effects. There are a number of possible ways in which probiotic bacteria could be used. In addition to integration into a detergent, a kind of probiotic detergent additive to be added to each load is also conceivable. Another possibility would be to introduce the probiotic bacteria into the washing machine itself (e.g. via a "contaminated" filter).

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# Appendix A – List of figures, tables, equations and abbreviations

## A.1 List of Figures

## A.2 List of Tables

## A.3 List of Equations

## A.4 List of Abbreviations

**Table A 1 List of Abbreviations (A-G)**

| Abbreviation (A- G) | Full form |
|---|---|
| 4M3H | 4-methyl-3-hexenoic acid |
| A-M1d | Malodor machine 1 detergent drawer |
| A-M1r | Malodor machine 1 rubber sealant |
| A-NO1d | Non-objectionable machine 1 detergent drawer |
| A-NO2r | Non-objectionable machine 2 rubber sealant |
| A.I.S.E | International Association for Soaps, Detergents and Maintenance Products |
| APG | Alkylpolyglycoside |
| AOB | active oxygen bleach |
| BAC | Benzalkonium chloride |
| Bp | Base pairs |
| $BC_{ij}$ | Bray-Curtis Dissimilarity |
| BSA | Bovine serum albumin |
| $c_W$ | weighted mean of viable count |
| cfu | colony forming units |
| *C. jeikeium* | *Corynebacterium jeikeium* |
| d | days |
| DDAC | dimethyldidecylammonium chloride |
| DSM | Deutsche Stammsammlung für Mikroorganismen und Zellkulturen (German collection of microorganisms and cell cultures) |
| DIN | Deutsches Institut für Normung (German institute for standardisation) |
| *e.g.* | *exempli gratia* (for example) |
| eDNA | extracellular DNA |
| EN | European Norm |
| EPS | extracellular polyeric substances |
| *et al.* | *et alii* (and others) |
| g | gram |
| gDNA | Total DNA |

**Table A 2 List of Abbreviations (G-Z)**

| Abbreviation (G-Z) | Full form |
|---|---|
| GC content | content of the bases guanine (G) and cytosine (C) |
| GPA | Generalized Procrustes Analysis |
| H´ | Shannon diversity |
| HRMA | High Resolution Melting Analysis |
| L | litre |
| LAS | linear alkyl benzene sulfonate |
| LR | logarithmic reduction |
| malodor | laundry associated odor |
| MEA | malt extract agar |
| MEB | malt extract broth |
| MIC | Minimal Inhibitory Concentration |
| min | minutes |
| *M. luteus* | *Micrococcus luteus* |
| *M. osloensis* | *Moraxella osloensis* |
| NaCl | sodium chloride |
| OTU | Operational Taxonomic Unit |
| *P. aeruginosa* | *Pseudomonas aeruginosa* |
| PBS | Phosphate-buffered saline |
| PCA | Principal Component Analysis |
| RH | relative humidity |
| PMA | propidium monoazide |
| QACs | quaternary ammonium compounds |
| rpm | revolutions per minute |
| s | seconds |
| *S. aureus* | *Staphylococcus aureus* |
| *S. epidermidis* | *Staphylococcus epidermidis* |
| SD | standard deviation |
| *S. hominis* | *Staphylococcus hominis* |
| TSA | tryptic soy agar |
| TSB | tryptic soy broth |
| VNBC | viable but not culturable |
| YOPI | young, old, pregnant, and immunocompromised people |

# Appendix B – Material and Media

## B.1 Microorganisms

**Table B 1 Bacterial test strains (DSM: Deutsche Sammlung von Mikroorganismen und Zellkulturen, ATCC: American Type Culture Collection)**

| Strain | Code | |
|---|---|---|
| *Bradyrhizobium japonicum* | DSM 30131 | ATCC 10324 |
| *Corynebacterium jeikeium* | DSM 7171 | ATCC 43734 |
| *Micrococcus luteus* | DSM 1790 | ATCC 10240 |
| *Moraxella osloensis* | DSM 6998 | ATCC 19976 |
| *Pseudomonas aeruginosa* | DSM 939 | ATCC 15442 |
| *Rhizobium flavum* | DSM 102134 | |
| *Rhizobium leguminosarum* | DSM 106839 | ATCC 10004 |
| *Rhizobium pisi* | DSM 30132 | |
| *Staphylococcus epidermidis* | DSM 1798 | ATCC 12228 |
| *Staphylococcus hominis* | DSM 20329 | ATCC 27845 |

## B.2 PCR-Primer

**Table B 2 Primer used for PCR and PCR standards and their characteristics**

| Target gene | Sequence forward-primer | Sequence reverse-primer | Amplicon length | Annealing-Temp. | Reference |
|---|---|---|---|---|---|
| ITS (bacteria) | 5’-TTGTACACACCGCCCG-3’ (ITS1f) | 5’-YGCCAAGGCATCCACC-3’ (ITS2r) | 600 bp | 55 °C | (Andini *et al.*, 2017) |

## B.3 Material

**Table B 3 List of other Materials and Devices (A-Cl)**

| article | description | supplier, manufacturer |
|---|---|---|
| 6-well cell culture plate | 83.3920.500 | Sarstedt AG & Co. KG, Nümbrecht, Germany |
| Adhesive film | 95.1999, optimized for PCR | Sarstedt AG & Co. KG, Nümbrecht, Germany |
| Agarose standard | 3810.4, for DNA/RNA electrophoresis, ROTI®Garose | Carl Roth GmbH + Co. KG, Karlsruhe, Germany |
| Alkyl polyglycoside | Nonionic tenside, C8 – 10, Glucopon® 215 UP, 50295370 | BASF SE, Ludwigshafen, Germany |
| Autoclave | Systec VX-150 resp. VX-65 | Systec GmbH, Linden, Germany |
| Beef tallow | | Brüggemeier Management GmbH, Kevelaer, Germany |
| Benzalkonium chloride | Benzalkonium chloride, B6296-100G | Sigma-Aldrich, St. Louis, USA |
| Bovines Serum albumin | Albumin Fraction V | AppliChem, Darmstadt, Germany |
| Bunsen burner | Labogaz® 206 | Camping Gaz (Deutschland) GmbH, Hungen-Inheiden, Germany |
| Centrifuge | Heraeus Multifuge X3R | Thermo Fisher Scientific, Waltham, US |
| Classic bead beating grinder and lysis system | 116004500, FastPrep-24™ | MP Biomedicals Germany GmbH, Eschwege, Germany |

**Table B 4 List of other Materials and Devices (Co-F)**

| **article** | **description** | **supplier, manufacturer** |
|---|---|---|
| Constant climate chamber | HPP110 | Memmert GmbH & Co. KG, Schwabach, Germany |
| Cotton fabric | CO DIN 53919 WFK 10 A, 100% cotton | wfk-Testgewebe GmbH, Brüggen, Germany |
| cotton swab | sterile | Copan Diagnostics Inc. Columbus, OH, USA |
| Cover glass | 637-1578P, round, Ø 18mm | VWR International GmbH, Darmstadt, Germany |
| Cuvette | 67.758, disposable, UV-transparent | Sarstedt AG & Co. KG, Nümbrecht, Germany |
| DNA Extraction-kit | Fast DNA SPIN Kit for Soil, Art. Nr: 116560-200 | MP Biomedicals Germany GmbH, Eschwege, Germany |
| DNA Ladder | 300003, 50 bp ready-to-use | GeneOn GmbH, Ludwigshafen am Rhein, Germany |
| Drigalski spatula | soda-lime glass, item no. T724.1 | Carl Roth GmbH, Karlsruhe, Germany |
| Erlenmeyer flasks | 100 mL, with caps | SCHOTT Duran Produktions GmbH & Co. OHG, Düsseldorf, Germany |
| Ethanol | 96%, denatured with 1% MEK | Carl Roth GmbH, Karlsruhe, Germany |
| Freezer | KS 9807 | Severin Elektrogeräte GmbH, Sundern (Sauerland), Germany |
| Freezer -86 °C | U9420-000X*, New Brunswick™ Innova®, U101 | Eppendorf AG, Hamburg, Germany |

**Table B 5 List of other Materials and Devices (G-O)**

| **article** | **description** | **supplier, manufacturer** |
|---|---|---|
| Gel Loading Dye, Purple (6X) | B7024S | New England Biolabs, Ipswich, England |
| Glass beads | soda lime glass, 3 mm diameter, GTIN 4250317312334 | Paul Marienfeld GmbH & Co. KG, Staufen Germany |
| Glycerin ≥ 99.5 % | 3783.3 | Carl Roth GmbH + Co. KG, Karlsruhe, Germany |
| High Pure PCR Product Purification-Kit | 11732668001 | Hoffmann-La Roche AG, Basel, Switzerland |
| Imaging System + Software | 170-8280, ChemiDoc MP™ | Bio-Rad Laboratories Inc., Hercules, USA |
| Incubator | HettCube 200R | Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany |
| Incubated shaker | Thermomixer comfort | Eppendorf AG, Hamburg, Germany |
| Infrared thermometer | Testo 805 | Thermo Fisher Scientific, Waltham, US |
| Inoculation loop | 10 µL, PS, blue, sterile, 86.1562.010 | Sarstedt AG & Co. KG, Nümbrecht, Germany |
| Lab timer | TR 112 | Carl Roth GmbH, Karlsruhe, Germany |
| Magnetic stirrer | RSM-04-H | Phoenix Instrument GmbH, Garbsen, Germany |
| Millipak Express 40 Filter | | Merck KGaA, Darmstadt, Germany |
| Orbital shaker | incubation of liquid cultures, MaxQ* 8000 | Thermo Fisher Scientific, Waltham, US |

**Table B 6 List of other Materials and Devices (P-Pi)**

| article | description | supplier, manufacturer |
|---|---|---|
| PCR 8s chain | 72.985.002 | Sarstedt AG & Co. KG, Nümbrecht, Germany |
| PCR cover chain | 65.989.002 | Sarstedt AG & Co. KG, Nümbrecht, Germany |
| PCR plate half rim | 72.1979 | Sarstedt AG & Co. KG, Nümbrecht, Germany |
| PCR-Kit | 06402712001, FastStart Essential DNA Green Master | Hoffmann-La Roche AG, Basel, Switzerland |
| PCR plate spinner | 521-1648 | VWR International GmbH, Darmstadt, Germany |
| PCR-Primer (diverse) | | Eurofins Genomics Germany GmbH, Ebersberg, Germany |
| Petri dishes | 92 16 mm, Polystyrene, with ventilation cams, sterile, Art. No. 1473 | Sarstedt AG & Co. KG, Nümbrecht, Germany |
| Photometer | BioPhotometer Plus | Eppendorf, Hamburg, Germany |
| Pipette 100 – 1000 µL | Research® plus, cataloge no.:3120000062 | Eppendorf AG, Hamburg, Germany |
| Pipette 20 – 200 µL | Research® plus, cataloge no.:3120000054 | Eppendorf AG, Hamburg, Germany |
| Pipette 0,5 – 5 mL | Reference®, cataloge no.: 4920000105 | Eppendorf AG, Hamburg, Germany |
| Pipette tips 200 µL | polypropylene, Art. No: 760002 | Sarstedt AG & Co. KG, Nümbrecht, Germany |
| Pipette tips 1000 µL | polypropylene, Art. No: 762 | Sarstedt AG & Co. KG, Nümbrecht, Germany |

**Table B 7 List of other Materials and Devices (Pi-SC)**

| article | description | supplier, manufacturer |
|---|---|---|
| Pipette tips 5000 µL | polypropylene, Art. No: 1183102 | Sarstedt AG & Co. KG, Nümbrecht, Germany |
| Pipetting aid | macro Pipettierhelfer | Brand GmbH & Co.KG, Wertheim, Germany |
| Precision balance | XA 105 | Mettler-Toledo AG, Greifensee, Switzerland |
| Reaction tube 1,5 mL | polypropylene, Art. No: 690001 | Sarstedt AG & Co. KG, Nümbrecht, Germany |
| Reaction tube 2.0 mL | polypropylene, Art. No: 72.691 | Sarstedt AG & Co. KG, Nümbrecht, Germany |
| Reaction tube 5.0 mL | polypropylene, Art. No: 72.701 | Sarstedt AG & Co. KG, Nümbrecht, Germany |
| Reaction tube 15.0 mL | polypropylene, Art. No: 62.554.502 | Sarstedt AG & Co. KG, Nümbrecht, Germany |
| Reaction tube 50.0 mL | polypropylene, Art. No: 62.547.254 | Sarstedt AG & Co. KG, Nümbrecht, Germany |
| Real-Time PCR System + Software | 15781198, Applied Biosystems QuantStudio™ 3 | Thermo Fisher Scientific Inc., Waltham, US |
| ROTI®fair Phosphate-buffered saline | 1112.2 | Carl Roth GmbH, Karlsruhe, Germany |
| ROTI®GelStain | 3865.1 | Carl Roth GmbH, Karlsruhe, Germany |
| Sampling tube 50 mL | polypropylene, 114 28 mm, sterile, conical base, Art. No: 547254 | Sarstedt AG & Co. KG, Nümbrecht, Germany |
| Safety workbench | Safe 2020 | Thermo Fisher Scientific, Wesel, Germany |
| Scale | XA 3002S, d = 0,01 g | Mettler Toledo AG, Greifensee, Switzerland |

**Table B 8 List of other Materials and Devices (Se-Z)**

| article | description | supplier, manufacturer |
|---|---|---|
| Serological pipette10 mL | plugged, sterile, Art. no.: 1254001 | Sarstedt AG & Co. KG, Nümbrecht, Germany |
| Serological pipette 25 mL | plugged, sterile, Art. no.: 1685001 | Sarstedt AG & Co. KG, Nümbrecht, Germany |
| Stearic acid | Saturated carbon and fatty acid, reagent grade, 95% | Sigma-Aldrich, St. Louis, USA |
| TEGO Care PS | emulgator | Evonik Industries AG, Essen, Germany |
| Thermal cycler | 1851196, C1000 Touch | Bio-Rad Laboratories Inc., Hercules, USA |
| Thermomixer | Thermomixer comfort | Eppendorf SE, Hamburg, Germany |
| Tweezers | 2801.1 | Carl Roth GmbH, Karlsruhe, Germany |
| Ultrapure water system | Q-POD® | Merck Millipore, Merck KGaA, Darmstadt, Germany |
| Vortex mixer | Lab dancer S40 | VWR, Darmstadt, Germany |

## B.4 Media and solutions

All media and solutions were prepared with high purity water (MQ water prepared with Q-POD, Merck Millipore, Darmstadt, Germany). All media and solutions were sterilized in an autoclave (121 °C, 15 min) or sterile filtered (pore size 0.2 µm).

Caso Bouillon (Trypton Soy Bouillon (TSB))

105459, Merck KGaA, Darmstadt, Germany

Ingredients: peptone from casein, peptone from soybean flour, D (+) glucose monohydrate, sodium chloride, di-potassium hydrogen phosphate.

Used as an additive of a nutrient medium to transfer a biofilm from the consumer household to the laboratory.

Cetrimid-Agar (CNA)

40-1024, Xebios Diagnostics GmbH, Düsseldorf, Germany

Ingredients: pancreatic hydrolysate from gelatin, magnesium chloride, dipotassium sulfate, cetrimide, glycerol, agar-agar.

Used for detection of pseudomonads.

Meat extract

X975.1, Carl Roth GmbH + Co. KG,

Ingredients: meat extract, powdered

Used as an additive of a nutrient medium to transfer a biofilm from the consumer household to the laboratory.

Potato starch

2045.3, Carl Roth GmbH + Co. KG,

Ingredients: starch from potatoes, soluble

Used as an additive of a nutrient medium to transfer a biofilm from the consumer household to the laboratory.

Malz Extract Agar (MEA), ready-to-use

105398, Merck KGaA, Darmstadt, Germany

Ingredients: malt extract, peptone from soy flour, agar-agar

Used as an additive of a nutrient medium to transfer a biofilm from the consumer household to the laboratory.

Malz Extract Bouillon (MEB), ready-to-use

105397, Merck KGaA, Darmstadt, Germany

Ingredients: malt extract

Used as an additive of a nutrient medium to transfer a biofilm from the consumer household to the laboratory.

Mannitol saline agar (MSA)

40-1166, Xebios Diagnostics GmbH, Düsseldorf, Germany

Ingredients: casein peptone (pancreatic digested), meat peptone (peptic digested), meat extract, D (-) -mannitol, sodium chloride, phenol red, agar agar.

Used for detection of *staphylococci*.

Physiological saline solution (0.9%)

NaCl, 27.808.297, VWR, Darmstadt, Germany

Used as dilution medium.

Tryptone Soy Agar (TSA)

105458, Merck KGaA, Darmstadt, Germany

Ingredients: casein peptone (pancreatic digested) casein, papaic digest of soybean, NaCl, agar agar.

Used for detection of live cell count of bacteria.

Milli-Q ultrapure water from extraction unit

ZMQSP0D01, Merck KGaA, Darmstadt, Germany

Used for the production of the media.

Rhizobium Medium

M408-500G, HiMedia Laboratories Pvt. Ltd. Mumbai, India

Ingredients: yeast extract, mannitol, dipotassium phosphate, magnesium sulphate, sodium chloride, agar agar

Used for detection of *Rhizobium spp.*

# Appendix C – Questionnaires and evaluation sheet

## C.1 Questionnaire 1 (Laundry hygiene questionnaire)

Dear participants,

Thank you for taking the time to participate in this study. The study is being conducted as part of my doctorate at the Rhine-Waal University of Applied Sciences / University of Duisburg-Essen and deals with the topic of textile bad odors and microbial odor formation in household washing machines.

Answering the questionnaire will take about 5 minutes. Read through the questions and statements at your leisure and then tick the answers that best apply to you personally.

The answers are anonymous and the data will only be used for scientific purposes. If you would like to support me beyond the questionnaire (e.g. sampling of the washing machine and the textile), I would be pleased to receive your contact details at the end of the questionnaire!

If you have any questions about this study or the questionnaire, please feel free to contact me.

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Marie-Curie-Str.1 47533 Kleve
Mail: Marc-Kevin.Zinn@hochschule-rhein-waal.de Phone:+49 2821 806739786

# Laundry hygiene questionnaire

1. **How many people live in your household? Please indicate the age and gender of the persons?**

2. **How old is the used washing machine (approx.)?**

Klicken oder tippen Sie hier, um Text einzugeben. years

3. **What kind of washing machine do you have ( brand, model, year of manufacture)?**

Klicken oder tippen Sie hier, um Text einzugeben.

4. **Are there any animals in your household? If yes, please indicate the number and type of animals?**

yes ☐ no ☐

If yes, which Klicken oder tippen Sie hier, um Text einzugeben. and number Klicken oder tippen Sie hier, um Text einzugeben.

5. **How often is washing done on average?**

☐ less frequently than 1x per week

☐ approx. 1x a week

☐ more often than 1x a week

6. **What type of detergent do you usually use (multiple choice possible)?**

☐ Heavy-duty detergents in solid form (powder, tabs, beads, etc.)
☐ Heavy-duty detergents in liquid form
☐ Solid colour detergents (powder, tabs, pearls, etc.)
☐ Liquid colour detergent
☐ Fabric softender
☐ other detergents/additives: Klicken oder tippen Sie hier, um Text einzugeben.

7. **Do you use hygienic rinse aids?**

☐ Yes, with every wash
☐ Yes, at regular intervals (e.g. once a month)
☐ When necessary (e.g. in case of illness)
☐ never

8. **How often do you wash at the following temperatures (multiple choice possible)?**

| | <30°C | 30°C | 40°C | 60°C | 95°C |
|---|---|---|---|---|---|
| At least 1x a week | ☐ | ☐ | ☐ | ☐ | ☐ |
| At least 1x per month | ☐ | ☐ | ☐ | ☐ | ☐ |
| At least 1x in three month | ☐ | ☐ | ☐ | ☐ | ☐ |
| rarer | ☐ | ☐ | ☐ | ☐ | ☐ |

9. **How to dry the laundry:**

☐ Tumble dryer
☐ Linen drying outdoors
☐ Linen drying indoors (heated room)
☐ Linen drying indoors (unheated room)

10. **Have you ever noticed unpleasant laundry odors? (Multiple choice possible):**

☐ Yes, before washing on the laundry
☐ Yes, before washing on damp laundry
☐ Yes, after drying on the laundry
☐ Yes, during/after ironing or wearing the textile
☐ Yes, before/after washing in the machine
☐ No

Please describe the smell: Klicken oder tippen Sie hier, um Text einzugeben.
If applicable, please describe the type of textile (type of garment, material, etc.):
Klicken oder tippen Sie hier, um Text einzugeben.

**If you would like to assist us with further investigations (e.g. sampling of the washing machine and the textile), please enter your name and contact details* in the following field:** Klicken oder tippen Sie hier, um Text einzugeben.

* Personal data will only be used to carry out this project and will not be passed on to third parties
For questions and return of questionnaires: Rhine-Waal University of Applied Sciences, attn.:
Marc-Kevin Zinn, Marie-Curie-Str. 1, 47533 Kleve, Mail: marc-kevin.zinn@hsrw.eu,
ph: +49 2821-806739786

### C.2 Questionnaire 2 (Information on household and towel use)

# Information on household and towel use

1. **How many people live in the household? Please indicate the age and gender of the members.** Klicken oder tippen Sie hier, um Text einzugeben.
2. **Are there any pets in the household? If yes, please indicate the number and type of pets.**

☐ yes ☐ no

If yes, type Klicken oder tippen Sie hier, um Text einzugeben. and number of pets Klicken oder tippen Sie hier, um Text einzugeben..

3. **Are there been any cases of illness among the residents in the household in the last four weeks?**

☐ yes ☐ no

If yes, which ones Klicken oder tippen Sie hier, um Text einzugeben. and period Klicken oder tippen Sie hier, um Text einzugeben..

4. **Did you have visitors during the one-week test phase?**

☐ yes ☐ no

If yes, please indicate the age Klicken oder tippen Sie hier, um Text einzugeben. and gender Klicken oder tippen Sie hier, um Text einzugeben. of the visitors.

**Did the visitors also use the test towel?**

☐ yes ☐ no

5. **Please indicate wether you have drinking or rainwater harvesting in your household.**

☐ drinking water ☐ rainwater

Thank you for your time and participation!

## C.3 Sensory evaluation sheet

**Table C 1 Sensory evaluation sheet (Part 1)**

| odour | Expert1 | | | Expert2 | | | Expert3 | | | Expert4 | | | Expert5 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | malodour | cheesy | pungent | malodour | cheesy | pungent | malodour | cheesy | pungent | malodour | cheesy | pungent | malodour | cheesy | pungent |
| positive control | | | | | | | | | | | | | | | |
| Benzalkoniumchloride | | | | | | | | | | | | | | | |
| sample 1 | | | | | | | | | | | | | | | |
| sample 2 | | | | | | | | | | | | | | | |
| sample 3 | | | | | | | | | | | | | | | |
| sample 4 | | | | | | | | | | | | | | | |
| sample 5 | | | | | | | | | | | | | | | |
| sample 6 | | | | | | | | | | | | | | | |
| sample 7 | | | | | | | | | | | | | | | |
| sample 8 | | | | | | | | | | | | | | | |
| sample 9 | | | | | | | | | | | | | | | |
| sample 10 | | | | | | | | | | | | | | | |
| sample 11 | | | | | | | | | | | | | | | |
| sample 12 | | | | | | | | | | | | | | | |
| sample 13 | | | | | | | | | | | | | | | |
| sample 14 | | | | | | | | | | | | | | | |
| sample 15 | | | | | | | | | | | | | | | |
| negative control | | | | | | | | | | | | | | | |

| odour | Expert1 | | | Expert2 | | | Expert3 | | | Expert4 | | | Expert5 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | malodour | cheesy | pungent | malodour | cheesy | pungent | malodour | cheesy | pungent | malodour | cheesy | pungent | malodour | cheesy | pungent |
| positive control | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! |
| Benzalkoniumchloride | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! |
| sample 1 | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! |
| sample 2 | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! |
| sample 3 | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! |
| sample 4 | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! |
| sample 5 | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! |
| sample 6 | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! |
| sample 7 | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! |
| sample 8 | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! |
| sample 9 | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! |
| sample 10 | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! |
| sample 11 | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! |
| sample 12 | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! |
| sample 13 | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! |
| sample 14 | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! |
| sample 15 | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! |
| negative control | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! |

**Table C 2 Sensory evaluation sheet (Part 2)**

| | | sample 1 | | | | sample 2 | | | | sample3 | | | | sample 4 | | | | sample 5 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | malodour | cheesy | pungent | | malodour | cheesy | pungent | | malodour | cheesy | pungent | | malodour | cheesy | pungent | | malodour | cheesy | pungent | |
| E1 | #DIV/0! | #DIV/0! | #DIV/0! | E1 | #DIV/0! | #DIV/0! | #DIV/0! | E1 | #DIV/0! | #DIV/0! | #DIV/0! | E1 | #DIV/0! | #DIV/0! | #DIV/0! | E1 | #DIV/0! | #DIV/0! | #DIV/0! | |
| E2 | #DIV/0! | #DIV/0! | #DIV/0! | E2 | #DIV/0! | #DIV/0! | #DIV/0! | E2 | #DIV/0! | #DIV/0! | #DIV/0! | E2 | #DIV/0! | #DIV/0! | #DIV/0! | E2 | #DIV/0! | #DIV/0! | #DIV/0! | |
| E3 | #DIV/0! | #DIV/0! | #DIV/0! | E3 | #DIV/0! | #DIV/0! | #DIV/0! | E3 | #DIV/0! | #DIV/0! | #DIV/0! | E3 | #DIV/0! | #DIV/0! | #DIV/0! | E3 | #DIV/0! | #DIV/0! | #DIV/0! | |
| E4 | #DIV/0! | #DIV/0! | #DIV/0! | E4 | #DIV/0! | #DIV/0! | #DIV/0! | E4 | #DIV/0! | #DIV/0! | #DIV/0! | E4 | #DIV/0! | #DIV/0! | #DIV/0! | E4 | #DIV/0! | #DIV/0! | #DIV/0! | |
| E5 | #DIV/0! | #DIV/0! | #DIV/0! | E5 | #DIV/0! | #DIV/0! | #DIV/0! | E5 | #DIV/0! | #DIV/0! | #DIV/0! | E5 | #DIV/0! | #DIV/0! | #DIV/0! | E5 | #DIV/0! | #DIV/0! | #DIV/0! | |
| Mean | #DIV/0! | #DIV/0! | #DIV/0! | Mean | #DIV/0! | #DIV/0! | #DIV/0! | Mean | #DIV/0! | #DIV/0! | #DIV/0! | Mean | #DIV/0! | #DIV/0! | #DIV/0! | Mean | #DIV/0! | #DIV/0! | #DIV/0! | |
| StabWN | #DIV/0! | #DIV/0! | #DIV/0! | StabWN | #DIV/0! | #DIV/0! | #DIV/0! | StabWN | #DIV/0! | #DIV/0! | #DIV/0! | StabWN | #DIV/0! | #DIV/0! | #DIV/0! | StabWN | #DIV/0! | #DIV/0! | #DIV/0! | |

| | | sample 6 | | | | sample 7 | | | | sample 8 | | | | sample 9 | | | | sample 10 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | malodour | cheesy | pungent | | malodour | cheesy | pungent | | malodour | cheesy | pungent | | malodour | cheesy | pungent | | malodour | cheesy | pungent | |
| E1 | #DIV/0! | #DIV/0! | #DIV/0! | E1 | #DIV/0! | #DIV/0! | #DIV/0! | E1 | #DIV/0! | #DIV/0! | #DIV/0! | E1 | #DIV/0! | #DIV/0! | #DIV/0! | E1 | #DIV/0! | #DIV/0! | #DIV/0! | |
| E2 | #DIV/0! | #DIV/0! | #DIV/0! | E2 | #DIV/0! | #DIV/0! | #DIV/0! | E2 | #DIV/0! | #DIV/0! | #DIV/0! | E2 | #DIV/0! | #DIV/0! | #DIV/0! | E2 | #DIV/0! | #DIV/0! | #DIV/0! | |
| E3 | #DIV/0! | #DIV/0! | #DIV/0! | E3 | #DIV/0! | #DIV/0! | #DIV/0! | E3 | #DIV/0! | #DIV/0! | #DIV/0! | E3 | #DIV/0! | #DIV/0! | #DIV/0! | E3 | #DIV/0! | #DIV/0! | #DIV/0! | |
| E4 | #DIV/0! | #DIV/0! | #DIV/0! | E4 | #DIV/0! | #DIV/0! | #DIV/0! | E4 | #DIV/0! | #DIV/0! | #DIV/0! | E4 | #DIV/0! | #DIV/0! | #DIV/0! | E4 | #DIV/0! | #DIV/0! | #DIV/0! | |
| E5 | #DIV/0! | #DIV/0! | #DIV/0! | E5 | #DIV/0! | #DIV/0! | #DIV/0! | E5 | #DIV/0! | #DIV/0! | #DIV/0! | E5 | #DIV/0! | #DIV/0! | #DIV/0! | E5 | #DIV/0! | #DIV/0! | #DIV/0! | |
| Mean | #DIV/0! | #DIV/0! | #DIV/0! | Mean | #DIV/0! | #DIV/0! | #DIV/0! | Mean | #DIV/0! | #DIV/0! | #DIV/0! | Mean | #DIV/0! | #DIV/0! | #DIV/0! | Mean | #DIV/0! | #DIV/0! | #DIV/0! | |
| StabWN | #DIV/0! | #DIV/0! | #DIV/0! | StabWN | #DIV/0! | #DIV/0! | #DIV/0! | StabWN | #DIV/0! | #DIV/0! | #DIV/0! | StabWN | #DIV/0! | #DIV/0! | #DIV/0! | StabWN | #DIV/0! | #DIV/0! | #DIV/0! | |

| | | sample 11 | | | | sample 12 | | | | sample 13 | | | | sample 14 | | | | sample 15 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | malodour | cheesy | pungent | | malodour | cheesy | pungent | | malodour | cheesy | pungent | | malodour | cheesy | pungent | | malodour | cheesy | pungent | |
| E1 | #DIV/0! | #DIV/0! | #DIV/0! | E1 | #DIV/0! | #DIV/0! | #DIV/0! | E1 | #DIV/0! | #DIV/0! | #DIV/0! | E1 | #DIV/0! | #DIV/0! | #DIV/0! | E1 | #DIV/0! | #DIV/0! | #DIV/0! | |
| E2 | #DIV/0! | #DIV/0! | #DIV/0! | E2 | #DIV/0! | #DIV/0! | #DIV/0! | E2 | #DIV/0! | #DIV/0! | #DIV/0! | E2 | #DIV/0! | #DIV/0! | #DIV/0! | E2 | #DIV/0! | #DIV/0! | #DIV/0! | |
| E3 | #DIV/0! | #DIV/0! | #DIV/0! | E3 | #DIV/0! | #DIV/0! | #DIV/0! | E3 | #DIV/0! | #DIV/0! | #DIV/0! | E3 | #DIV/0! | #DIV/0! | #DIV/0! | E3 | #DIV/0! | #DIV/0! | #DIV/0! | |
| E4 | #DIV/0! | #DIV/0! | #DIV/0! | E4 | #DIV/0! | #DIV/0! | #DIV/0! | E4 | #DIV/0! | #DIV/0! | #DIV/0! | E4 | #DIV/0! | #DIV/0! | #DIV/0! | E4 | #DIV/0! | #DIV/0! | #DIV/0! | |
| E5 | #DIV/0! | #DIV/0! | #DIV/0! | E5 | #DIV/0! | #DIV/0! | #DIV/0! | E5 | #DIV/0! | #DIV/0! | #DIV/0! | E5 | #DIV/0! | #DIV/0! | #DIV/0! | E5 | #DIV/0! | #DIV/0! | #DIV/0! | |
| Mean | #DIV/0! | #DIV/0! | #DIV/0! | Mean | #DIV/0! | #DIV/0! | #DIV/0! | Mean | #DIV/0! | #DIV/0! | #DIV/0! | Mean | #DIV/0! | #DIV/0! | #DIV/0! | Mean | #DIV/0! | #DIV/0! | #DIV/0! | |
| StabWN | #DIV/0! | #DIV/0! | #DIV/0! | StabWN | #DIV/0! | #DIV/0! | #DIV/0! | StabWN | #DIV/0! | #DIV/0! | #DIV/0! | StabWN | #DIV/0! | #DIV/0! | #DIV/0! | StabWN | #DIV/0! | #DIV/0! | #DIV/0! | |

**Table C 3 Sensory evaluation sheet (Part 3)**

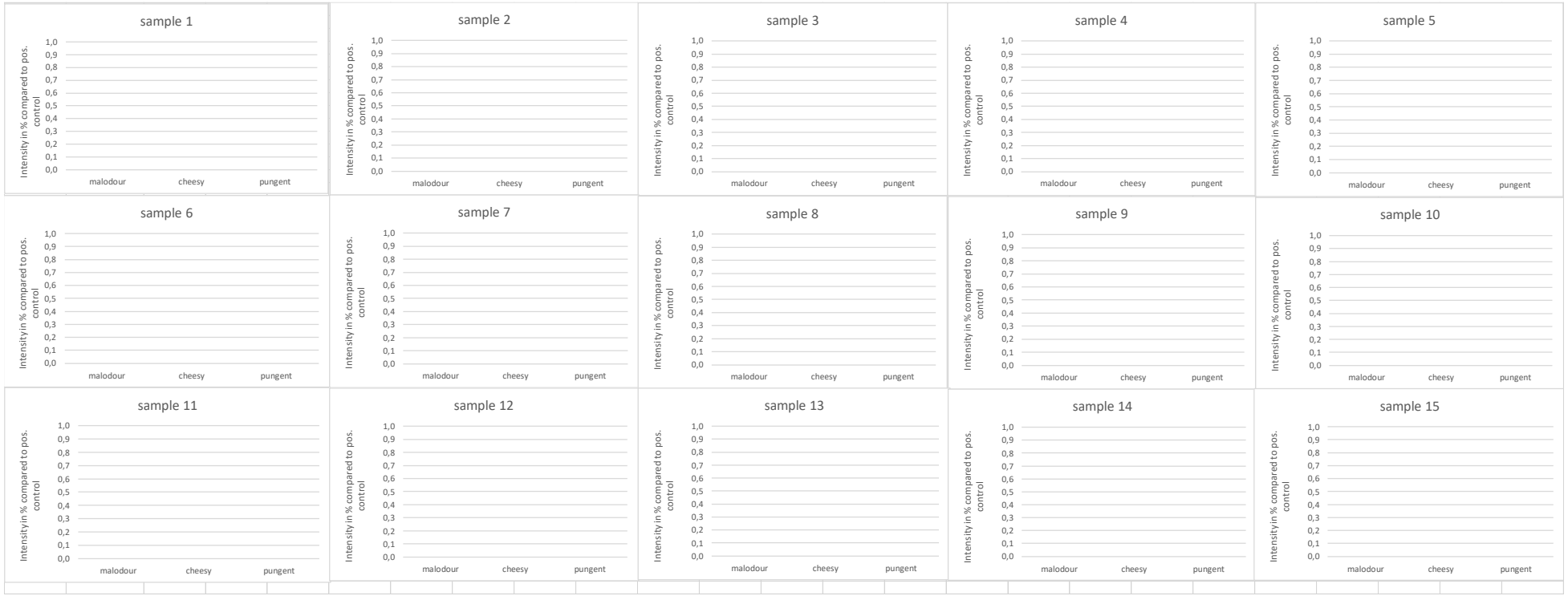

**Table C 4 Sensory evaluation sheet (Part 4)**

| | | malodour | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | sample 1 | sample 2 | sample 3 | sample 4 | sample 5 | sample 6 | sample 7 | sample 8 | sample 9 | sample 10 | sample 11 | sample 12 | sample 13 | sample 14 | sample 15 |
| E1 | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! |
| E2 | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! |
| E3 | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! |
| E4 | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! |
| E5 | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! |
| Mean | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! |
| StabWN | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! |
| | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! |
| | | cheesy | | | | | | | | | | | | | |
| | sample 1 | sample 2 | sample 3 | sample 4 | sample 5 | sample 6 | sample 7 | sample 8 | sample 9 | sample 10 | sample 11 | sample 12 | sample 13 | sample 14 | sample 15 |
| E1 | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! |
| E2 | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! |
| E3 | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! |
| E4 | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! |
| E5 | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! |
| Mean | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! |
| StabWN | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! |
| | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! |
| | | pungent | | | | | | | | | | | | | |
| | sample 1 | sample 2 | sample 3 | sample 4 | sample 5 | sample 6 | sample 7 | sample 8 | sample 9 | sample 10 | sample 11 | sample 12 | sample 13 | sample 14 | sample 15 |
| E1 | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! |
| E2 | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! |
| E3 | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! |
| E4 | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! |
| E5 | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! |
| Mean | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! |
| StabWN | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! |
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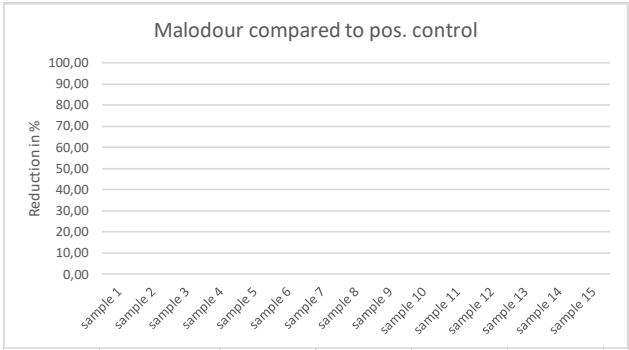


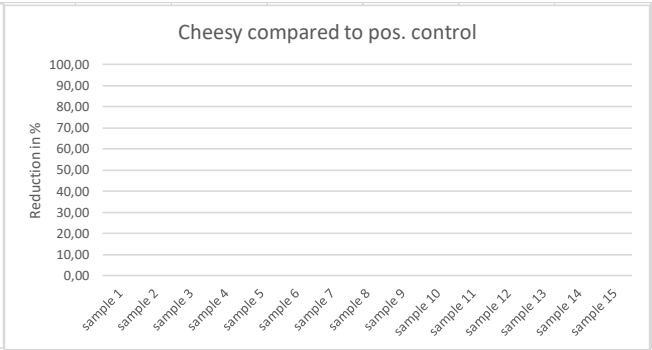


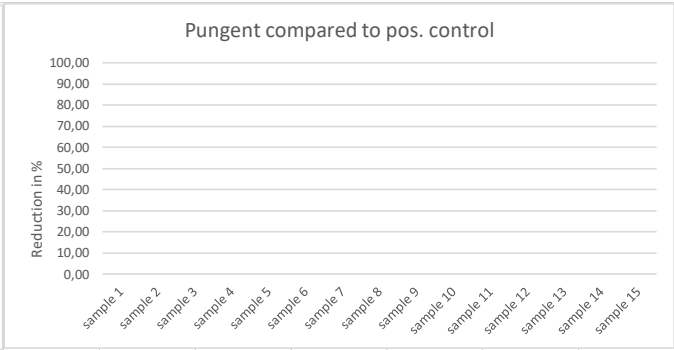

# Appendix D – research contributions and curriculum vitae

## D.1 research contributions

### Scientific Papers

- D Klapper, **M-K Zinn**, S Schulze-Struchtrup, B von Esmarch-Rummler, R Stamminger (2018) *Micrococcus luteus* – An Alternative Test Germ for Testing the Hygienic Performance of Commercial Freshwater Dishwashers. Tenside Surfactants Detergents 55(5):369-375
- **M-K Zinn**, D Klapper, B von Esmarch-Rummler, D Bockmühl (2018) Development of a Test Method for Analyzing the Hygienic Performance of Commercial Dishwashers Operating on the Fresh Water Principle. Tenside Surfactants Detergents 55(5):376-382
- D Bockmühl, B Brands, R Lucassen, J Schages, **M-K Zinn** (2020) Lifelike experimental approaches to evaluate hygiene-related effects in laundry and home care. Household and Personal Care Today – vol. 15(1)
- **M-K Zinn**, D Bockmühl (2020) Did Granny know best? Evaluating the Antibacterial, Antifungal and Antiviral Efficacy of Acetic Acid for Home Care Procedures. BMC Microbiology (2020) 20:265
- **M-K Zinn**, L Schages, D Bockmühl (2020) The toothbrush microbiome: Impact of user´s age, period of use and bristle material on the microbial communities of toothbrushes. Microorganisms 2020, 8, 1379
- **M-K Zinn**, M Singer, D Bockmühl (2021) Smells like Teen Spirit – A model to generate laundry-associated malodor *in vitro*. Microorganisms 2021, 9, 974
- **M-K Zinn**, H-C Flemming, D Bockmühl (2022) A comprehensive view on microbial communities in the laundering cycle suggests a preventive effect of soil bacteria on malodor formation. Microorganisms 2022, 10 1465

- M Rademacher, **M-K Zinn**, R Beinio, D Bockmühl (2022) A new model to investigate the effects of cosmetics on skin microorganisms *in vitro.* Cosmetics 2022, 9, 88
- N van Leuven, **M-K Zinn**, R Lucassen, A Lipski, H-C Flemming, D Bockmühl (2023) High Resolution Melting Analysis as a tool to analyse microbial communities of household biofilms in *ex-situ* models. (submitted)

**Journalistic activity and media knowledge transfer (on the dissertation topic)**

**Radio**

- WDR Cosmo 01.03.2023
- DeutschlandRadio Nova 18.03.2023
- WDR 5 Morgenecho 01.03.2023
- 1 Live 02.03.2023

**Newspaper**

- BILD-Zeitung “Er ist Dr. Waschmaschine” 02.12.2022
- Laborjournal “Mikroben gegen Waschmaschinen-Muff” 29.08.2022
- Rheinische Post “Schützen diese Bakterien vor schlecht riechender Wäsche?” 18.08.2022
- NRZ “Forscher in Kleve klären, warum Waschmaschinen stinken” 23.01.2023

**TV**

- WDR Fernsehen “Lokalzeit Ruhr” 15.02.2023
- WDR Fernsehen “Lokalzeit am Samstag” 04.03.2023

## D.2 Curriculum vitae

The Curriculum Vitae is not included in the online version for data protection reasons.

# Declaration of Originality

I hereby certify that I have submitted the present work entitled

*“Protective biofilms to prevent the colonization of household water systems by harmful organisms, exemplified by the washing machine”*

I have written the thesis myself and have not used any auxiliary materials or sources other than those indicated. The thesis has not been submitted in this or a similar form to any other university. I have not undergone a failed doctoral procedure in this or any other subject.

Essen, 27.06.2023

Marc-Kevin Zinn

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