Design and optimization of antimicrobial peptides combating gram-negative biofilm-forming bacteria

Dissertation
for the degree of doctor of natural sciences (Dr. rer. nat.)

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Justus Liebig University Giessen
Giessen, July 2024

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Declaration of authorship

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Giessen, July 2024

Lena Stillger

Every day may not be good,
but there is something good in every day.

Alice Morse Earle

Abstract

The accumulation of undesirable microorganisms in form of biofilms, the so-called biofouling, represents a significant challenge in numerous fields, including industry, where the presence of biocorrosive bacteria can cause in a wide range of material damage. Antimicrobial peptides (AMPs) have demonstrated potential as an alternative for the usage of antibiotics in the medical technology and pharmaceutical sectors, and increase in importance as new drug candidates. The objective of this thesis is to expand the application range of AMPs to the industrial sector, with a focus on water-bearing systems. Here, AMPs are intended to replace the current biocide treatment. For this aim, membranolytic peptides were initially selected from peptide databases based on their characteristics (charge, hydrophobicity, and amphiphilicity), and then tested for their planktonic activity against various biocorrosive and other biofilm-forming bacteria. Inhibitory concentrations of 15 µM were observed against *Desulfovibrio vulgaris*, the primary representative of biocorrosive bacteria. Subsequent optimization of the peptide sequences by modifying the amino acid composition and modifying the peptide termini by amidation and lipidation led to an eight-fold increase in planktonic activity. An increase in activity through the use of two AMPs could only be demonstrated in combination with one peptide (P5) due to different modes of action. The development of a modified Calgary biofilm assay enables simple and rapid screening of the antibiofilm activity of the peptides, both in terms of (long-term) biofilm inhibition and removal. The analysis is based on the determination of microbial growth and the characterization of the biofilm mass by crystal violet staining. By extending the growth surfaces to polished steel surfaces, this test could also be carried out under realistic conditions. The microbiological tests show advantages of an early AMP treatment, preferably already in the planktonic stage. Comparative experiments with two common biocides, glutaraldehyde and tetrakis hydroxymethylphosphonium sulfate, in terms of activity, toxicity and resistance rate demonstrate the huge potential of AMPs, as they are more active, less toxic and less resistant against *Desulfovibrio vulgaris* at the same concentration. On this basis, it is possible to extend the scope of application of AMPs from the medical to the industrial sector for the control of biocorrosive bacteria and thus represents a promising alternative to the current biocide treatment.

Zusammenfassung

Die Ansammlung unerwünschter Mikroorganismen in Form von Biofilmen, das so genannte Biofouling, stellt in vielen Bereichen eine große Herausforderung dar, z.B. in der Industrie, wo biokorrosive Bakterien vielfältige Materialschäden verursachen können. Antimikrobielle Peptide (AMPs) haben sich bereits als vielversprechende Alternative zum Einsatz von Antibiotika im medizintechnischen und pharmazeutischen Bereich gezeigt und gewinnen als neue Wirkstoffkandidaten immer mehr an Bedeutung. Ziel dieser Thesis ist es, den Anwendungsbereich von AMPs auf den industriellen Sektor, insbesondere wasserführende Systeme auszuweiten. Hier sollen die AMPs die derzeitigen Biozidbehandlung ersetzen. Zu diesem Zweck wurden zunächst membranolytische Peptide anhand ihrer Charakteristika (Ladung, Hydrophobizität und Amphiphilizität) aus Peptiddatenbanken ausgewählt und anschließend auf ihre planktonische Aktivität gegen verschiedene biokorrosive und weitere biofilmbildende Bakterien getestet. Hemm-Konzentrationen von <15 µM konnten gegen *Desulfovibrio vulgaris*, dem Hauptvertreter der biokorrosiven Bakterien, nachgewiesen werden. Eine nachfolgende Optimierung der Peptidsequenzen durch Änderung der Aminosäurezusammensetzung und Modifizierung der Peptidtermini durch Amidierung und Lipidierung führte bis zu einer acht-fachen Steigerung der planktonischen Aktivität. Eine Aktivitätssteigerung durch die Verwendung von zwei AMPs konnte aufgrund unterschiedlicher Wirkungsweisen nur in Kombination mit einem Peptid (P5) nachgewiesen werden. Die Entwicklung eines modifizierten Calgary-Biofilm-Assays ermöglicht ein einfaches und schnelles Screening der Antibiofilm-Aktivität der Peptide, sowohl hinsichtlich (langfristigen) Biofilmhemmung als auch -entfernung. Die Analyse basiert dabei auf der Bestimmung des Mikroorganismenwachstums und der Charakterisierung der Biofilmmasse durch Kristallviolettfärbung. Durch die Erweiterung der Wachstumsoberflächen auf polierte Stahloberflächen konnte dieser Test auch unter realitätsnahen Bedingungen durchgeführt werden. Die mikrobiologischen Tests zeigen Vorteile einer frühzeitigen AMP-Behandlung, vorzugsweise bereits im planktonischen Stadium. Vergleichsexperimente mit zwei gängigen Bioziden, Glutaraldehyd und Tetrakis-Hydroxymethylphosphoniumsulfat, in Bezug auf Aktivität, Toxizität und Resistenzrate zeigen das große Potenzial der AMPs, da diese bei gleicher Konzentration aktiver, weniger toxisch und weniger resistent gegenüber *Desulfovibrio vulgaris* sind. Auf dieser Grundlage ist eine Ausweitung des Anwendungsbereichs der AMPs vom medizinischen auf den industriellen Bereich zur Bekämpfung von biokorrosiven Bakterien möglich und stellt somit eine vielversprechende Alternative zur derzeitigen Biozidbehandlung dar.

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Abbreviations

AMP antimicrobial peptide
CBD calgary biofilm device
DMF dimethylformamide
DNA desoxyribonucleic acid
eDNA extracellular desoxyribonucleic acid
EPS extracellular polymeric substance
FIC fractional inhibitory concentration
fmoc 9-fluorenyl-methoxy-carbonyl
GA glutaraldehyde
HC hemolytic concentration
IC inhibitory concentration
LPS lipopolysaccharide
MBEC minimum biofilm eradication concentration
MBIC minimum biofilm inhibitory concentration
CMIC chemical microbiologically influenced corrosion
EMIC electical microbiologically influenced corrosion
MIC minimum inhibitory concentration
MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NRB nitrate reducing bacterium
PBS phosphate-buffered saline
QS quorum sensing
RBC red blood cell
SFB slime forming bacterium
SI selective index
SIcyto. selective index of the cytotoxicity-assay
SIery. selective index of the hemolysis-assay

SOB sulfur oxidizing bacterium

SPPS solid-phase peptide synthesis

SRB sulfate reducing bacterium

TFA trifluoroacetic acid

THPS tetrakis hydroxymethyl phosphonium sulfate

List of amino acids

amino acid	three letter code	one letter code
alanine	Ala	A
arginine	Arg	R
asparagine	Asn	N
aspartic acid	Asp	D
cysteine	Cys	C
glutamic acid	Glu	E
glutamine	Gln	Q
glycine	Gly	G
histidine	His	H
isoleucine	Ile	I
leucine	Leu	L
lysine	Lys	K
methionine	Met	M
phenylalanine	Phe	F
proline	Pro	P
serine	Ser	S
threonine	Thr	T
tryptophan	Trp	W
tyrosine	Tyr	Y
valine	Val	V

1. Introduction: Opportunities and challenges of antimicrobial peptides against biofilm forming bacteria

The discovery of penicillin by Fleming almost 100 years ago was the start of the golden antibiotics era [1, 2]. However, penicillin-resistant *Staphylococcus aureus* could be reported less than 20 years later [3]. To circumvent penicillin resistance, the antibiotic methicillin was introduced in the year 1959 [4]. Nevertheless, only two years later in 1961, strains of *Staphylococci* got resistant against this new antibiotic [5]. The rapid development and expansion of resistance in combating microorganisms is still a significant challenge at the present. By 2050, 10 million deaths are expected each year due to infections with multi-resistant pathogens [6]. In addition, bacteria often accumulate as a biofilm, making it more difficult to combat them. Numerous undesirable biofilms, so-called biofouling, can occur in the medical field due to colonization of implants or medical devices, often by ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter* spp.) organisms. Biofouling could also be found in the industrial sector, such as within pipelines or tanks, usually caused by biocorrosive bacteria [7]. For example, biocides represent a common method for eliminating microorganisms from technical equipment. However, their high resistance rate necessitates higher biocide concentration and higher application rate for an effective treatment [8]. Furthermore, the usage of biocides can results in significant environmental damage due to their high cytotoxicity [9]. The development of new substances as alternatives against the current ones is crucial. In the field of medicine, antimicrobial peptides (AMPs) have gained importance as potential new drug candidates in the recent years [10]. Currently, there are ten peptide-based antibiotics available on the market, including polymyxin B, gramicidin D, and daptomycin. These substances are predominantly used to treat skin infections. Additionally, several further peptide substances are undergoing clinical trials and thus represent promising antibiotic alternatives [2]. The advantage of AMPs is their broad spectrum of activity compared to antibiotics, which are often only active against a certain bacterial species due to specific mechanisms. Antibiotics often act on one specific target or target group, which is why a change in the target leads to a loss of activity. In contrast, AMPs can act through different mechanisms. In

particular, the non-specific mechanism of action of membranolytic AMPs should be mentioned. Since a change in the bacterial membrane represents an immense modification in bacterial structure, the maintenance of activity is guaranteed for a much longer period of time than with antibiotics [11]. Although AMPs have increased in importance in the medical and pharmaceutical fields in the recent years, their use in other areas is still not widespread, despite their advantages. Only in food technology, a further application of AMPs is possible. The AMPs nisin and polylysine have currently been approved by the food and drug administration as substitutes for food preservatives [12]. A technical application of AMPs has not been established yet. Promising results have been observed in initial trials conducted with AMPs from snow crab [13] and magainin II [14] against marine biofilms.

1.1. Antimicrobial peptides

Since AMPs play an important role as new drug candidates in the control of microorganism, this chapter will discuss the structural and functional properties of AMPs, especially membranolytic ones.

1.1.1. Structure and function

AMPs consist of a short series of amino acids (typically <40). Due to a high number of different AMPs, a classification is necessary. They can be classified according to various properties such as origin, biological function, sequence characteristics or 3D structure (figure 1.1). Over 3500 natural AMPs exist and could be found in a lot of different species, like human, animals or microorganisms, with the majority of natural AMPs being of animal origin (>2600), particularly from amphibians or insects [15]. They are part of the innate immune system and provide their host with competitive advantages in the face of selection pressure from other species [16]. Synthetic AMPs can be chemically produced using solid-phase peptide synthesis solid-phase peptide synthesis (SPPS) as a common method for laboratory and industrial scale. The advantage of this AMP production is the flexibility in sequence design and the simplicity of sequence modification, such as the incorporation of non-natural amino acids or terminus modifications [17]. However, the disadvantage of SPPS is the high consumption of organic solvents, but in the recent past, the development of water-soluble protective groups and thus a clean peptide technology has been advanced [18, 19].

In general, AMPs can be divided into three structural groups (figure 1.1). The first subgroup represents α-helical peptides. While these peptides have no structure in aqueous environment, the α-helical structure is formed through contact with surface-active substances such as surfactants, micelles or bacterial membrane. This right-handed structure requires 3.6 amino acids per turn, whereby the respective amino acid residues extend from the helix and are stabilized by the formation of hydrogen bonds between the carbonyl oxygen and the amide proton of two amino acids. An example of an α-helical AMP is the human peptide LL-37. The second subgroup are β-sheet structures, such as human defensin. Unlike α-helices, β-sheets are already formed in aqueous environment. The peptide sequence of this structure type contains a high concentration of cysteine, which stabilize the sheet structure via disulfide bridges. The last subgroup are the extended coil peptides. These peptides have an elongated, twisted structure. AMPs of these group are often proline-rich like indolicidin [20]. The amide group in the ring structure of proline prevents the formation of hydrogen bonds, why this amino acid is also known as a helix-breaker. Nevertheless, these proline properties contribute significantly to the extended coil structure [21, 22].

AMPs show a variety of different activities. One mechanism is the direct killing,

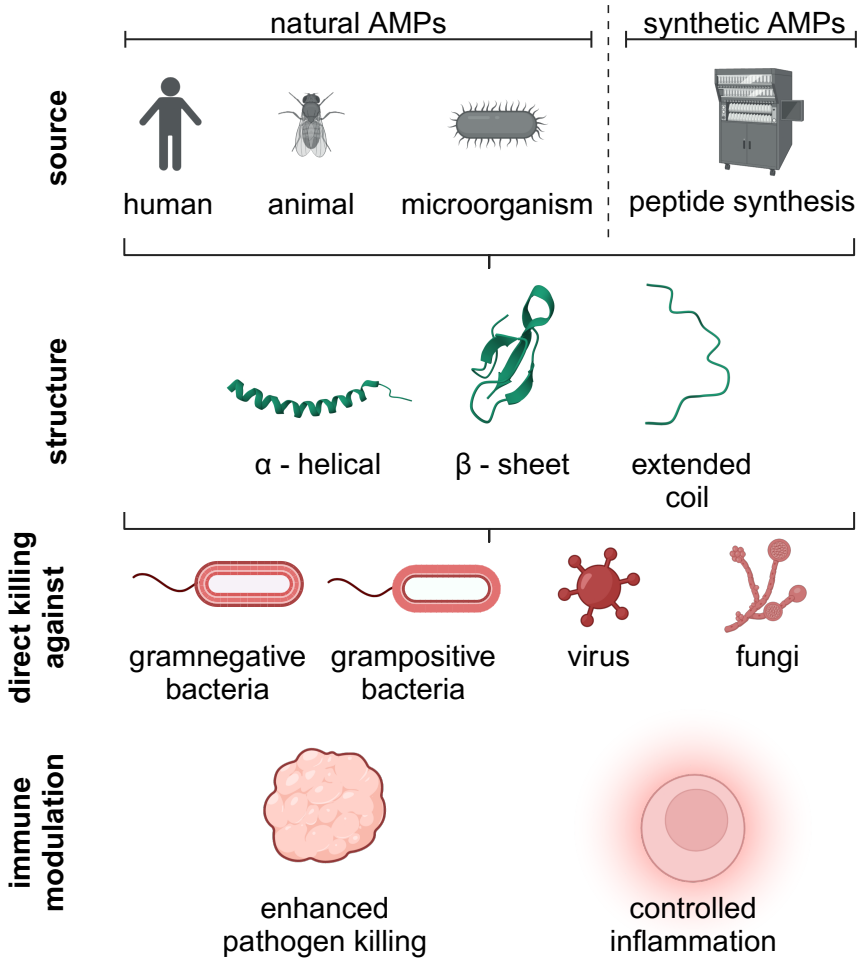

Figure 1.1.: Overview of source, structure (α-helical: human LL-37 PDB ID 2K6O, β-sheet: human defensin PDB ID 6MJV, extended coil: bovine indolicidin PDB ID 5ZVF), activity – direct killing and immune modulation of AMPs; created with BioRender.com

which could be differentiated into two sub-activities, against a huge range of different microorganism such as gram-negative and gram-positive bacteria, viruses or fungi. The majority of AMPs (90 %) show antibacterial properties [15]. One sub-group is the membranolytic effect via the interaction between the peptide and the bacterial membrane, such as magainin-2 [23]. A detailed description of this mode of action could be found in the section 1.1.2. The second sub-group is the intracellular killing, which is based on the inhibition of various metabolic pathways in the cell. The AMP indolicidin can act intracellularly by interfering with several metabolic pathways, like the inhibition of desoxyribonucleic acid (DNA) synthesis and nucleic acid synthesis in *Escherichia coli* [20]. Nearly 40 % of AMPs also show antifungal activities [15], which are also based on the interaction with the membrane. The plant defensin DmAMP1 binds to sphingolipids of *Saccharomyces cerevisiae*, result-

ing in membrane permeabilization [24]. Viruses and especially antiviral agents have been in people´s awareness at least since the Covid-19 pandemic. Almost 5 % of AMPs exhibit antiviral activities with diverse targets [15], including spike protein, capsid, envelope, DNA and RNA replication. [12] Another mechanism of AMPs is the modulation of the immune system. In this process, AMPs stimulate the immune system, resulting in enhanced microbial killing activity and controlled inflammatory responses. The peptide IDR-1018, although it has no direct effect, has anti-malarial activity by influencing the immune system by reducing inflammatory responses in malaria disease, for example by suppressing the production of the pro-inflammatory cytokine tumor necrosis factor TNF-α. Thereby, AMPs can influence both the innate immune system, such as macrophages, as well as the adaptive immune system, like T cells [25].

Since a large number of different bacterial species are involved in biocorrosion (chapter 1.3), the membranolytic mode of action is suitable for killing all the different bacteria involved. For this purpose, the membranolytic mode of action is described in more detail in the following chapter. Thereby, the group of amphiphilic peptides with α-helical conformation represents an important class of membrane-interacting AMPs [26], wherefore the focus is on this structural class.

1.1.2. Mode of action and structural characteristics of membranolytic AMPs

The membranolytic mode of action takes place in three sections [27], as can be seen in figure 1.2.

In the first step, an attraction between the AMP and the bacterial membrane occurs via electrostatic interactions. The gram-positive bacterial membrane consists of a plasma membrane followed by a thick murein layer. The plasma membrane anchored lipoteichoic acid and the murein layer anchored teichoic acid represent the targets for interaction with AMPs in gram-positive bacteria. The membrane of gram-negative bacteria is composed of two plasma membranes separated by a thin layer of murein. The outer layer of the second plasma membrane is made up of lipid A, which is a part of the lipopolysaccharide (LPS). In addition to the lipid A moiety and a polysaccharide component, LPS is characterized by numerous negatively charged phosphate groups. These provide the first electrostatic interactions with AMPs, but also represent a major diffusion barrier for hydrophobic drug candidates [12]. Due to their complex membrane structure, gram-negative bacteria are harder to kill than gram-positive ones [28]. Since biocorrosion bacteria are usually gram-negative, gram-positive bacteria will be disregarded going forward.

After penetrating the LPS layer, the peptide interacts with anionic phospholipids in the membrane, resulting in the formation of the α-helical structure (second step).

In the third stage, the membrane permeabilization is carried out, differentiating between models with pore formation (barrel-stave and toroidal-pore) and models

without pore formation (carpet). In the barrel-stave model, multiple helices form a pore, with peptide orientation of the hydrophobic moiety toward the lipid bilayer and the hydrophilic moiety toward the pore lumen. The AMP alamethicin acts across this membrane model to form a pore with a pore lumen diameter of ~18 Å [29]. In contrast to the previous model, in the second model (toridal-pore) the hydrophilic peptide moieties interact continuously with the hydrophilic headgroups of the phospholipids, resulting in a curvature of the bacterial membrane at the pore. An example of this membrane model is the AMP magainin, which forms a pore with a diameter of 30 Å [29]. Instead of pore formation like in the previous two models, peptides can act via the carpet model without pore formation. Instead, AMPs accumulate on the membrane due to electrostatic interactions until a certain threshold concentration is reached. Afterward, the cell wall is disrupted with a release of micelles, causing the AMPs to react in an all-or-nothing manner. Numerous peptides, particularly at high concentrations, act through this model, such as melittin [30], cecropin P1 [31], and dermaseptin S [32].

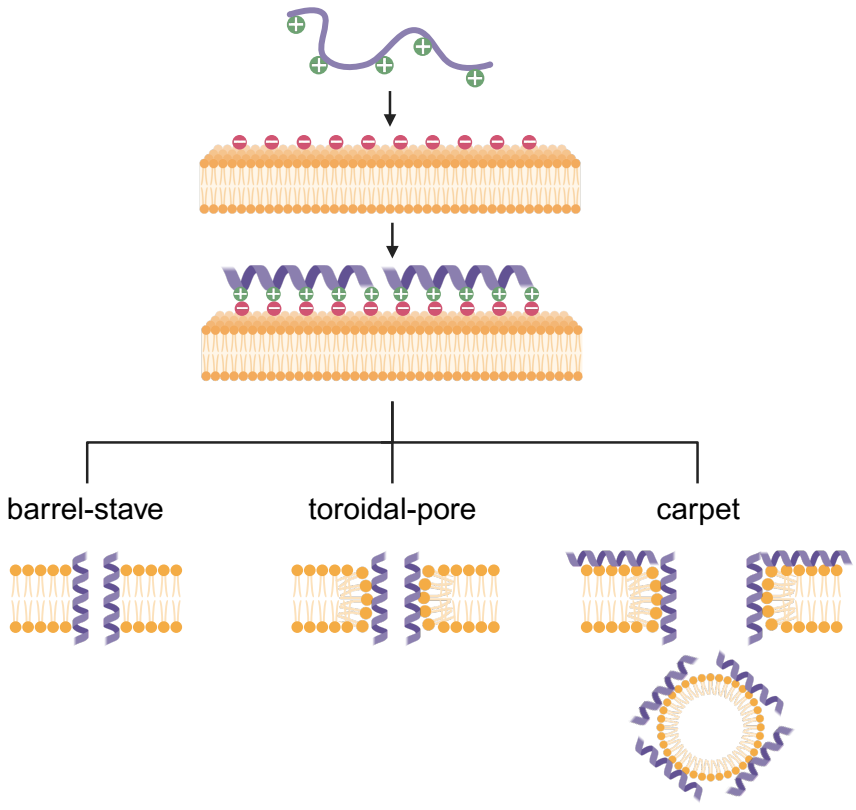

Figure 1.2.: Electrostatic interaction of cationic AMP with anionic bacterial membrane, formation of α-helical peptide structure and incorporation of peptide in bacterial bilayer via barrel-stave, toroidal-pore or carpet model; created with BioRender.com

For an improved membranolytic activity of AMPs, certain properties should be present in the sequence [27]:

- Net charge: Firstly, it is essential to mention the positive net charge of the peptide sequence as it is necessary for electrostatic interaction with the bacterial membrane. Increasing the net charge enhances the activity not only against the target organism but also against other biological components such as erythrocytes [33]. To achieve a balance between activity against the target bacterium and the protection of the environment, a net charge of +4 to +6 is considered to be optimal [34].
- Hydrophobicity: Another aspect to consider is the hydrophobicity of the peptide, which allows the interaction with the lipid tails and the incorporation into the bacterial membrane, especially in the barrel-stave model. However, high hydrophobicity results in high cytotoxicity, as hydrophobic AMPs penetrate deeper into the membrane causing to increased membrane disruption [35]. A hydrophobicity level of 40-60 % is considered optimal for membranolytic AMPs [34].
- Amphiphilicity: The distribution of hydrophobic and charged amino acids within the α-helical structure is crucial, in contrast to their total amount. As the so-called amphiphilicity of the AMP increases, the hydrophobic and hydrophilic amino acids are more strictly separated in the top view of the helical structure. Thereby, the hydrophobic amino acids are located on one side of the helix and the hydrophilic ones on the other side, resulting in maximum interaction with the corresponding bacterial components. The peptides with the greatest activity exhibited the highest amphiphilicity among a group of peptides characterized by identical amino acid composition and therefore identical hydrophobicity, but different sequence and therefore different amphiphilicity. This suggests that amphiphilicity is a critical factor for AMP activity and is therefore preferable to hydrophobicity [36].
- Sequence length: The sequence length is also an important factor. A minimum of seven to eight amino acids are necessary to form an amphiphilic structure. Besides, a minimum length is crucial for the complete bacterial membrane perforation, depending on the composition of the bacterial membrane [37]. The advantages of the length must be balanced against the disadvantages. As the peptide length increases, hemolysis activity significantly increases [37] as well as the cost of peptide synthesis and the probability of synthesis problems.
- Special amino acids: Finally, it is important to note the presence of certain amino acids in the peptide sequence, specifically the amino acid tryptophan. Due to the large aromatic ring, tryptophan can form numerous interactions with membrane components, such as pi-pi interactions, hydrophobic interactions, or hydrogen bonds, resulting in anchoring, stabilization and correct orientation of AMPs into the membrane [38]. Additionally, it is important to mention the role of the amino acid cysteine, which has the ability to build

a disulfide bridge with another cysteine, significantly changing the structural properties of the peptide sequence. Bactenecin is a peptide that shows two different forms depending on the environment: a linear form in reduced environment [39], and a ring-closed structure with a hydrophobic ring and a hydrophilic tail in oxidized milieu [40].

1.1.3. Bacterial mechanisms against AMPs

Resistance refers to the inability of a substance to be present at the microbiological target in sufficient concentration to be active [41]. Despite the expected minor resistance rate due to the non-specific mode of action of membranolytic AMPs, bacteria have developed numerous protection mechanisms against external substances like AMPs, rendering their application challenging [42].

Firstly, a change in the target can contribute to a reduced effectiveness. A change in the bacterial membrane represents a major modification and therefore only occurs slowly [11]. Nevertheless, bacteria have developed methods to modify their membrane structure. Many bacteria have the regulatory systems for modification of their LPS layer, which is present in gram-negative bacteria membrane (chapter 1.1.2). Contact with external substances, such as the AMP polymyxin B, activates these regulatory systems and result in diverse changes to the LPS structure. This could cause a modification of the phosphate group located in the core region of the LPS, which leads to decreased electrostatic interaction between AMP and LPS [43]. Such regulatory system could be found in many bacteria, such as in *P. aeruginosa* [44], *Salmonella* spp. [45], and *E. coli* [46].

The second method of bacterial control against external substances is to reduce them. Proteases degrade proteins and peptides through interaction between the respective active enzyme side and the peptide bond [47]. Proteases are classified based on their structural properties in the active side. These can either be amino acids like serine or cysteine, e.g. trypsin or papain, as well as other amino acids such as aspartic acid, threonine, glutamic acid and asparagine, or metal ions, usually zinc like in pseudolysin. Exopeptidases split dipeptides or single amino acids from the end of the peptide chain - either from N-terminal (amino peptidase) or from C-terminal (carboxyl peptidase). Endopeptidases cut within the amino acid chain [48, 49]. Many bacteria produce both extracellular and intracellular proteases for protection. *P. aeruginosa* is an example for a bacterium that produces various extracellular proteases, such as pseudolysin [50], aeruginolysin [51], staphylolysin [52], and protease IV [53]. These proteases prefer different cleavage sites and can therefore rapidly degrade peptides and destroy their activity, leading to immense protection and high virulence of *P. aeruginosa* [54]. However, high stability is crucial for effective application of AMPs. Consequently, methods have been developed to reduce or prevent protease attachment to the peptide sequence, including the insertion of D-amino acids, acetylation of the N-terminus, amidation of the C-terminus, and

cyclization [55]. Another possibility is the removal of interfering substances from the periplasm and cytoplasm by efflux pumps [56]. Seven different efflux pump categories exist, with the resistance-nodulation-division and the ATP-binding cassette variants representing the main ones for AMP resistance in gram-negative bacteria [57]. For this first transporter subfamily, the resistance-nodulation-division, the efflux system AcrAB-TOlC is an example for the elamination of the AMP 4DK5L7 in *Salmonella typhimurium* [58] as well as Polymyxin B and human defensine in *K. pneumoniae* [59]. The second mentioned efflux subclass, the ATP-binding cassette, could removes the AMPs protamine and melittin from *S. typhimurium* [60]. Furthermore, bacteria have the ability to aggregate as a biofilm. These bacteria are protected by the formation of a layer of extracellular polymeric substances (EPSs), which acts as a diffusion barrier, as well as the interaction of AMPs with negatively charged component of the EPS matrix, such as extracellular desoxyribonucleic acid (eDNA) [61]. Detailed information could be found in next chapter.

1.2. Biofilm

The majority of bacteria grow as a biofilm, which describes the accumulation of bacteria within a protection layer [7]. As a result, this section will discuss the biofilm and its characteristics in more detail.

1.2.1. Biofilm formation

Since biofilms provide immense protection for bacteria against external substances, e.g. AMPs, they typically grow together as a biofilm. The process of biofilm formation can be classified into five phases, see figure 1.3 [22, 62].

During the initial phase, microorganisms attach reversible to a surface. This is the point at which bacteria first encounter the surface and weak interactions, including van-der-Waal forces, hydrophobic interactions, and electrostatic attraction, begin to form. Microbiological attachments, including pili, and adhesion surface proteins, such as fibrinogen, also allow bacteria to attach to a surface.

In the second step, a primary layer of bacteria develops on the surface, combined with covalent interactions with the surface (irreversible attachment). Furthermore, the production of EPS initiates, shielding the initial bacteria.

The following steps include the bacteria grow (proliferation), leading to increased EPS production and the formation of a mature biofilm (maturation). The EPS matrix consists mainly of extracellular proteins, DNA and polysaccharides. Interactions among them, including van-der-Waal forces, hydrogen bonding, and electrostatic attraction, lead to stability of the EPS. The individual EPS components can be involved in various functions: EPS components could also be involved in the bacterial adhesion to a surface. Additionally, the EPS serves as a diffusion barrier and provides numerous points of interaction with external substances, such as the electrostatic attraction between cationic AMP (LL-31) and anionic eDNA, resulting in a reduced AMP concentration within the biofilm and thus a reduced AMP activity and consequently the protection of the bacteria [61]. Furthermore, the EPS facilitates intercellular communication by serving as a transport pathway for quorum sensing (QS) molecules and serves as a nutrient source [63]. During these biofilm formation stages, bacteria adapt to living within the biofilm and could develop persister cells with low cell metabolism. These cells are able to survive for extended periods of time, as the metabolic processes targeted by traditional antibiotics are inactive, making it impossible to combat them. Persister cells can be reactivated and begin to regrow under certain internal, e.g. toxin-antitoxin molecules, and external conditions, e.g. reduction in environmental stress or change in carbon source availability. Substances that interact with the membrane, such as membranolytic AMPs, are suitable for killing persister cells and enabling long-term treatment of biofilms [64, 65].

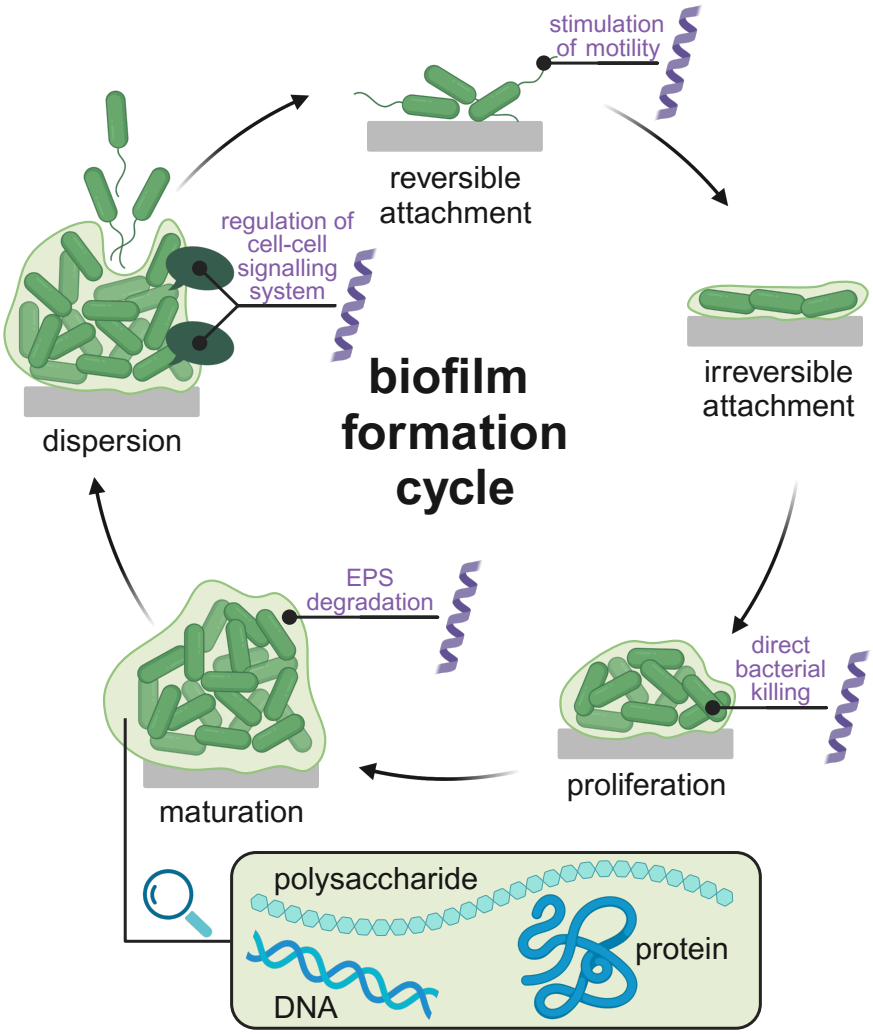

Figure 1.3.: Biofilm formation cycle: reversible attachment of planktonic bacteria to surface, irreversible attachment via formation of a first biofilm layer and first production of EPS, bacterial growth (proliferation) and increase in EPS production, existing of extracellular polysaccharide, protein, DNA (maturation), and dispersion of bacteria for formation of a new biofilm, overview about interaction points of AMPs with the biofilm (purple): stimulation of bacterial motility, direct bacterial killing, EPS degradation and inhibition of cell-cell signalling system; created with BioRender.com

In the final stage of the biofilm formation process, bacteria are released into the environment and become mobile again, leading to the formation of a completely new biofilm or the integration into the individual stages of biofilm formation.

Since bacteria in the biofilm are immensely protected by the EPS matrix and therefore make treatment significantly more difficult, it is preferable to prevent the formation of biofilm by using various surface coating processes (figure 1.4).

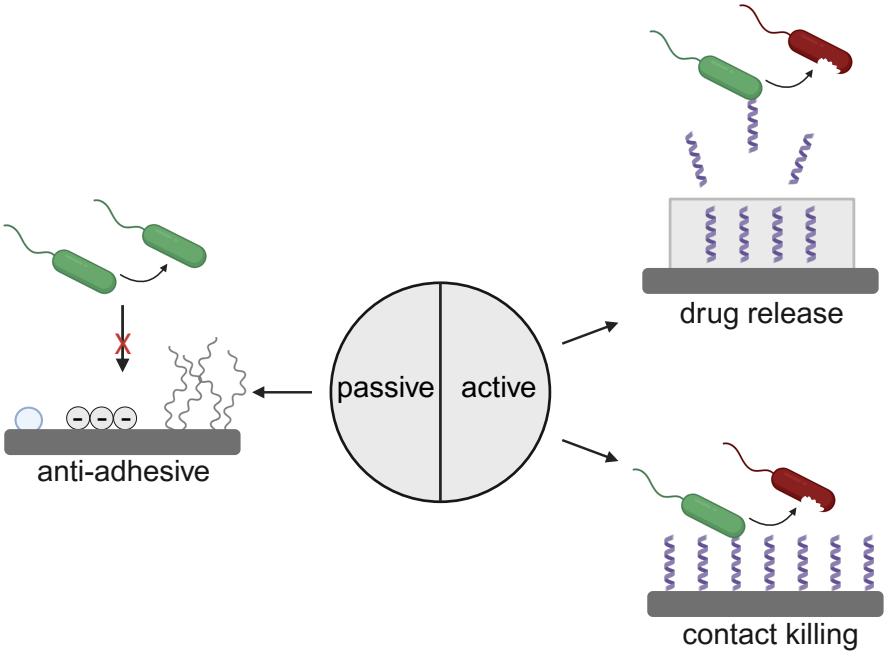

Figure 1.4.: Mechanisms of coating: passive action with prevention of bacterial adhesion by increased hydrophobicity, anionic surface, or steric shielding, active action with bacteria killing (red bacteria) by AMP release or direct contact with AMP; created with BioRender.com; adapted from [66] CC-by 4.0

Passive coatings can prevent bacterial adhesion while maintaining bacteria viability (figure 1.4 left). To achieve this, highly hydrophobic surfaces can be used, preventing the interaction between the hydrophilic lipid head group from the lipid bilayer of the bacterial membrane with the surface. Negatively charged surfaces can also prevent bacterial attachment by electrostatic repulsion with the negatively charged bacterial membrane. Furthermore, the surface can be shielded with polymers [67].

In contrast to passive coatings, active coatings lead to bacterial killing (figure 1.4 right). Drugs, like AMPs, can be constantly released from a matrix, such as polymethyl methacrylate [68] or polyelectrolyte multilayers [69], to kill bacteria. These free peptides are highly active compared to tightly bound AMPs. However, AMPs within this coating are released at any time, even when they are unnecessary and the storage capacity is limited. The alternative method of active protection describes the contact killing, wherein the AMP is bound to the surface and kills the bacterium by contact. This approach provides the advantages of preventing unwanted

drug release, but the AMP flexibility is limited, necessitating the usage of linkers to maintain activity [70].

Nevertheless, if a biofilm is still forming, AMPs provide further antibiofilm activity in addition to combating bacteria, which is described in more detail in the following chapter.

1.2.2. Antibiofilm-peptides

Over 100 antibiofilm-active AMPs are available in the APD3 database [15], as well as over 200 ones in the specialized biofilm database BaAMPs [71]. There are various interaction points for AMPs within the biofilm (figure 1.3 purple).

The first point to consider is the direct killing similar to planktonic bacteria. However, this process is more difficult within the biofilm. Experiments with the AMP BP100 against *P. aeruginosa* demonstrate that at minimum inhibitory concentration (MIC) only 60 % of biofilm could be inhibited, and for a 60 % biofilm eradication an eight-fold MIC is necessary [72]. Studies with other peptides also show a significant decrease in biofilm activity compared to their planktonic effectiveness [73].

Besides direct contact with bacterial membrane, AMPs could also interact via other points, such as the bacteria´s motility. These include the peptides LL-37 and 1037. On the one hand, these peptides can cause an upregulation of the gene fimX responsible for type IV pili in *P. aeruginosa* and thus for twitching motility. Twitching motility is the capacity of a bacterium to move across a surface, so an increased activity leads to a decreased bacterial accumulation. On the other hand, these peptides can cause a downregulation of the genes flgB, flgC, flgD, and flgF, which are important for flagella and therefore the motility of the bacterium. If the motility of the bacterium is restricted, bacteria released from the biofilm are no longer able to form a new biofilm, and interactions within the biofilm may also be hindered, as flagella could be involved in the formation of and interaction with the EPS matrix [74].

Although some components of the EPS, like eDNA, can interact with AMPs and therefore hinder their activity, there are also many AMPs that can disrupt the EPS matrix. For example, the AMP piscidin 3 enables copper-dependent cleavage of eDNA [75]. Additionally, other AMPs, such as hepcidin 20 [76] or human β-defensin 3 [77], can interact with the polysaccharide intercellular adhesin or inhibit its synthesis by downregulating the icaA, icaR, and icaD expression genes. Furthermore, EPS can be reduced by biosurfactants such as the cyclic AMP viscosin, as experiments on *Pseudomonas fluorescens* have shown [78].

Another potential target for AMPs is the inhibition of the cell-cell communication system. In *P. aeruginosa*, the AMP LL-37 leads to downregulation of the two QS systems Las and Rhl. An identical mode of action can be observed with the AMP

indolicidin [79]. Through interaction of AMPs with the biofilm, instead of direct bacterial elimination, AMP activity against the biofilm can be significantly increased, resulting in a better biofilm effect compared to their planktonic effect, such as the AMP 1018, which blocks two important signaling molecules (ppGpp and pppGpp) in *E. coli* [80].

1.3. Microbiologically influenced corrosion

Corrosion is typically the result of electron transfer from elemental iron to iron ions and binding with other molecules such as oxygen or sulfur [81]. While in classical oxygen corrosion the electron transfer is based on reactions with oxygen in aqueous solution, biocorrosion describes corrosion caused by microorganisms [82]. Thereby, the involvement of microorganisms in causing corrosion damages could be described for the first time in 1910 [83]. Since then, there have been several instances of microbiologically influenced damage, such as the leak in the Alaska oil pipeline in the year 2006 [84].

1.3.1. Biocorrosive bacteria

Numerous bacteria species can be involved in biocorrosion and could damage the material each on their own way: sulfate reducing bacterium (SRB), sulfur oxidizing bacterium (SOB), iron reducing bacteria, iron oxidizing bacteria, and slime forming bacterium (SFB) [85–87]. The focus in this thesis is on SRB and SOB, because these two bacteria species can built up a so-called sulfur cycle (figure 1.5). Depending on the water level and the resulting oxygen demand, either SRBs or SOBs are active and produce reactants for the other one, maintaining biocorrosion processes at all time [81].

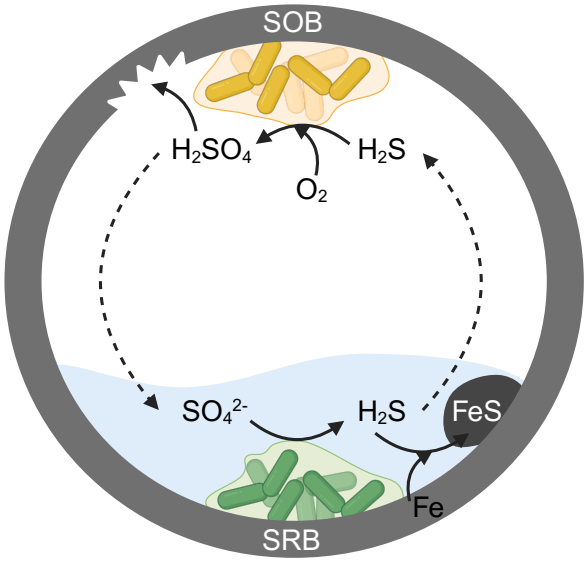

Figure 1.5.: Sulfur cycle: reduction of sulfur through SRB (green) with production of hydrogen sulfide and iron sulfide, oxidation of hydrogen sulfide in the present of oxygen to sulfuric acids through SOB (yellow); created with BioRender.com

Sulfur of higher oxidation states, such as sulfate, functions as an electron acceptor for the SRBs and reacts with hydrogen to form hydrogen sulfide, which can further

react with iron to iron sulfide. There are two types for the electron source for this reaction: chemical microbiologically influenced corrosion (CMIC) and electical microbiologically influenced corrosion (EMIC). In the first one, organic compounds or hydrogen serve as electron donors. In the latter, the electrons are taken from metallic materials, such as elemental iron by the bacteria itself through direct electron transfer by cell extensions and outer membrane cytochromes or electron shuttle molecules like mediators. The resulted iron(II) ions can react with hydrogen sulfide and minerals such as carbonates [81, 88, 89]. As a result, deposits are formed on the metal. The majority are iron minerals and other inorganic precipitates, and only a small proportion being actual biomass [90]. SOBs oxidize hydrogen sulfide in the presence of oxygen, producing aggressive products such as sulfuric acid, which damage the metal surface (acid corrosion). Further, sulfuric acid can serve as sulfate source for SRBs [81].

SFBs are characterized by a high rate of EPS formation. As biocorrosive bacteria live as a consortium in nature, it leads to the protection of other biocorrosive bacteria. At the same time, the EPS matrix has usually a high amount of acidic components, which leads to acid corrosion and therefore also metal damage [86]. An example for these SFBs is *P. aeruginosa*, whose EPS matrix is characterized by a high amount of alginate [91]. Since *P. aeruginosa* is a crucial bacteria, both in medical technology as a member of the ESKAPE organisms and in industrial sector as a biocorrosive bacteria, the development of AMPs in this thesis also includes this bacteria.

1.3.2. Current treatment

Maintaining a clean system is crucial in preventing biocorrosion. This involves removing all unwanted materials from the system and regularly inspecting connecting parts to prevent unwanted materials entering the system from the outside [87]. Furthermore, the system should be designed to minimize areas with low or stagnant flow since these areas are highly affected by deposition, which can cause strong corrosion effects [92, 93]. In addition, modification of the material surface can minimize bacterial adhesion (figure 1.4).

Despite the preventive techniques, biocorrosion may occur. Therefore, there are various approaches for combating biocorrosion:

During pigging (mechanical treatment), a cylindrical device is inserted into the pipeline to remove accumulated deposits. This technique is a popular method for cleaning pipelines because the pig can slide through the pipe with product flow, which prevents process shutdown. However, it is limited to pipelines and these have to conform to certain criteria, such as no major changes in pipe diameter or no T and Y connections. Furthermore, this approach is relatively inefficient and is typically utilized in combination with another method, such as the application of biocides [92, 94].

For the chemical treatment, inhibitors can be used to influence anodic, cathodic, or both types of reactions. However, the disadvantage here is that the biocorrosion reactions are only blocked. Alternatively, biocides can be applied to kill the bacteria. Oxidizing biocides such as chlorine, bromine, ozone, and hydrogen peroxide lead to oxidation of numerous substances, including oxygen, which can result in an acceleration of corrosion processes. As a result, non-oxidizing biocides have now been established [87, 95]. Glutaraldehyde (GA) and tetrakis hydroxymethyl phosphonium sulfate (THPS) are two typical non-oxidizing biocides (figure 1.6), which are often used in the oil and gas industry due to low cost and a broad spectrum of activity [96]. GA is characterized by two aldehyde groups, which can form imine with amino groups in peptides, proteins, and nucleic acids, resulting in their inactivation [97]. 100 ppm GA results in a reduction of living cells of an oilfield consortium in biofilm prevention test [98]. However, bacteria like *E. coli* can produce the enzyme aldehyde reductase, which catalyze the reaction of GA to non-toxic 1,5-pentanediol. By upregulating the yghD gene and overproduction of the aldehyde reductase, *E. coli* is able to withstand the presence of GA [99]. THPS has numerous modes of action like the damage of membranes, the interruption of proton flux and the inhibition of sulfate reduction [97].

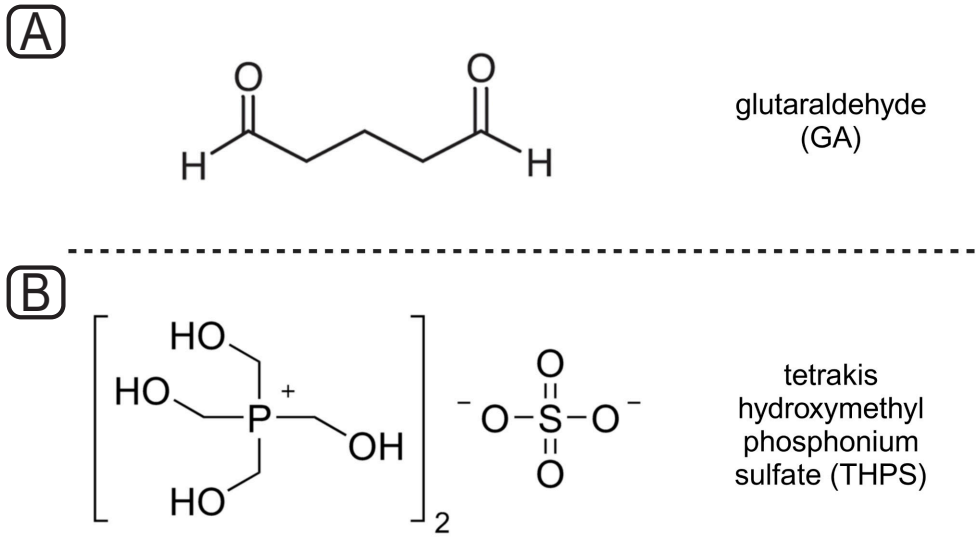

Figure 1.6.: Structure of two typical non-oxidizing biocides: (A) GA, PubChem ID 3485, (B) THPS, PubChem ID 41478

The physical treatment includes the exposure to ultraviolet light and the usage of ultrasonic cleaning, which are less common in industrial plants than the previous methods [95].

Biological treatment consists the usage of bacteria, like nitrate reducing bacterium (NRB). These could be used, competing with SRBs for the carbon source as an

electron donor. As the free enthalpy for nitrate reduction is more negatively than for sulfate reduction, NRBs win in comparison to SRBs. Additionally, NRBs are able to produce biosurfactants that can also inhibit the growth of SRBs. Other biosurfactant-producing bacteria, like *Pseudomonas stutzeri*, can support this [87, 100]. Using this method, a 1000-fold decrease in the number of SRBs on the oil platform Gullfaks could be demonstrated [101]. Another possibility is to utilize bacteria that produce AMPs, like a genetically engineered strain of *Bacillus subtilis*, which can produce two AMPs - indolicidin and bactenecin. These AMPs provide SRB inhibition, but they also attack their host organisms, making this method unsuitable [102].

2. Objective of the thesis

AMPs are gaining increasing importance as new drug candidates in medical technology and pharmaceutical field. However, the current applications are only focused on this area. An expansion of the application area to the industrial sector is absolutely necessary to avoid biocide usage, minimize resistance formation and promote environmental safety. This thesis addresses the expansion of the application range of AMPs by investigating the usage of AMPs against the industrially relevant biocorrosive bacteria SRB, SOB, and SFB and their biofilms. Therefore, this thesis is split into four main sections:

The initial chapter focuses on the selection of suitable peptide sequences from common peptide databases with following planktonic testing (MIC) against SRB, SOB, and SFB, as well as further biofilm forming bacteria. Cytotoxicity, hemolysis and stability tests conclude this chapter.

The second chapter addresses the improvement of AMPs against planktonic SRB by peptide optimization according to common sequence optimization methods and the combination of different AMPs with the calculation of the fractional inhibitory concentration (FIC)-index to indicate the activity improvement in combination experiments compared to individual usage.

The third chapter focuses on the adaption of an antibiofilm-assay to the growth conditions of the biocorrosive bacteria *Pseudomonas aeruginosa* (SFB) and *Desulfovibrio vulgaris* (SRB) through partially modification of the growth surface. Thus, the corresponding determination of minimum biofilm inhibitory concentration (MBIC) and minimum biofilm eradication concentration (MBEC) is carried out with the previously selected AMPs and the respective optimized versions.

Finally, this new AMP treatment is compared to two current biocides, GA and THPS, through different parameters like bacterial activity, cytotoxicity, hemolysis and resistance rate and the impact of this new developed AMP treatment is evaluated.

3. Selection of AMPs against planktonic biocorrosive bacteria

This chapter is part of the following publication: adapted from L. Stillger, L. Viau, L. Kamm, et al. "Optimization of antimicrobial peptides for the application against biocorrosive bacteria". In: Applied Microbiology and Biotechnology 107 (2023), pp. 4041–4049. doi: 10.1007/s00253-023- 12562-9. [103], CC-by 4.0

3.1. Introduction

Microbiologically influenced corrosion is a common problem due to the damage of metals in the presence of various microorganisms, such as anaerobic SRBs like *Desulfovibrio spp.* and aerobic SOBs like *Thiomonas spp.* (for more details see chapter 1.3). The current method of treatment involves the use of biocides (chapter 1.3.2). However, the disadvantage here is the rapid resistance formation. This leads to the necessity to apply higher and higher concentrations in ever shorter time intervals. This is mainly enhanced through the low number of suitable biocides, with THPS and GA being the main ones [96]. Recent efforts to reduce the biocide concentration through additives have already been successfully demonstrated against SRBs. By adding a mix of eight different D-amino acids to THPS, the obtained effect was comparable to the double concentration of THPS alone [104]. Identical results could be achieved with the addition of a cyclic peptide [105]. The addition of chelate can also significantly improve the effect of the biocide GA against two SRBs, *D. desulfuricans* [106] and *D. vulgaris* [107]. Further researches are focusing on herbal additives such as D-limonene, which also demonstrates an enhanced effect in combination with GA [108]. The problem with all these modifications is that the additives alone have no antimicrobial properties. Thus, biocide application is still necessary. An alternative treatment strategy could be the use of AMPs. These naturally occurring peptides have already demonstrated their antimicrobial impact in medical applications [109]. Specific applications of AMPs against biocorrosive bacteria, specifically SRB or SOB, are rarely described. Initial experiments were performed by expressing the AMP in a host organism (*Bacillus subtilis* BE1500) to circumvent the diffusion processes into the SRB biofilm. The disadvantage of this method was that the AMPs also attacked the host organism and thus reducing the applicability of the in-situ production of AMPs. Furthermore, the application of a

genetically modified organism will be only possible in a limited number of applications. Nevertheless, the AMPs of this study showed antimicrobial property against *D. vulgaris* and *D. gigas* [102].

This chapter includes preliminary experiments in form of MIC determination on general biofilm forming bacteria *(E. coli*, *P. fluorescens*) and on biocorrosion bacteria (SRB: *D. vulgaris*, *D. desulfuricans*, *D. indonesiensis*, SOB: *T. intermedia*, SFB: *P. aeruginosa*), toxicity of these peptides and stability studies to demonstrate the potential of AMPs against biocorrosion bacteria.

3.2. Material and methods

3.2.1. Peptide selection

Based on two existing peptide databases, BaAMPs [71] and APD3 [15], suitable AMP sequences with the following characteristics were searched: positive charge of approximately +5, amphiphilicity as high as possible, hydrophobicity around 50 %, sequences as short and simple as possible. Thereby, suitable sequences were identified, prioritized according to the above criteria, and were synthesized and tested.

In table 3.1, the four favorites AMPs are listed. A complete table of all the selected AMPs could be found in the appendix, figure A.1.

Table 3.1.: Four selected AMPs used in this thesis with sequence and source

peptide number	(analogous) peptide	sequence	source
P1	L5K5W	KKLLKWLKKLL	AP00143 [15]
P2	S6L3-33	FKKFWKWFRRF	ID40 [71]
P3	DASamP1	FFGKVLKLIRKIF	ID146 [71]
P4	bactenecin	RLCRIVVIRVCR	[102]

3.2.2. Solid-phase peptide synthesis

All peptides were synthesized by solid-phase using microwave-assisted peptide synthesizer LibertyBlue™ (CEM GmbH, Kamp-Lintfort, Germany) with 9-fluorenylmethoxy-carbonyl (fmoc)-chemistry in 0.1 mM scale. Solvents were purchased from Carl Roth GmbH & Co.KG (Karlsruhe, Germany), amino acids and resins from Merck KGaA (Darmstadt, Germany) and Iris Biotech GmbH (Marktredwitz, Germany). First, swelling of the resin (preloaded) in 10 mL dimethylformamide (DMF) for 300 s was performed. The following steps were performed cyclically for each amino acid: First, deprotection with 3 mL of 20 % piperidine in DMF, 90 °C, 65 s, 30 W followed by a washing step with 4 mL of DMF three-times, and repeating these two steps. Then, coupling of 2.5 mL of 0.2 M amino acid at 90 °C, 125 s with 1 mL of N,N´-diisopropylcarbodiimid (0.5 M in DMF) and 0.5 mL of Oxyma Pure (1 M in DMF) and washing two-times. Afterwards, final deprotection using 4 mL of 20 % piperidine in DMF, 90 °C, 65 s followed by washing three-times with 3 mL of DMF each. These last two steps were repeated twice. For the amino acid arginine, coupling was first performed at 25 °C, 150 s and then at 75 °C, 120 s, 30 W in duplicate, interrupted by one washing step. After synthesis, the peptide was removed from the device and rinsed twice with DMF and three-times with dichloromethane followed

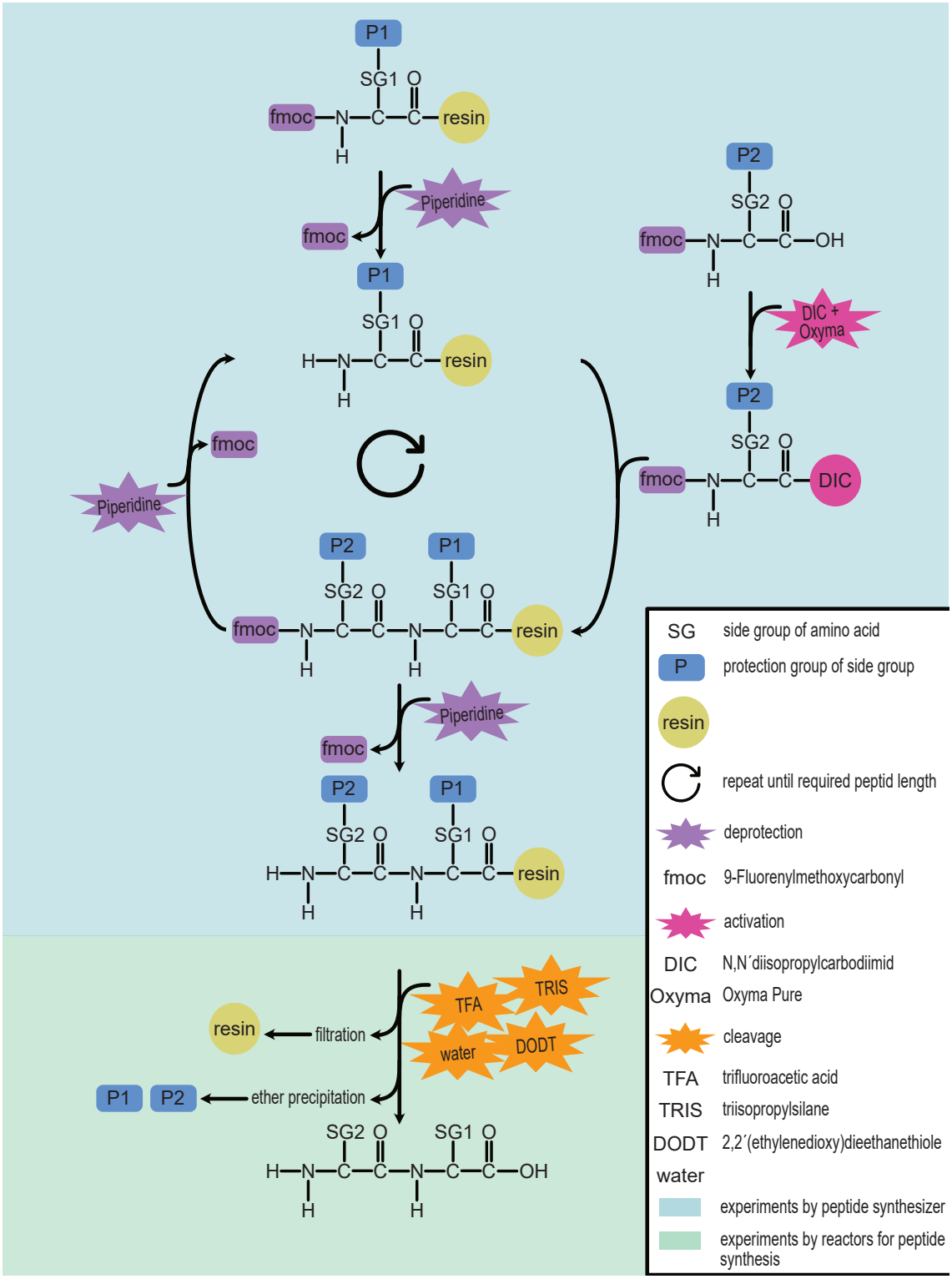

Figure 3.1.: Overview of the individual steps of SPPS; [103] CC-by 4.0

by overnight lyophilization. The cleavage from the resin was incubated with 4 mL solution consisting of 85 % trifluoroacetic acid (TFA), 5 % triisopropylsilane, 5 % 2,2´-(ethylenedioxy)diethanethiole, 5 % milliQ water (v/v/v/v) at room temperature for 3 h at 300 rpm. Filtration was used to separate the resin from the peptide. Precipitation of the peptide was performed with five-fold volume of cleavage-solution with ice-cold diethylether followed by centrifugation for 5 min at 4024 g (2x). Subsequently, the peptide pellet was dried under nitrogen for approximately 5 min, and lyophilized using freeze-drying. An overview of the individual steps of the peptide synthesis can be observed in the figure 3.1.

The analysis of the peptides was performed on a RP-HPLC (Agilent 1100, detector: diode array detector G1315A, column: Phenomenex®-Aeris™ 2.6 µm Peptide XB-C18 100 Å,150 x 2.1 mm) and on an ESI-LC-MS (Knauer, detector: Surveyor MSQ, column: Eurospher II 100-2 C18A, 100 x 2 mm, 2 µm). The gradient was performed with a flow rate of 0.25 mL/min from 99 % of mobile phase A (0.1 % TFA in milliQ) over 15 min to 95 % of mobile phase B (0.1 % TFA in acetonitrile), kept at 95 % with B for 2 min and equilibrated at 99 % with A for another 3 min. 20 µL (RP-HPLC) and 10 µL (ESI-LC-MS) peptide solution (concentration: 250 µg/mL in milliQ) was injected.

3.2.3. Strains and cultivation

In the following table, the different bacterial strains used in this thesis and their media as well as their parameters for subcultivation and incubation are listed.

Table 3.2.: Strains and cultivation parameters of bacteria used in this thesis

bacteria	medium	subcultivation	incubation
	SRB		
Desulfovibrio vulgaris DSM 644	DSMZ medium 63 with modification 0.004 g/L $FeSO_4$ x 7 H_2O and 0.3 g/L tri-sodium citrate	1:100	48 h, 37 °C, 0 rpm
Desulfovibrio desulfuricans DSM 642	DSMZ medium 63 with modification 0.004 g/L $FeSO_4$ x 7 H_2O and 0.3 g/L tri-sodium citrate	1:100	48 h, 30 °C, 0 rpm

Table 3.2 continued: Strains and cultivation parameters of bacteria used in this thesis

bacteria	medium	subcultivation	incubation
Desulfovibrio indonesiensis DSM 15121	DSMZ medium 63 with modification 0.004 g/L $FeSO_4$ x 7 H_2O and 0.3 g/L tri-sodium citrate + 2 % or 3.5 % NaCl	1:100	48 h, 37 °C, 0 rpm
	SOB		
Thiomonas intermedia DSM 18155	DSMZ medium 35a	1:100	48 h, 30 °C, 115 rpm
	SFB		
Pseudomonas aeruginosa DSM 1117	LB Lennox medium	1:1000 in Mueller-Hinton broth	24 h, 37 °C, 115 rpm
Pseudomonas aeruginosa DSM 22644 (PAO1)	LB Lennox medium	1:1000 in Mueller-Hinton broth	24 h, 37 °C, 115 rpm
Pseudomonas aeruginosa DSM 19882 (PA14)	LB Lennox medium	1:1000 in Mueller-Hinton broth	24 h, 37 °C, 115 rpm
	general biofilm forming bacteria		
Escherichia coli DSM 5698 (K12)	LB Lennox medium	1:1000 in Mueller-Hinton broth	18 h (for over-night culture), 24 h (during assay), 37 °C, 115 rpm
Pseudomonas fluorescens DSM 289	LB Lennox medium	1:1000 in Mueller-Hinton broth	24 h, 37 °C, 115 rpm

3.2.3.1. MIC-assay

A stock solution of 1 mg/mL in milliQ from the respective peptide and a concentration range of 1 mg/mL-1.95 µg/mL was prepared by serial two-fold dilution. 20 µL of each test substance (n=3) per well was added to a 96-well microtiter plate, U-bottom polystyrene (TPP Techno Plastic Products AG, Trasadingen, Switzerland). 100 µL of each bacteria suspension was added to the well with test substance, as well as 120 µL as growth control (n=35) and 120 µL medium (n=1) as sterile control to an empty well. To avoid evaporation, all microtiter plates were cultivated in air-tight containers during the incubation period. Microtiter plates for testing on SRB were prepared in the anaerobic chamber (Coy Laboratory Products Inc., Grass Lake, United States of America) and cultivated in air-tight containers including Oxoid anaerobic bag (Thermo Fisher Scientific Inc., Waltham, United States of America) to ensure anaerobic conditions. The microtiter plates were measured before and after incubation at OD600 InfiniteM200Pro plate reader (Tecan Group AG, Männedorf, Switzerland); for anaerobic bacteria, the bacterial suspension was measured n=3 and used as OD600 t=0. The growth inhibition in % was determined with the following equation 3.1:

$$inhibition[\%] = \frac{OD600_{test\,substance} - OD600_{sterile\,control}}{OD600_{growth\,control} - OD600_{sterile\,control}} * 100\,\% \tag{3.1}$$

The MIC describes the lowest concentration where no bacterial growth could be detected. Besides the MIC100, where a complete reduction of bacterial growth could be detected, other partial inhibitions such as MIC95 or MIC50, where 95 % or 50 % growth inhibition occurs, can be specified.

3.2.4. Cytotoxicity-assay

For the cytotoxicity-assay, TFA-free peptides were used where TFA was replaced with HCl. Therefore the peptides were solved in 0.01 mM HCl (c=1 mg/mL), frozen over night with followed lyophilization (3x). The whole process was repeated three-times with milliQ.

The cytotoxicity of the peptides was analyzed on human keratinocytes, HaCaT cells, (AddexBio, San Diego, United States of America) using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-assay. For this purpose, HaCaT cells were cultured in Dulbecco's Modified Eagle Medium (4.5 g/L glucose), supplemented with 200 mM L-glutamine solution and 100 mM sodium pyruvate solution and with 10 % fetal bovine serum in T75 flasks, polystyrene at 37 °C and 8.5 % CO_2. The medium was changed every two days by washing with 10 mL phosphate-buffered saline (PBS) with Ca^{2+}/Mg^{2+} and adding 15 mL of fresh culture medium. The

cells were passaged at a confluence of approximately 80 % by washing with 10 mL of PBS without Ca^{2+}/Mg^{2+}, adding 4 mL of trypsin/ethylenediaminetetraacetic acid, and incubating for 10 min. 12 mL culture medium was added to solution, the whole volume was transferred to a centrifuge tube and centrifugation for 5 min at 500 g was performed. The supernatant was discard, the cell pellet was resuspended in 10 mL cell medium and the cell count was determined using Neubauer-counting-chamber and trypan blue staining, 1:1 cell culture and trypan blue solution. The corresponding cell culture volume was inoculated into 15 mL culture medium for a new T75 bottle (350.000-550.000 cells/ml, depending on the incubation time) or in 12 ml culture medium (100 µL per well) for a 96-well microtiter plate, polystyrene (20.000 cells/ml) and 100 µL culture medium (without cells) were added as sterile control (n=2). The 96-well microtiter plate was incubated for 24 h. A stock solution of 666 µg/mL in culture medium from the respective peptide and a concentration range of 666 µg/mL-2.6 µg/mL was prepared by serial two-fold dilution. 100 µL of each test substance (n=3) per well was added as well as 100 µL culture medium (n=34) and the plate was incubated for 24 h again. Each well was washed twice time with PBS with Ca^{2+}/Mg^{2+}, 100 µL MTT-working solution (1:20 in culture medium of MTT-working solution: 5 mg/mL in PBS with Ca^{2+}/Mg^{2+}) per well was added under light exclusion and the plate was incubated for 1 h. 100 µL dimethyl sulfoxide was added on each well and the plate was incubated for 1 h, 100 rpm at 22 °C. Afterwards, the OD at 570 nm and at 650 nm was measured and the difference OD570-650 was used for the calculation of the cytotoxicity according to equation 3.2:

$$cytotoxicity[\%] = \frac{OD570\text{-}650_{test\,substance} - OD570\text{-}650_{negative\,control}}{OD570\text{-}650_{positive\,control} - OD570\text{-}650_{negative\,control}} * 100\,\% \quad (3.2)$$

The selective index of the cytotoxicity-assay (SIcyto.) was calculated by the quotient of inhibitory concentration (IC) at 50 % cytotoxicity and MIC95 (equation 3.3).

$$SI_{cyto.} = \frac{IC50}{MIC95} \quad (3.3)$$

The previous solution was pipetted out of the wells or T-flask in above mentioned steps before adding the next solution, unless explicitly the directly addition of the next solution was mentioned. All materials used for this purpose were supplied by Capricorn Scientific GmbH, Ebsdorfergrund, Germany, unless stated otherwise.

3.2.5. Hemolysis-assay

In addition to the cytotoxicity-assay, TFA-free peptides were also used for the determination of hemolysis. Defibrinated sheep blood (No. SR0051E by Thermo Fisher Scientific Inc. Waltham, United States of America) was rinsed with PBS by diluted 1:4 with followed centrifugation at 700 g for 8 min. The supernatant was discarded and the process was repeated three-times (the last centrifugation was at 1000 g for 8 min). Afterwards, a 0.5 % (v/v) suspension of sheep red blood cells (RBCs) was prepared with PBS. A stock solution of 1 mg/mL in PBS from the respective peptide and a concentration range of 1 mg/mL-15.63 µg/mL was prepared by serial two-fold dilution. 20 µL of each test substance (n=3) per well was added to a 96-well microtiter plate, U-bottom polystyrene (TPP Techno Plastic Products AG, Trasadingen, Switzerland), as well as 20 µL PBS as negative control (n=6) and 20 µL 1 % (v/v) Triton X-100 (Carl Roth GmbH & Co.KG, Karlsruhe, Germany) as positive control (n=6). 100 µL of the sheep RBC suspension was added in each well and the plate was incubated for 1 h at 37 °C. The plate was centrifuged at 1000 g for 10 min, 60 µL of the supernatant of each well was transferred into a new 96-well microtiter plate and was measured at 414 nm. The hemolysis in % was calculated by the following equation 3.4:

$$hemolysis[\%] = \frac{OD414_{test\,substance} - OD414_{negative\,control}}{OD414_{positive\,control} - OD414_{negative\,control}} * 100\,\% \tag{3.4}$$

The selective index of the hemolysis-assay (SIery.) was calculated by the quotient of hemolytic concentration (HC) at 50 % hemolysis and MIC95 (equation 3.5).

$$SI_{ery.} = \frac{HC50}{MIC95} \tag{3.5}$$

3.2.6. Stability-assay

For the stability tests, a bacteria suspension (seven-days old) of *D. vulgaris* was centrifuged at 4024 g for 10 min. 1 mg/mL peptide solution was prepared within the supernatant and incubated at 37 °C for 7 d. The respective aliquots (t=0, 48, 72, 168 h) were mixed 1:10 with quenching solution (milliQ + 0.08 % TFA) and analyzed in triplicates according to the protocol described above for RP-HPLC.

3.3. Results and discussion

The selection of AMPs focused on the membranolytic mode of action to address all the different species involved in biocorrosion. The selection criteria were explained in chapter 1.1.2. In addition to peptide selection from common databases, APD3 [15] and BaAMPs [71], peptides were also selected from literature, specifically the AMPs bactenecin and indolicidin, which showed activity against two SRBs in preliminary experiments [102]. In the following, only the four pre-selected peptides that showed the best effectiveness against the tested bacteria will be further discussed. For a complete list of selected AMPs, please refer to the appendix A.1.

3.3.1. Planktonic killing activity

All measured MIC values could be found in table 3.3.

Table 3.3.: MIC [µM] at 100 % (MIC100), 95 % (MIC95) or 50 % (MIC50) inhibition against two biofilm-forming bacteria (*E. coli* DSM 5698 (K12), *P. fluorescens* DSM 289), three SRBs (*D. vulgaris* DSM 644, *D. desulfuricans* DSM 642, *D. indonesiensis* DSM 15121 2 % NaCl), one SRB (*T. intermedia*) and three SFBs (*P. aeruginosa* DSM 1117, *P. aeruginosa* DSM 22644 (PAO1), *Pseudomonas aeruginosa* DSM 19882 (PA14)) for the four pre-selected peptides; n=3; adapted from [103] CC-by 4.0 and completed

peptide number	*E. coli*	*P. fluorescens*	*D. vulgaris*	*D. desulfuricans*	*D. indonesiensis*	*T. intermedia*	*P. aeruginosa*	*P. aeruginosa* PAO1	*P. aeruginosa* PA14
	MIC100	MIC100	MIC95	MIC50	MIC95	MIC50	MIC100	MIC100	MIC100
P1	4	4	15	>118	>118	59	4	7	30
P2	12	6	6	50	12	6	6	12	25
P3	26	6	13	52	52	13	13	6	52
P4	56	56	7	28	4	7	>112	n.d.	n.d.

A MIC100 of 4 µM against *E. coli, P. fluorescens*, and *P. aeruginos*a was detected for peptide P1, while higher concentrations were necessary to achieved the MIC against SRBs and SOB. P2 and P4 showed the lowest MIC against SRBs and SRB, especially P4 against *D. desulfuricans* (MIC50 of 28 µM) and *D. indonesiensis* (MIC95 of 4 µM). This peptide had the highest MIC100 above 112 µM against *P. aeruginosa.* A required concentration range between 6 µM (against *P. fluorescens* and *P. aeruginosa* PAO1) and 52 µM (against *D. desulfuricans* and *P. aeruginosa* PA14) was detected for peptide P3.

A membranolytic mode could be assumed for these four peptides due to their peptide characteristics. While a concrete mode of action is currently unknown for P1 [110, 111], P2 [112] and P3 [113], Barrel-Stave is assumed for linear P4, as is present here due to reduced conditions in the medium [39]. The high activity of these selected four AMPs compared to the other ones in table A.1 could be explain by their high amphiphilicity. The hydrophobic moment of these four AMPs is above 0.83 (calculated with HeliQuest [114]). This is consistent with the conclusion of Fernández-Vidal et al., who identified amphiphilicity as a crucial parameter for peptide structure. They analyzed peptides with the same amino acid composition and thus with the same hydrophobicity but different amphiphilicity. Thereby, a stronger helix formation was detected for peptides with a higher amphiphilicity, which is associated with an increased membrane effect, resulting that the amphiphilicity is more crucial for the peptide activity than the hydrophobicity [36]. Nevertheless, the results of the MIC tests indicate that the activity of AMPs is influenced by both the specific bacterial strain and the sequence's individual characteristics, as further studies have also shown [115].

3.3.2. Cytotoxicity and hemolysis

The results of the cytotoxicity and hemolysis experiments are listed in table 3.4. The selective index (SI) was calculated using only activity data against *D. vulgaris*, as this is a major model organisms for biocorrosion studies, and consequently peptide optimization were primarily performed regarding to this stain. A calculation via the geometric mean of all determined MIC values is listed in table A.2 in the appendix.

The SIcyto. was $\geq$1 for all tested peptides, which P4 standing out with a SIcyto. greater than 64. For the peptides P1 and P2 a SIcyto. of 8 was determined. For P3 a SIcyto. of 1 was determined. The SIery. was $\geq$8 for all tested peptides. The highest SIery. was determined for the peptide P4 with >64.

All tested peptides have a SI greater than or equal to 1, indicating they are non-toxic against skin cells (HaCaT) and RBC. However, the SI should be greater than or equal to 10 for potential drug candidate to ensure that no cell damage can be expected at the concentration required for bacterial killing [116]. This criteria could only be achieved by P4, which shows a high SI due to its structural properties with a low amphiphilicity of this cyclic peptide, comparable to a high SIery. of

Table 3.4.: MIC [µM] at 95 % (MIC95) for *D. vulgaris* DSM 644 (detailed MIC values could be found in table 3.3 and 4.4), IC [µM] at 50 % (IC50) against HaCaT cells with calculated SIcyto.=IC50/MIC95, HC [µM] at 50 % (HC50) against sheep RBC with calculated SIery.=HC50/MIC95 for the four selected AMPs; n=3

peptide number	MIC95	IC50	SIcyto.	HC50	SIery.
P1	15	118	8	>118	>8
P2	6	50	8	50	8
P3	13	13	1	104	>8
P4	7	>449	>64	>112	>16

54 reported in another study [117]. The lower SI of the peptides P1-P3 could be explained by their assumed helical structure with a high amphiphilicity above 0.82 and a hydrophobicity above 0.53, calculated with HeliQuest [114], which leads to an immense increase in cytotoxicity due to a good incorporation of the hydrophobic part of the peptide into the lipid bilayer of mammalian cells [118]. P3 has the highest hydrophobicity of 0.74 and P2 has the highest amphiphilicity of 0.95 of these peptides (calculated with HeliQuest [114]), explaining their lowest SIcyto. or SIery..

3.3.3. Stability tests in the supernatant of *D. vulgaris*

The results of this stability studies are shown in figure 3.2.

Thereby, the peptides P1, P2, and P3 demonstrated only a low degradation, and even after an incubation time of 168 h, a peptide content of more than 80 % was still present for P1 and P2. P4 had a significant stability loss after only 48 h (figure 3.2). No peptide loss could be detected in stability studies in medium (Postgate C), except for P4 (see A.1).

The peptides P1, P2, and P3 can be considered as stable, and long-term applications with these peptides could be envisaged, especially as these peptides also exhibit nearly consistent stability in the medium. In comparison to the other peptides, P4 is the only peptide that contains cysteines. These two cysteines can be oxidized to form a disulfide bridge, which stabilizes the peptide structure [119]. However, since *D. vulgaris* is cultivated in reduced medium, the cysteines are present in their reduced form with thiol groups. Since stability was not performed under anaerobic conditions, atmospheric oxygen may cause oxidation of the cysteines over time. Thus, the seeming loss of stability could also be caused by a change in the peptide conformation. This was consistent with the stability experiments of P4 in the medium, where it also resulted in peptide loss (figure A.1).

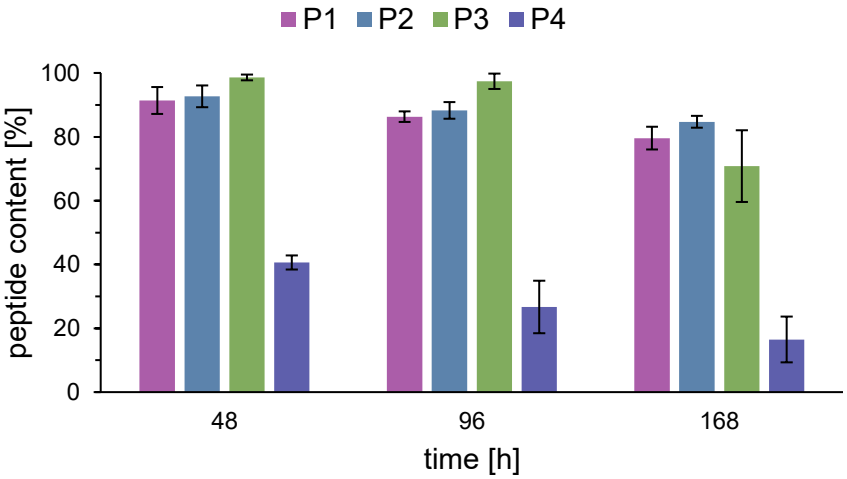

Figure 3.2.: Stability in the supernatant of a seven-day-old culture of *D. vulgaris* of the four favorites peptides P1 (pink), P2 (blue), P3 (green) and P4 (purple) over 168 h, analyzed with RP-HPLC, 100 % peptide content corresponds with the peptide content at 0 h; values shown as means with standard deviation by error propagation, n=3; adapted from [103] CC-by 4.0

3.4. Conclusion

The current strategy to control microbiologically influenced corrosion is based on biocides, which lead to fast resistance, requiring higher and higher concentrations to kill the bacteria. Alternative treatment strategies are based on additives like D-amino acids or chelates to biocides to reduce the required biocide concentration by a synergistic effect. However, these additives are not inhibitory on their own, and although a reduced biocide concentration is required, the presence of biocide is still necessary [104, 106, 107]. In this chapter, a new method for the treatment of biocorrosion is described: the application of AMPs. It could be shown that peptide sequences from APD3 and BaAMPs databases were selected based on certain characteristics and successfully applied against biocorrosion bacteria with a MIC mostly less than 20 µM against these selected biofilm-forming bacteria. However, there exists some limitation in the peptide effect. Membranolytic AMPs have lower effectiveness in saline environments due to the interaction of salt ions with the charged peptide. This could be observed for the activity of the peptides against the marine SRB strain *D. indonesiensis*, where the MIC was too high to be established as a new strategy and is therefore not competitive to the existing biocide treatment. Further optimizations are necessary to improve the activity of the peptides, especially for marine bacteria stains. Cytotoxicity and hemolysis experiments show that the peptide effect is only limited to the bacterial activity and damage of the environment could be avoided. Stability studies showed that the peptides remained stable in the supernatant of *D. vulgaris* and thus long-term applications of the peptides are possible.

Even though this chapter presents a novel approach to combating biocorrosion bacteria, these experiments represents the initial research and multiple investigations are still needed to establish AMPs as a protection method against biocorrosion. This includes further experiments such as the determination of the antibiofilm activity of these AMPs (chapter 5). Additionally, the further procedure for the successful establishment of AMPs as a biocide alternative is discussed in chapter 7. Nevertheless, this chapter demonstrates an alternative treatment in form of AMPs against biocorrosion bacteria using SRB, SOB and SFB, as well as other biofilm forming bacteria.

4. Improvement of AMP activity through sequence optimization and combination

This chapter is part of the following publication: adapted from L. Stillger, L. Viau, L. Kamm, et al. “Optimization of antimicrobial peptides for the application against biocorrosive bacteria”. In: Applied Microbiology and Biotechnology 107 (2023), pp. 4041–4049. doi: 10.1007/s00253-023- 12562-9. [103], CC-by 4.0

4.1. Introduction

High potential drugs are necessary for developing and establishing new candidates for biologically treatments in medicine, pharmaceuticals and industry to achieve a maximum effect with a minimum of concentration. AMPs have recently gained immense importance as a new treatment option [10]. Therefore, two approaches have been established to increase the activity of AMPs: The first approach is the optimization of the peptide sequence. By modifying the peptide sequence through the incorporation of different amino acids and altering the peptide ends, the peptides can be changed simple and fast via SPPS. Two modifications have been developed to increase mainly the activity - the amidation of the N-terminus and the attachment of specific molecules like fatty acid, saccharide, and polyethylene glycol to the C-terminus. Li et al. [120] compared these different modifications, lipidation, glycosylation, and addition of polyethylene glycol, at peptide SAMP-A4. Thereby, lipidation seems to be the best C-terminal modification with an improved planktonic and biofilm inhibitory activity as well as an improved stability. Additionally, amidation and lipidation of the AMP K12 leads to a significant increase in activity against ESKAPE organisms, especially *S. aureus* [121]. An increase could also be achieved for two short cationic peptides A1 and B1 by lipidation with a lipid length between eight and fourteen carbon atoms as well as by various glycosylations including glucose, mannose, and galactose [122]. The second approach for an increase AMP activity is the combination of different drugs to reduce the required drug concentration, resulting additionally in a reduced toxicity and a prolonged time in resistance formation. There are different combination strategies: i) AMP + antibiotic or biocide, ii) AMP + AMP, and iii) AMP + biological molecules

[118]. So far, most studies have focused on the first strategy, such as the combination of peptide CRAMP with vancomycin, of which some combinations should achieve a strong synergy with a FIC below <0.1 [123–125]. Additional experiments have already been done with the biocide THPS and a cyclic peptide, halving the required biocide concentration [105]. In principle, it is also possible to combine two AMPs, for example a synergy against *P. aeruginosa* can be detected with gramicidin S and polymyxin B (FIC=0.375), which can be explained by targeting different cell membrane structures [126].

This chapter includes both approaches - the optimization of the peptide sequence through alanine-scan of peptide P1, following by amidation and lipidation of the peptides P1 and P2 and MIC determination against SRBs as well as combinations of two AMPs against planktonic *D. vulgaris*.

4.2. Material & methods

The stains and cultivation parameters as well as the procedure of MIC determination is described in chapter 3.2.3 and 3.2.3.1.

4.2.1. Alanine-scan and peptide optimization

For the alanine-scan, each amino acid of P1 was individually replaced with alanine. The peptides obtained were termed P1-A1 to P1-A11. In addition, both position 4 and 6 in P1 were replaced with the amino acid alanine (P1-A4I6) as a double alanine scan and with the amino acid tryptophan (P1-W4I6). The C-terminus was amidated and the N-terminus was modified by the addition of lipids with different lengths (see table 4.1). The synthesis of the peptides is described in the chapters 3.2.2. Thereby, the modified peptides were similarly synthesized to the normal peptides: For the coupling of lipids, the normal coupling protocol of amino acids was applied (fmoc-6-aminohexan acid and fmoc-8-aminooctane acid), but without final deprotection for not fmoc-protected lipids (C2, C10, C12, C14). Rink-amid resin was used instead of preloaded resin for the amidation of the C-terminus.

Table 4.1.: Modified peptides of P1 with sequence after alanine-scan (modification colored in blue) and after terminus-modification (modifications colored in red), * amidated lipid

peptide number	sequence
P1	KKLLKWLKKLL
modification of amino acid composition after alanine-scan	
P1-W4I6	KKLWKWLKKLL
modification of the C-terminus	
P1-W4I6-1.1	KKLWKWLKKLL-NH_2
modification of the N- and C-terminus	
P1-W4I6-2.1	C2-KKLWKWLKKLL-NH_2
P1-W4I6-2.2	C6-KKLWKWLKKLL-NH_2*
P1-W4I6-2.3	C8-KKLWKWLKKLL-NH_2*
P1-W4I6-2.4	C10-KKLWKWLKKLL-NH_2
P1-W4I6-2.5	C12-KKLWKWLKKLL-NH_2
P1-W4I6-2.6	C14-KKLWKWLKKLL-NH_2

Identically to P1, the N- and C-terminus of the peptide P2 was modified. The individual sequences could be found in the table 4.2.

Table 4.2.: Modified peptides of P2 with sequence after terminus-modification (modifications colored in red), * amidated lipid

peptide number	sequence
P2	FKKFWKWFRRF
modification of the C-terminus	
P2-1.1	FKKFWKWFRRF-NH_2
modification of the N- and C-terminus	
P2-2.1	C2-FKKFWKWFRRF-NH_2
P2-2.2	C6-FKKFWKWFRRF-NH_2*
P2-2.3	C8-FKKFWKWFRRF-NH_2
P2-2.4	C10-FKKFWKWFRRF-NH_2
P2-2.5	C12-FKKFWKWFRRF-NH_2

The modified peptides were analyzed via MIC assay and the peptides P1-W4I6, P1-W4I6-1.1 and P1-W4I6-2.2 were also analyzed via cytotoxicity-assay (description see 3.2.4) and hemolysis-assay (description see 3.2.5).

4.2.2. Stability-assay with MIC determination

The stability study of the modified peptides was identically to the stability study in the chapter before, see 3.2.6 for a detailed description. In addition, the peptide was analyzed for activity using the MIC assay after incubation in bacterial supernatant. For this purpose, the peptide (1 mg/mL) was first incubated in the supernatant for 168 h, wherefrom the dilution series for the MIC assay was subsequently prepared.

4.2.3. Synergy-assay

The synergy-assay was based on the MIC-assay from chapter 3.2.3.1. Eleven dilution steps of AMP 1 were added horizontally (figure 4.1 purple) and seven dilution steps of AMP 2 were added vertically (figure 4.1 green) in a 96-well microtiter plate (10 µL per well). Each combination experiment with the respective concentration range of each peptide is listed in table 4.3.

Table 4.3.: Overview about combination experiments with concentration range of each peptide

number	AMP 1	conc. range of AMP 1 [µg/mL]	AMP 2	conc. range of AMP 2 [µg/mL]
A	P2-2.3	1000-3.91	P1-W4I6-2.2	1000-15.63
B	P1-W4I6-2.2	1000-3.91	P3	1000-15.63
C	P2-2.3	1000-3.91	P3	1000-15.63
D	P1-W4I6-2.2	500-1.95	P5	250-3.91
E	P2-2.3	500-1.95	P5	250-3.91

Row H contained only AMP 1, while column 12 contained only AMP 2, allowing for individual MIC determination. 120 µL medium in well H12 served as sterile control. Each plate was repeated three times (n=3). The wells of the non-grown

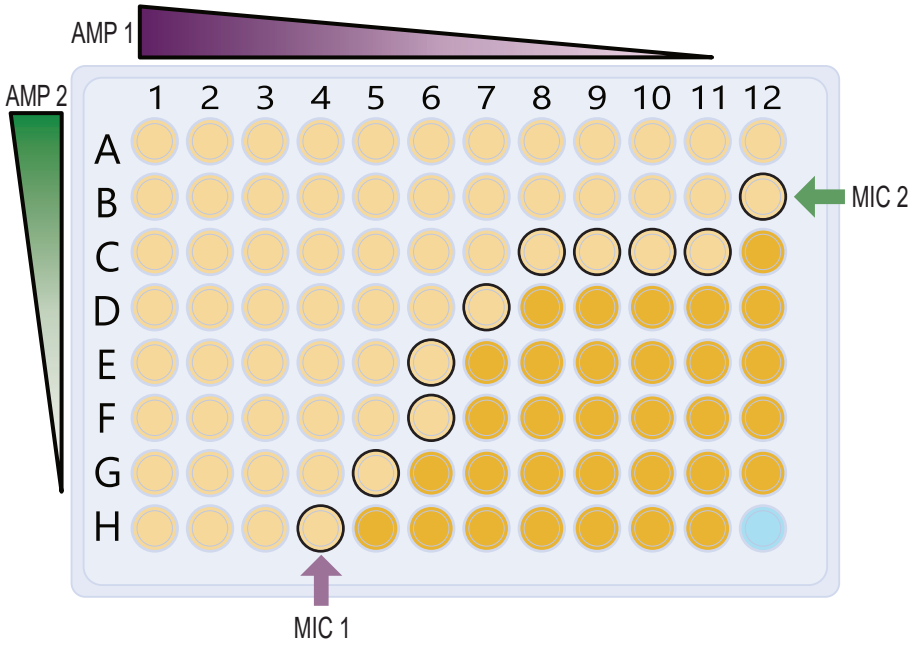

Figure 4.1.: Overview of the synergy study of two AMPs for MIC development in a 96-well microtiter plate with wells with bacterial growth (dark orange) and without bacterial growth (light orange), marked individual MIC for AMP 1 (purple) and AMP 2 (green), sterile control (blue), and wells used for FIC determination (black circle)

bacteria culture, which are located directly at the interface to wells with grown bacteria culture (figure 4.1 black circle), were used to determine the FIC index. The FIC index was calculated by the addition of the respective individual FIC of two substances A and B, which is calculated by the quotient of the MIC in combination and the MIC alone (equal 4.1).

$$FIC = FIC_A + FIC_B = \frac{MIC_{Acombination}}{MIC_{Aalone}} + \frac{MIC_{Bcombination}}{MIC_{Balone}} \tag{4.1}$$

Depending on the value of the FIC index, this leads to the following interpretations:

- FIC index $\leq$0.5: synergy
- FIC index 0.5-1: weak synergy
- FIC index 1-4: additive
- FIC index >4: antagonism

4.3. Results & discussion

Since SRBs are the initiator of the sulfur cycle, the main focus is on these bacteria, especially on *D. vulgaris*, since this bacteria represents a standard bacterium for the investigation of biocorrosion [127].

4.3.1. Optimization of the peptide L5K5W

The peptides P1 and P2 were selected for further optimization based on their good activity against all of the different biofilm-forming bacteria (chapter 3.3), their high SI against HaCaT and RBC (chapter 3.4) and high stability (chapter 3.3.3), as well as the simplicity of their sequence with a minimum number of different amino acids and simple amino acids to reduce synthesis costs.

The helical structure of the peptide P1 is shown in figure 4.2 A as well as the MIC values for the individual peptides of the alanine-scan in figure 4.2 B.

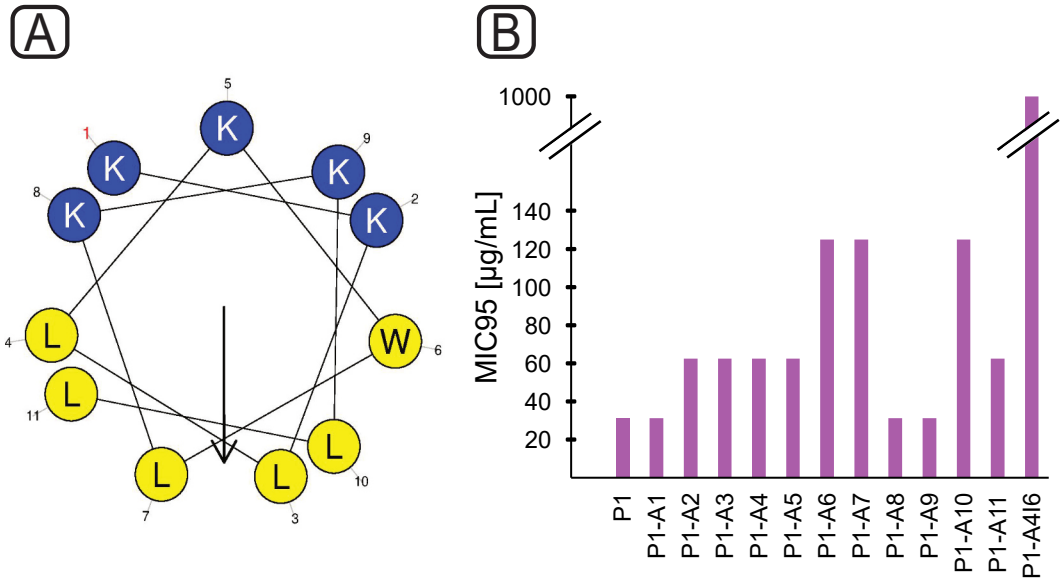

Figure 4.2.: (A) Helical structure of P1, the arrow symbolizes the hydrophobic moment, simulated with HeliQuest [114]; (B) MIC95 [µg/mL] of the original peptide P1, peptides of the alanine-scan P1-A1 to -A11 and peptide P1-A4I6 of the double alanine-scan (pink), tested against *E. coli* DSM 5698 (K12); adapted from [103] CC-by 4.0

Replacement of the amino acid (figure 4.2 B) at positions 6, 7 and 10 resulted in an increase in the required inhibitory concentration from 31.25 µg/mL to 125 µg/mL. The exchange at positions 2 - 5 and 11 caused a reduction of the activity up to a MIC95 of 62.5 µg/mL, while the exchange at positions 1, 8, 9 did not lead to any

loss of activity. A double alanine-scan was performed (P1-A4I6) where the amino acid alanine was inserted at both positions 4 and 6. This resulted in an immense loss of activity (MIC95: 1000 µg/mL).

The exchange at positions 6, 7 and 10 was classified as critical by the alanine-scan. Assuming an alpha-helix structure of P1, positions 7 and 10 are in the hydrophobic range (figure 4.2 A) and a substitution significantly reduces the hydrophobicity and the hydrophobic moment of the peptide and thus the incorporation of the peptide into the bacterial membrane. Tryptophan (position 6) is described to be crucial as a amphiphilic amino acid for anchoring the peptide in the bacterial membrane, enabling interactions of especially its residues with both hydrophobic and hydrophilic components of the membrane and thus stabilize the amphiphilic properties of peptide helices [38, 128]. That is the reason why tryptophan has a decisive role especially at the interface between hydrophobic and hydrophilic regions [110]. The alanine-scan carried out here proves this, as a significant loss of activity can be detected when position 6 was replaced. To confirm the role of tryptophan at the amphiphilic interface, a double alanine-scan was performed (P1-A4I6) where the amino acid alanine was inserted at both positions 4 and 6, resulting in an immense loss of bacterial activity.

Since SRBs are considered to initiate biocorrosion through their reduction of sulfur compounds, the following experiments will henceforward only focus on these bacteria. As a result of the alanine-scan, tryptophan was determined as crucial at the amphiphilic interface in alpha-helical peptides, resulting in a further addition of tryptophan at the other interface position, the amino acid number 4 (P1-W4I6). In addition, modification of the N- and C-terminus was performed. The results of these optimizations are listed in table 4.4.

More than a two-fold reduction of MIC95 against *D. vulgaris* was detected for the optimized peptide with the additional insertion of tryptophan at position 4 (P1-W4I6). Amidation of the C-terminus of the peptide (P1-W4I6-1.1) resulted in a MIC95 of 4 µM against *D. vulgaris*, MIC95 of 28 µM against *D. desulfuricans* and MIC95 of 14 µM against *D. indonesiensis.* By modifying the C- and N-terminus, a MIC of 2 µM against *D. vulgaris* was achieved, which means a almost eight-fold increase in activity of the original sequence. The enhancement of this modification was more crucial for the other SRBs, where the lowest MIC of 6 µM (*D. desulfuricans*) and 3 µM (*D. indonesiensis*), respectively, was achieved while no antibacterial effect could be detected with the original sequence. The lowest MIC against *D. vulgaris* was detected by P1-W4I6-2.2 and P1-W4I6-2.3, whereas longer lipids resulted again in higher MIC values. The lowest MIC was measured for P1-W4I6-2.5 against *D. desulfuricans* and P1-W4I6-2.4 and P1-W4I6-2.5 against *D. indonesiensis.*

In contrast to P1-A4I6 in figure 4.2, the double insertion of tryptophan at position 4 and 6 (P1-W4I6) reduced the required concentration to 7 µM against *D. vulgaris.* This further confirms the enormous influence of tryptophan at this interface and provides the basis for the further optimization of the peptide termini. Amidation

Table 4.4.: MIC [µM] at 95 % (MIC95) or 50 % (MIC50) inhibition against three SRBs (*D. vulgaris* DSM 644, *D. desulfuricans* DSM 642, *D. indonesiensis* DSM 15121 2 % NaCl) for the modified peptides of P1; adapted from [103] CC-by 4.0

peptide number	***D. vulgaris***	***D. desulfuricans***	***D. indonesiensis***
	MIC95	**MIC50**	**MIC95**
P1	15	>118	>118
P1-W4I6	7	> 118	>118
P1-W4I6-1.1	4	28	14
P1-W4I6-2.1	3	55	14
P1-W4I6-2.2	2	26	13
P1-W4I6-2.3	2	26	6
P1-W4I6-2.4	3	13	3
P1-W4I6-2.5	6	6	3
P1-W4I6-2.6	12	12	6

of the C-terminus of the peptide (P1-W4I6-1.1) results in an addition of a positive charge, leading to enhanced electrostatic interaction between cationic peptide and anionic bacterial membrane, resulting in an improved binding to the bacterial membrane, which is reflected in the enhanced activity. Furthermore, coupling of lipids of different lengths (C2, C6, C8, C10, C12 and C14) to the N-terminus results in a significant increase in hydrophobicity, allowing the peptide to be better incorporated into the hydrophobic bacterial lipid bilayer [129]. This is shown by a significant reduction of the MIC (table 4.4), especially against the marine bacterium *D. indonesiensis*, which can be attributed to the increased salt tolerance of the peptides due to the huge increase in hydrophobicity through lipidation, resulting in increased hydrophobic forces [130]. Since peptides with different lipid lengths demonstrated the best activity for each of the three SRBs, it was shown that not only the lipid length is crucial for lipidation, but also the peptide sequence and the bacterium. Grimsey et al. confirmed this with respective modifications of two peptides on two gram-positive, *S. aureus*, *E. faecalis*, and three gram-negative bacteria, *S. typhimurium*, *E. coli*, *P. aeruginosa* [122]. But nevertheless, increasing the lipid length beyond the optimum point results in an decreased activity again. This can be explained by a higher self-assembly rate in aqueous solution resulting by the increase in hydrophobicity. Similar results have been reported in other studies, indicating that a lipid length of eight carbon atoms provides the greatest increase in activity, while longer lipids decrease the activity again [121].

In addition to the MIC determination, the modified version P1-W4I6, P1-W4I6-1.1 and P1-W4I6-2.2 were used in the cytotoxicity-assay and hemolysis-assay, see table 4.5.

Table 4.5.: MIC [µM] at 95 % (MIC95) for *D. vulgaris* DSM 644 (detailed MIC values could be found in table 3.3 and 4.4), IC [µM] at 50 % (IC50) against HaCaT cells with calculated SIcyto.=IC50/MIC95, HC [µM] at 50 % (HC50) against sheep RBC with calculated SIery.=HC50/MIC95 for the modified versions of peptide P1

peptide number	MIC95	IC50	SIcyto.	HC50	SIery.
P1	15	118	8	>118	>8
P1-W4I6	7	28	4	56	8
P1-W4I6-1.1	4	14	4	28	7
P1-W4I6-2.2	2	7	4	13	7

The SIcyto. of the modified peptides was halved (SIcyto. of 4) in comparison to the original peptide P1 (SIcyto. of 8). While a SIery. of above 8 was detected for P1, the SIery. was decreased for P1-W4I6 to 8 and for P1-W4I6-1.1 and P1-W4I6-2.2 to 7. MIC95, IC50 and HC50 decreased with increasing optimization.

Despite the decrease in IC50 and HC50, all modifications resulted in a SI greater than 1 against HaCaT and RBC due to the simultaneous increase in MIC95, enabling the respective peptides to be classified as non-toxic. The change of the amino acid leucine by tryptophan at position four of P1 leads to an increase in hydrophobicity up to 0.58 and in amphiphilicity up to 0.88 (calculated with HeliQuest [114]), resulting in a better incorporation into the lipid bilayer with its hydrophobic part, which is shown in a decrease of IC50 and HC50. Since the cytotoxicity is more influenced than the bacterial activity by this change, the SIs reduce. A decrease in IC50 and HC50 can be observed with increasing degree of modification: P1-W4I6 > P1-W4I6-1.1 > P1-W4I6-2.2. The lowest IC50 and HC50 for P1-W4I6-2.2 could be explained by the immense increase in hydrophobicity due to lipidation and thus the highly incorporation of this peptide into the lipid bilayer. Since activity and cytotoxicity are changed by the same amount, the SI of these three modified peptides remains almost unchanged. For this modification by lipidation to maintain a SI greater than 1, a significant increase in activity is required, as demonstrated by the peptide presented here. Studies show that this is the case for a lipid length up to eight carbon atoms, while the cytotoxicity increases significantly from ten carbon atoms onward with a partially decreasing activity [121], as can also be seen with P1 in table 4.4, which leads to toxic AMPs (SI<1).

In addition to the stability study of P1 (figure 3.2), the modified peptides P1-W4I6 and P1-W4I6-2.2 were also tested for stability in the supernatant of *D. vulgaris*

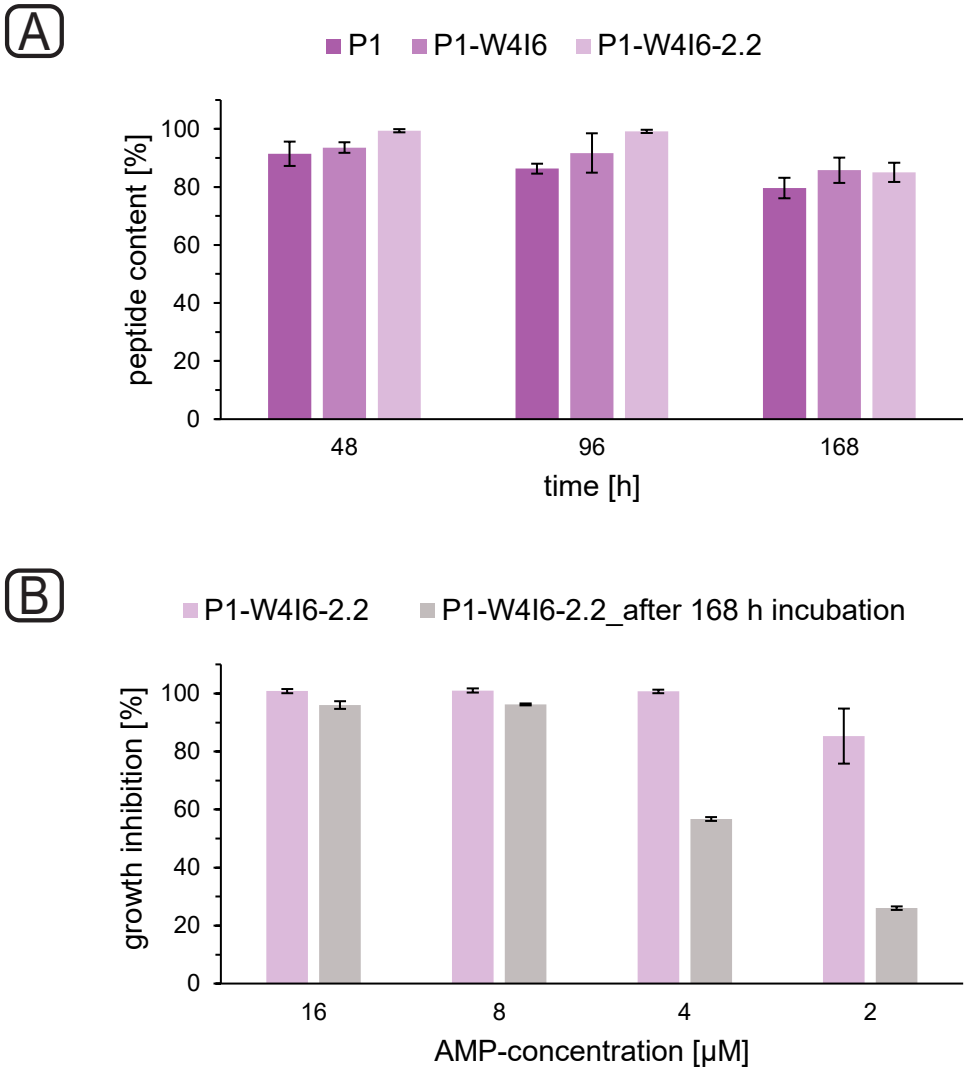

Figure 4.3.: (A) Stability in the supernatant of a seven-day-old culture of *D. vulgaris* of P1 (pink) and its modified version P1-W4I6 (light pink) and P1-W4I6-2.2 (ultra light pink), analyzed with RP-HPLC, 100 % peptide content corresponds with the peptide content at 0 h; (B) growth inhibition of *D. vulgaris* [%] of P1-W4I6-2.2 (ultra light pink) and after incubation for 168 h in the supernatant (ultra light gray); values shown as means with standard deviation n=3; adapted from [103] CC-by 4.0

(figure 4.3 A) and in cultivation medium Postgate C (figure A.2), as well as P1-WI6-2.2 for activity after incubation (figure 4.3 B).

A complete maintenance of stability for up to 96 h and afterwards only a low decrease to 85 % peptide content was detected for both optimized peptides P1-W4I6 (figure 4.3 A light pink) and P1-W4I6-2.2 (figure 4.3 A ultra light pink). P1-W4I6-2.2 showed for the direct MIC determination even at low concentrations (up to 2 µM) a growth inhibition up to 95 % (figure 4.3 B ultra light pink). The growth inhibition for the peptide after 168 h incubation (figure 4.3 B ultra light gray) was only around 30 % at 2 µM and around 50 % at 4 µM. A concentration of 8 µM was required to achieve a 95 % growth inhibition.

The optimization of the selected peptide P1 resulted in an increased stability, which could be explained by a better protection against proteases. In particular, due to lipidation of the N-terminus and amidation at the C-terminus of the optimized peptide P1-W4I6-2.2, the peptide ends are protected against exopeptidases, thus preventing degradation of the peptide by them. The activity of P1-W4I6-2.2 was four-times lower after the incubation compared to the activity before incubation. However, the decrease in peptide content after 168 h was only 15 %. This should result in higher activity, leading to the assumption that peptide molecules are still present but no longer active. The reduced activity may also be explained by interactions of peptide fragments with the original peptide. To achieve a more precise evaluation of the remaining peptide activity, it would be useful to fractionate the active peptide and test only this fraction instead of the entire peptide solution of the stability test. Nevertheless, the high stability and the retention of activity of the peptides seems really promising for long-term application.

4.3.2. Optimization of the peptide S6L3-33

Since peptide P2 already contains tryptophan at both interface positions between hydrophilic and hydrophobic regions (figure A.3), amidation of the C-terminus and lipidation of the N-terminus was carried out directly (table 4.6)

Amidation of the peptide (P2-1.1) resulted in an increased effectiveness against *D. indonesiensis*. Lipidation of the N-terminus (P2-2.1 to P2-2.5) resulted in a four-fold increase of activity against *D. desulfuricans* and an eight-fold increase of activity against *D. indonesiensis*. As a result of the lipidation, a MIC of 6 µM was detected against the marine strain *D. indonesiensis* despite 3.5 % NaCl. The activity of the initial peptide P2 exhibited a significant decrease of activity at a NaCl content of 3.5 % in comparison to 2 % NaCl, as shown in table 3.3. No improvement in the activity against *D. vulgaris* was measured between the original peptide and the modified peptides.

The MIC values of peptide P2 demonstrate a decreased activity at higher salt concentrations, consistent with the results of Olli et al. who reported a loss of activity with increasing NaCl concentration [131]. This is due to the shielding of the peptide

Table 4.6.: MIC [µM] at 95 % (MIC95) or 50 % (MIC50) inhibition against three SRBs (*D. vulgaris* DSM 644, *D. desulfuricans* DSM 642, *D. indonesiensis* DSM 15121 3.5 % NaCl) for the modified peptides of P2

peptide number	***D. vulgaris***	***D. desulfuricans***	***D. indonesiensis***
	MIC95	**MIC50**	**MIC95**
P2	6	50	50
P2-1.1	6	50	25
P2-2.1	12	97	49
P2-2.2	6	47	47
P2-2.3	6	12	6
P2-2.4	6	23	6
P2-2.5	6	22	6

charge by salt ions, resulting in the prevention of electrostatic interaction between the charged AMP and the bacterial membrane [130, 132]. Lipidation has proven to be a suitable tool against the activity loss due to an increased hydrophobicity [130], which is also represented in the results here, especially despite a high salt concentration. In agreement with the results of the optimization of peptide P1 (chapter 4.3.1), a lipid length of six and eight carbon atoms is also considered optimal for the optimization of this AMP. However, the activity depends on the sequence as well as on the bacterium [115]. This indicates that various optimizations, particularly lipid length, provide the best MIC depending on the bacterium. In addition, no increase in activity against *D. vulgaris* can be achieved in the modification of peptide P2 discussed here, although the modification results in an optimization against the two other SRBs and an optimization of peptide P1 against *D. vulgaris* can also be achieved with these modifications.

4.3.3. Combination of two AMPs

Additionally to the optimization of the peptide sequence, the combination of different drugs like two AMPs could be another point for improving the AMP activity. These results could be found in figure 4.4. Thereby, the best optimized versions of peptide P1 and P2 were used to minimize the required concentration.

The combination of P1-W4I6-2.2 and P2-2.3 (figure 4.4 A) as well as P2-2.3 and P3 (figure 4.4 C) resulted in a lowest FIC of 1, while the other points indicated FIC indices up to 1.5. The FIC values for the combination P1-W4I6-2.2 and P3 (figure 4.4 B) were slightly above 1, exactly 1 and even slightly below 1 at 0.75. For the combination P1-W4I6-2.2 (figure 4.4 D) and P2-2.3 (figure 4.4 E) with P5,

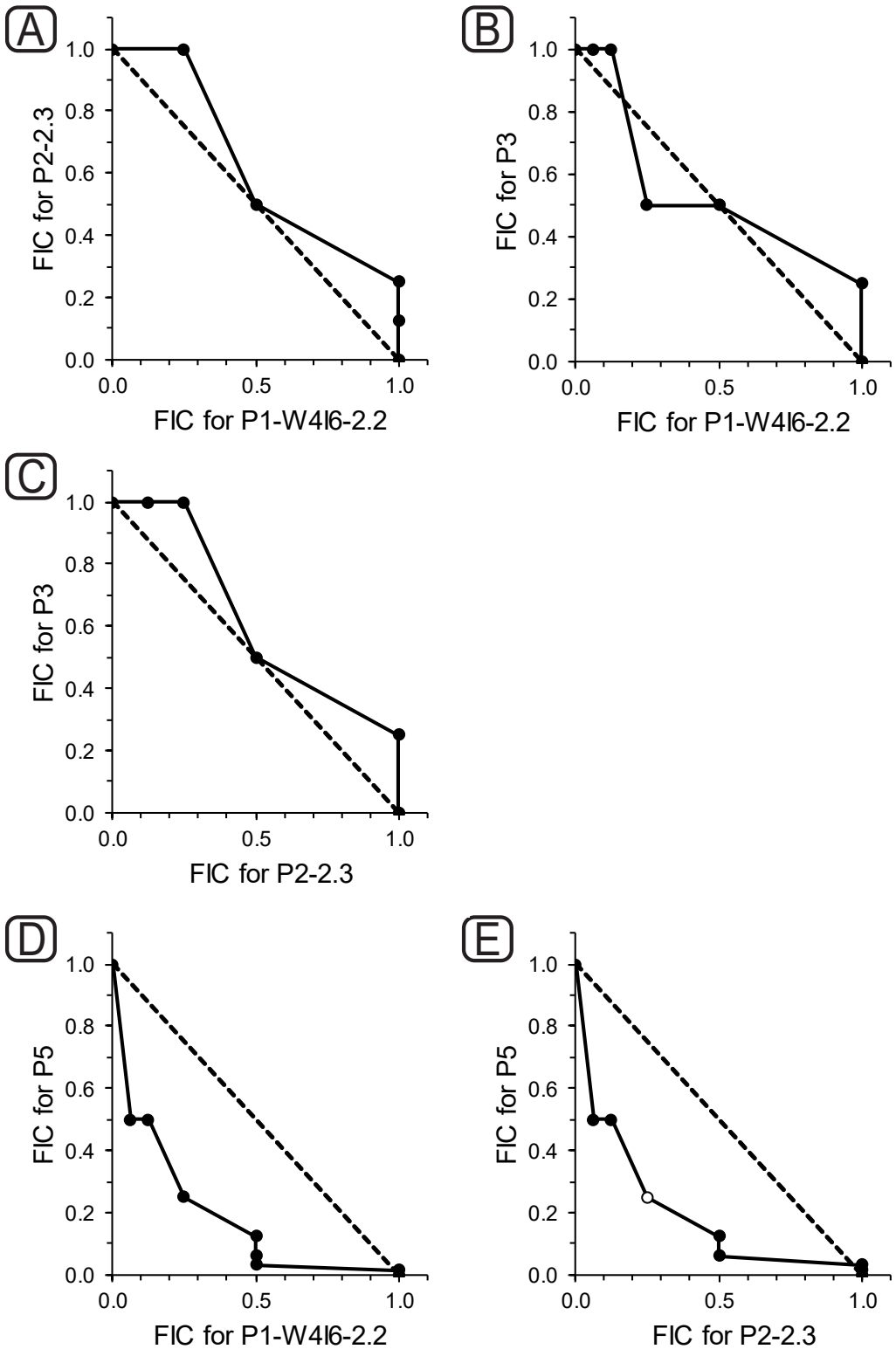

Figure 4.4.: Individual FIC for MIC95 (black dots) and MIC80 (white dot) determination against *D. vulgaris* DSM 644 for the combination of two AMPs: (A) P2-2.3 and P1-W4I6-2.2, (B) P1-W4I6-2.2 and P3, (C) P2-2.3 and P3, (D) P1-W4I6-2.2 or (E) P2-2.3 and P5, dotted line indicates synergy limit; values calculated by the mean of three independent runs n=3

all FIC values were below 1 with the lowest FIC at 0.5 for both combinations; for combination E, only 80 % instead of 95 % growth inhibition in wells was measured for determined FIC of 0.5 (figure 4.4 gray dot).

Figure 4.4 demonstrates that the combinations A-C are not successful in enhancing activity, but do not lead to antagonism either, so the required concentration of the combined dose is almost identical to the single dose. Due to the peptide characteristics such as charge, hydrophobicity and amphiphilicity, a membranolytic mode of action can be assumed for these peptides. The combination of two drugs with identical mode of action seems to be not promising due to the identical target. Different targets are useful for activity improvement as experiments with gramicidin S and polymyxin B have already shown, where the first AMP interact mainly with the inner membrane while the target of the second AMP is the lipid A and core region of LPS [126]. Since the peptides were selected based on their potential membranolytic effect, further combination experiments would not be beneficial. Only peptide P5 (indolicidin) could show both membranolytic and intracellular activity, why this peptide was choose for further combination. At high concentrations, this cationic AMP permeabilizes the cell membrane and leads to filamentation, while at low concentrations it inhibits DNA synthesis by binding to DNA in *E. coli*, and addresses further intracellular targets like binding to proteins, as well as other biosynthesis pathways [20, 133]. For the combinations D and E, a significant reduction of the required concentration (FIC<1) was observed compared to the combinations A-C. This suggests a intracellular activity of peptide P5 in *D. vulgaris* within the concentration range used here. This combination leads to an improved membrane permeabilization through the membranolytic AMP P1-W4I6-2.2 or P2-2.3 and consequently to an improved uptake of AMP P5 into the cell, resulting in an enhanced activity of it. The combination of different mechanisms of action to increase the bacterial effect is thus crucial. However, since no further intracellular AMPs are currently known against *D. vulgaris*, additional combination experiments are not promising at this time.

4.4. Conclusion

Two classical methods for the improvement of AMP activity were used here: optimization of the peptide sequence, in particular by modification of the peptide termini and combination of two AMPs. Changes of the amino acid composition and the position within the sequence as well as modifications of the C- and N-terminus resulted in a significant increase in activity. Thereby, the insertion of the amino acid tryptophan at both amphiphilic interface positions, amidation of the N-terminus and lipidation of the C-terminus with a optimal length of six or eight carbon atoms was determined as the best for increasing bacterial activity, resulting in a finally MIC of 2 µM for the optimized P1 peptide against *D. vulgaris*. The activity improvement was particularly obvious for the marine bacterium *D. indonesiensis* at high salinity, as lipidation leads to an increase in hydrophobic forces and thus activity is maintained despite interfering salt ions. Additionally to the enhanced activity, an increase in stability was detected due to the protection of the peptides against proteolytic digestion. Thus, the optimization of the peptide sequence can be considered as very effective. Nevertheless, an increase in bacterial activity leads also to an increase killing amount against other cells like HaCaT and RBC. Although activity and toxicity increased in the same manner, a lower SI was avoided. Experiments of the combination of two AMPs were less successful than the optimization of the sequence, because combining two membranolytic AMPs did not lead to an improved activity due to their identical target. However, the usage of an intracellular AMP, in this case P5, showed the potential of combination effects of different AMP targets to increase the activity with only a half of the required concentrations compared to single dose (FIC=0.5). Since no other intracellular AMPs against *D. vulgaris* are currently known, further research is necessary to follow up synergy tests with membranolytic and intracellular AMPs.

5. Antibiofilm-assay for AMPs against biocorrosive bacteria

This chapter is part of the following publication: adapted from L. Stillger, L. Viau, D. Holtmann, et al. "Antibiofilm assay for antimicrobial peptides combating the sulfate–reducing bacteria *Desulfovibrio vulgaris*". In: MicrobiologyOpen 12.4, e1376 (2023). doi: 10.1002/mbo3.1376. [134], CC-by 4.0

5.1. Introduction

The majority of microorganisms live as aggregates forming a biofilm [7]. Thereby, biofouling describes the phenomenon of undesirable biofilms [135]. Here, it can lead to numerous disruptions of processes, either in the medical field and human health due to the colonization of implants (usually titanium) or medical devices (usually stainless steel) through numerous multi-resistant bacteria like ESKAPE pathogens and their associated potential for infection [136], or in industrial plants such as water circuits or the oil and gas industry [135]. In industrial processes, biocorrosion bacteria have an important role in attacking metals through various mechanisms [87]. For more details see chapter 1.3. The problem in combating biofilms is that in contrast to the planktonic mode, sessile cells are better protected through their production of EPS, which act as a diffusion barrier against extrinsic substances [137]. Additionally, the so-called persister cells, which are characterized by resting cell metabolism, are immune to antibiotics [138]. These problems result in a higher required biocide concentration compared to combating suspension bacteria [139]. Due to high resistance and high cytotoxicity [8, 9], the application of biocides is limited, and the development of new antimicrobial and antibiofilm candidates is crucial. In recent years, AMPs have emerged as a new treatment strategy [140]. In addition to killing cells directly, AMPs can act on any state of biofilm formation (see figure 1.3).

Analysis of the complete biofilm is not possible due to its complex composition. Nevertheless, various analytical methods (colorimetric, microscopic, etc.) and different assays (BioFilm Ring Test, flow chamber, etc.) are available to characterize the different biofilm aspects [141]. A limitation of these methods is often insufficient throughput, which is important for the development of new compounds. To overcome this, the calgary biofilm device (CBD), a 96-well biofilm assay, has been

developed [142]. The optimization of this assay, with the use of microcentrifuge tubes as growth surfaces in the respective wells, ensures flexibility to the respective conditions, as well as high throughput at the same time. Additionally, this method is more economical than the CBD and can be implemented with common laboratory equipment [143]. It can be used to determine the MBIC and MBEC.

This chapter thematized the establishment of this antibiofilm-assay to determine the MBIC and MBEC on biocorrosive bacteria and quantify the activity of AMPs in a high-throughput method. Therefore, the AMPs are tested against the aerobic biocorrosive bacteria *P. aeruginosa* as a representative of SFB. For the first time, the application of AMPs against a representative of anaerobic SRB, *D. vulgaris*, for inhibition and eradication of biofilm is presented.

5.2. Material & methods

The synthesis of the peptides as well as the strains and cultivation conditions is described in the chapters 3.2.2 and 3.2.3.

5.2.1. Biofilm growth on different surfaces

Microcentrifuge tubes (Eppendorf SE, Hamburg, Germany) served as growth surface for *P. aeruginosa* and were autoclaved for sterilization before use. Steel rivets DIN660 4x15 mm (Würth-Gruppe, Künzelsau, Germany) were employed as growth surface for *D. vulgaris*. These were applied in the following pretreated stages: i) mechanically untreated ii) roughened: two turns with silicon carbide sandpaper with a grit size of 180 iii) polished: two turns with silicon carbide sandpaper with a sequential grit size of 320, 400, 600, 800, 1200. The steel rivets were then cleaned in acetone, 2-propanol, and ethanol (Carl Roth GmbH & Co.KG, Karlsruhe, Germany) for 5 min each using an ultrasonic bath, frequency 47 KHz (Branson Ultrasonic Corporation, Danbury, United States of America). The process was repeated a second time before sterilization using a UV lamp for 1 h. After 30 min, the rivets were rotated to ensure uniform exposure. 120 µL bacterial culture per well (*P. aeruginosa*: n=3, *D. vulgaris*: n=80 for microcentrifuge tubes and n=12 for each steel rivet) and 120 µL medium per well as sterile control (*P. aeruginosa*: n=1, *D. vulgaris*: n=8 for microcentrifuge tubes and n=4 for each steel rivet) was added in a 96-microtiter plate (polystyrene). Pegs were removed after 24 h (*P. aeruginosa*) or 72 h (*D. vulgaris*) and the biofilm formation was characterized by crystal violet staining: For crystal violet staining, the pegs were removed and washed with 0.9 % NaCl. Subsequently, the biofilm was fixed using 99 % methanol (incubation 5 min) and dried for 60 min. Subsequently, staining was performed using 0.5 % crystal violet (incubation 30 min), washing three-times with water, and drying for 120 min. De-staining was performed with ethanol/acetone (80/20) for 20 min and detection at 570 nm. The respective steps were realized by transferring the pegs into a well plate with the appropriate solution (140 µL per well).

5.2.2. Minimum biofilm inhibitory concentration-assay

120 µL bacterial culture per well was added as growth check (n=14), 100 µL bacterial culture per well for AMP test (n=3 per AMP concentration), and 120 µL medium per well as sterile control (n=4) to a 96-microtiter plate (polystyrene). Subsequently, each well was fitted with a peg. Pre-incubation for 2 h (*P. aeruginosa*) or 6 h (*D. vulgaris*) was done. Afterwards, 20 µL of AMP solution (sequential two-fold dilution of 1 mg/mL - 1.95 µg/mL) was added to the corresponding well. After a further incubation time of 22 h (*P. aeruginosa*) or 66 h (*D. vulgaris*), bacterial growth

was measured by OD600 and the biofilm formation was characterized by crystal violet staining (OD570), for description of crystal violet staining see 5.2.1. Except for the crystal violet staining, the assay was carried out for *D. vulgaris* in an anaerobic chamber (Coy Laboratory Products Inc., Grass Lake, United States of America). Anaerobic cultivation was realized through air-tight containers including an Oxoid anaerobic bag (Thermo Fisher Scientific Inc., Waltham, United States of America) to ensure anaerobic conditions. Routine measurements of OD600 and OD570 were performed using the InfiniteM200Pro plate reader (Tecan Group AG, Männedorf, Switzerland).

An overview of the individual steps of the MBIC assay is shown in figure 5.1.

For the long-term inhibition of *D. vulgaris* biofilm, 120 µL bacterial culture per well was added as growth check (n=14), 120 µL bacterial culture per well without rivets (n=2), 100 µL bacterial culture per well for AMP test (n=3 per AMP concentration) + 20 µL peptide solution after 6 h of pre-incubation, 120 µL medium per well as sterile control (n=7), and 120 µL medium per well without rivets (n=1) to a 96-microtiter plate (polystyrene). The OD600 was measured in the anaerobic chamber of the whole plate with a Stratus microplate reader (Cerillo Inc., Charlottesville, United States of America). This enables the determination of long-term MBIC effects via OD600. For the OD570 determination, three samples as well as two biofilm growth checks and a sterile control were taken at each time point and were analyzed (description of crystal violet straining in chapter 5.2.1).

5.2.3. Minimum biofilm eradication concentration-assay

The preparation of the MBEC assay was similar to the MBIC. 120 µL bacterial suspension was added per well as growth check (n=8) or as AMP test (n=6 per AMP concentration) and 120 µL medium as sterile control (n=8). The microtiter plate was incubated for 24 h (*P. aeruginosa*) or 72 h (*D. vulgaris*). Afterward, the pegs were transferred to the challenge plate consisting of 120 µL AMP solution (1 mg/mL - 1.95 µg/mL), 120 µL medium for growth check (n=8), 120 µL medium (n=4) or 120 µL water (n=2) or 120 µL AMP with a concentration of 1 mg/mL (n=2) for sterile control. Incubation was performed for 24 h. The pegs were then washed with 0.9 % NaCl and half of them was stained with crystal violet (description in chapter 5.2.1). The other half was transferred to the recovery plate containing 120 µL medium per well and placed in the ultrasonic bath for 30 min. The pegs were removed and the microtiter plate was incubated for 24 h (*P. aeruginosa*) or 48 h (*D. vulgaris*). After the incubation period OD600 was measured.

An overview of the individual steps of the MBEC assay is shown in figure 5.1.

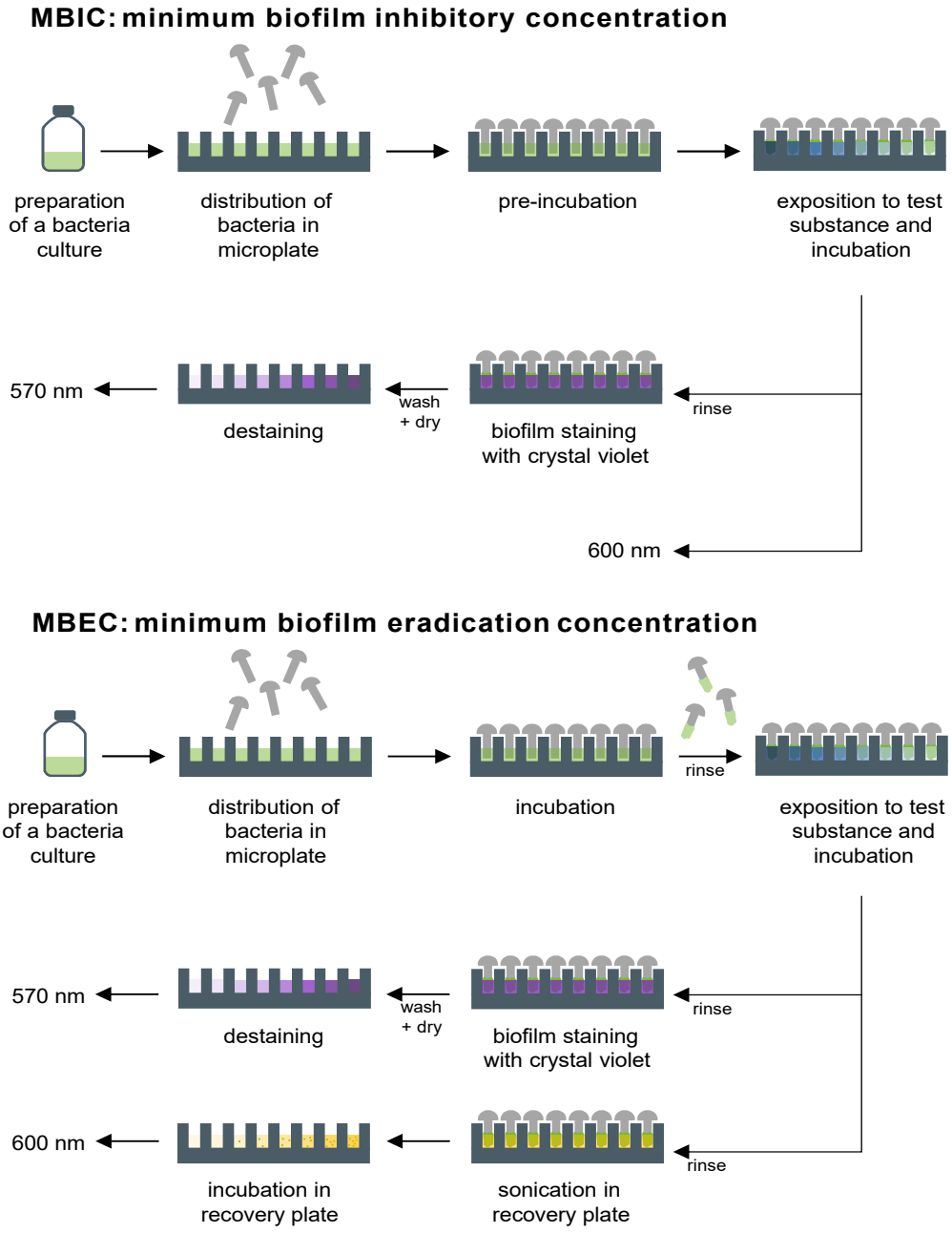

Figure 5.1.: Overview of the individual steps of MBIC and MBEC-assay; adapted from [134] CC-by 4.0

5.3. Results & discussion

5.3.1. Adaptation of antibiofilm-assay to biocorrosive bacteria

For the development of the antibiofilm-assay to the biocorrosive bacteria *P. aeruginosa* (SFB) and *D. vulgaris* (SRB), the biofilm was first analyzed on different growth surfaces. The results are presented in table 5.1 and in figure 5.2.

Table 5.1.: Biofilm of *P. aeruginosa* DSM 1117 after 24 h and *D. vulgaris* DSM 644 after 72 h on different growth surfaces: microcentrifuge tubes, rivets: mechanically untreated, roughened: silicon carbide sandpaper (grit size: 180), polished: silicon carbide sandpaper (grit size: 320, 400, 600, 800, 1200); staining by crystal violet and measurement OD 570 nm; determination of mean and standard deviation by error propagation; adapted from [134] CC-by 4.0

	micro-centrifuge tube	**steel rivets**		
		mechanically untreated	**roughened**	**polished**
P. aeruginosa	1.39 ± 0.23	n.d.	n.d.	n.d.
D. vulgaris	0.24 ± 0.12	0.59 ± 0.23	0.95 ± 0.14	1.30 ± 0.21

Based on table 5.1, the OD570 of the biofilm of *P. aeruginosa* reached a value of 1.39 with a standard deviation of ± 0.23 after 24 h on microcentrifuge tubes. The amount of *D. vulgaris* biofilm on microcentrifuge tubes indicated a low concentration of biomass with a high standard deviation of 50 %. When steel rivets were used, the OD570 increased significantly. Additionally, OD570 increased with increasing pretreatment – OD570 of the polished rivets (iii) higher than roughened rivets (ii) higher than mechanically untreated rivets (i). The polished steel rivets showed the highest concentration of biofilm with an OD570 of 1.30 simultaneously with a low variation.

For the polished rivets, a constant biofilm over the entire contact surface between the rivet and the bacterial suspension was observed (figure 5.2 B). This was in distinct contrast to the untreated rivets, where the biofilm was mainly confined to the edge (figure 5.2 A).

The selection of growth medium and temperature was based on the optimal conditions for planktonic growth of these bacteria. The incubation period for *P. aeruginosa* biofilms was oriented to the growth curve of Alayande et al., where a huge biofilm matrix could be detected after 24 h incubation. The results presented here demonstrate a high total biofilm mass on microcentrifuge tubes after 24 h, which is comparable to the findings of the previously mentioned study that used microtiter plates as growth surface [144]. In addition to transferring the assay to anaerobic conditions for

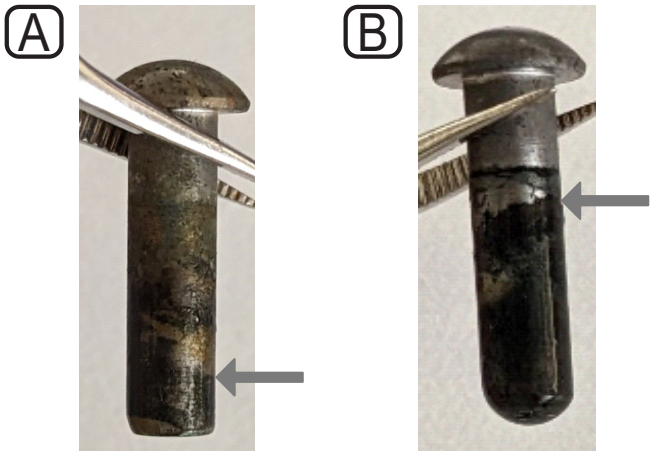

Figure 5.2.: (A) Mechanically untreated and (B) polished rivets with *D. vulgaris* biofilm after 72 h incubation, gray lines indicate the height of the biofilm; adapted from [134] CC-by 4.0

D. vulgaris, the incubation periods were also adjusted. This adjustment was oriented to the growth curve of Zhang et al., where the mean exponential phase of sessile growth was be reached after an incubation period of 72 h [145]. A modification of the materials adhesion surface is necessary due to very low and heterogeneous biofilm formation on previously used polypropylene tubes (see table 5.1). A large number of different materials have been described in the literature for the colonization of SRB biofilms, which are mostly based on metals such as steel, titanium, and aluminum [98, 146]. For further assay development, steel rivets were used. This offers realistic material conditions for the usage of the biocorrosive SRB. The mechanical pretreatment of the rivets was based on Andrade et al. [147]. Here, the increase of the surface area by treatment with silicon carbide sandpaper leads to an increased roughness, which would explain the improved biofilm accumulation, as seen in table 5.1 for an increased OD570 with pretreatment as well as in figure 5.2 for visual control, where the biofilm is colored black through the production of iron(II) sulfide. Comparable studies were also performed with mechanically pretreated metals [98, 146].

5.3.2. Biofilm inhibition

The peptides that were tested here exhibited good effectiveness against planktonic bacteria (see 3.1) and were therefore used for further studies against biofilm presented in this chapter. The optimized form of P1-W4I6 was used instead of the original peptide P1, although the lipidated and amidated optimizations were avoided due to better comparability of the individual four peptides. Nevertheless, the best version of the peptides P1 and P2 were also tested with this antibiofilm-assay.

Table 5.2.: MBIC [µM] at 90 % (MBIC90) biofilm inhibition for one SRB (*D. vulgaris* DSM 644) and one SFB (*P. aeruginosa* DSM 1117) for the four preselected peptide, analyzed of total biofilm matrix via crystal violet staining of the pegs (OD570); n=3; adapted from [134] CC-by 4.0 and completed

peptide number	*D. vulgaris*		*P. aeruginosa*	
	MBIC90 OD600	**MBIC90 OD570**	**MBIC90 OD600**	**MBIC90 OD570**
P1-W4I6	>112	>112	112	112
P1-W4I6-2.2	>103	>103	n.d.	n.d.
P2	99	99	>99	>99
P2-2.3	46	46	n.d.	n.d.
P3	26	26	n.d.	n.d.
P4	>112	>112	n.d.	n.d.

The peptides P1-W4I6 and P4 showed no biofilm inhibitory effects against *D. vulgaris*. The modified version of P1-W4I6 (P1-W4I6-2.2) had also no effectiveness against biofilm inhibition. The peptide P2 had a MBIC90 of 99 µM, whereas the modified version of it (P2-2.3) had a MBIC90 of 46 µM. The peptide P3 had the lowest minimum biofilm inhibitory concentration with a MBIC90 of 26 µM. The peptide P1 showed at 112 µM an inhibition of *P. aeruginosa* biofilm, whereas peptide P2 had no activity against the inhibition of *P. aeruginosa* biofilm (see 5.2).

Peptide P3 is the most effective one against *D. vulgaris*, requiring only the double MIC for biofilm inhibition. In further studies, this peptide was also able to inhibit an early biofilm stage of multi-resistant *S. aureus* [113] and consequently has great potential as a new drug for inhibiting biofilms. All tested peptides, especially P1 and P2 against *P. aeruginosa*, showed a significant loss of activity regarding to biofilm inhibition compared to their effectiveness against planktonic cells. This can be explained by the time gap of peptide addition in MBIC-assay compared to MIC determination, since biofilm inhibition is mainly focused on killing of planktonic bacteria [148]. The peptides were added directly at the beginning of the MIC-assay,

enabling the initial growth inhibition of planktonic bacteria while the peptides were added after a pre-incubation in the MBIC-assay, resulting in a higher bacterial amount and thus it is more difficult to kill all of these bacteria with the same peptide concentration. This effect was particularly observed in the experiments against *P. aeruginosa*, where both peptides had a low MIC of 4 µM and 6 µM (table 3.3), while their MBIC was more than 20-fold higher. *P. aeruginosa* expresses a large number of proteases that significantly reduce peptide lifetime [149]. In contrast to the MIC-assay, the protease concentration was higher in the MIC-assay, explaining the reduce peptide activity. The higher ability of the optimized peptides to kill planktonic bacteria is also reflected in an increased biofilm inhibition for peptide P2. Although the concentration range selected for P1 was probably too low to detect an increase in activity of the optimized version, difference between the original peptide P1 and the optimized peptide P1-W4I6-2.2 are expected with increasing peptide concentration. Additionally, the challenges of the beginning biofilm structure also prevent the peptide effect. In particular, the EPS matrix acts as a diffusion barrier, and therefore higher concentrations are often necessary to combat biofilms [137, 139]. Why the peptide P3 was the best to overcome these challenges is currently unexplored and more detailed analyses of biofilm mechanism are needed.

5.3.3. Long-term inhibition against *D. vulgaris*

Peptide P3 was selected for long-term study based on its best activity in the previous MBIC-assay. Therefore, both MBIC (26 µM) and double MBIC (56 µM) were used as peptide concentrations (figure 5.3).

For the uninhibited sample (black diamonds) and the growth check without rivet (black triangles), an increase in OD600 (figure 5.3 A) was initially detected within the first two days, remaining constant thereafter. At a peptide concentration of 26 µM (green dots), no bacterial growth was detected up to day 9. Subsequently, an increase in OD600 was measured and, from day 11 onward, remained almost constant until the end of the study. While no positive OD570 (figure 5.3 B) was measured until day 7, an increase in the OD570 was detected on day 10, which remained almost constant over the further period. It should be mentioned that both OD600 and OD570 of the inhibited samples did not reach the identical values of the uninhibited sample during the investigated period. Instead, they reached approximately half the values of the uninhibited sample. In contrast, no increase in OD600 was detected over the complete period investigated at a peptide concentration of 56 µM (dark green squares), see figure 5.3 A. This was also visible in the crystal violet staining, where no increase in OD570 was detected (figure 5.3 B).

Up to ten days, the bacterial growth and biofilm formation could be inhibited using MBIC (26 µM). Afterwards, the identical OD600 and OD570 could not be reached for inhibited and uninhibited samples. Instead, the uninhibited samples are double as high as the uninhibited samples, explaining by a partial efficacy of the AMP. The

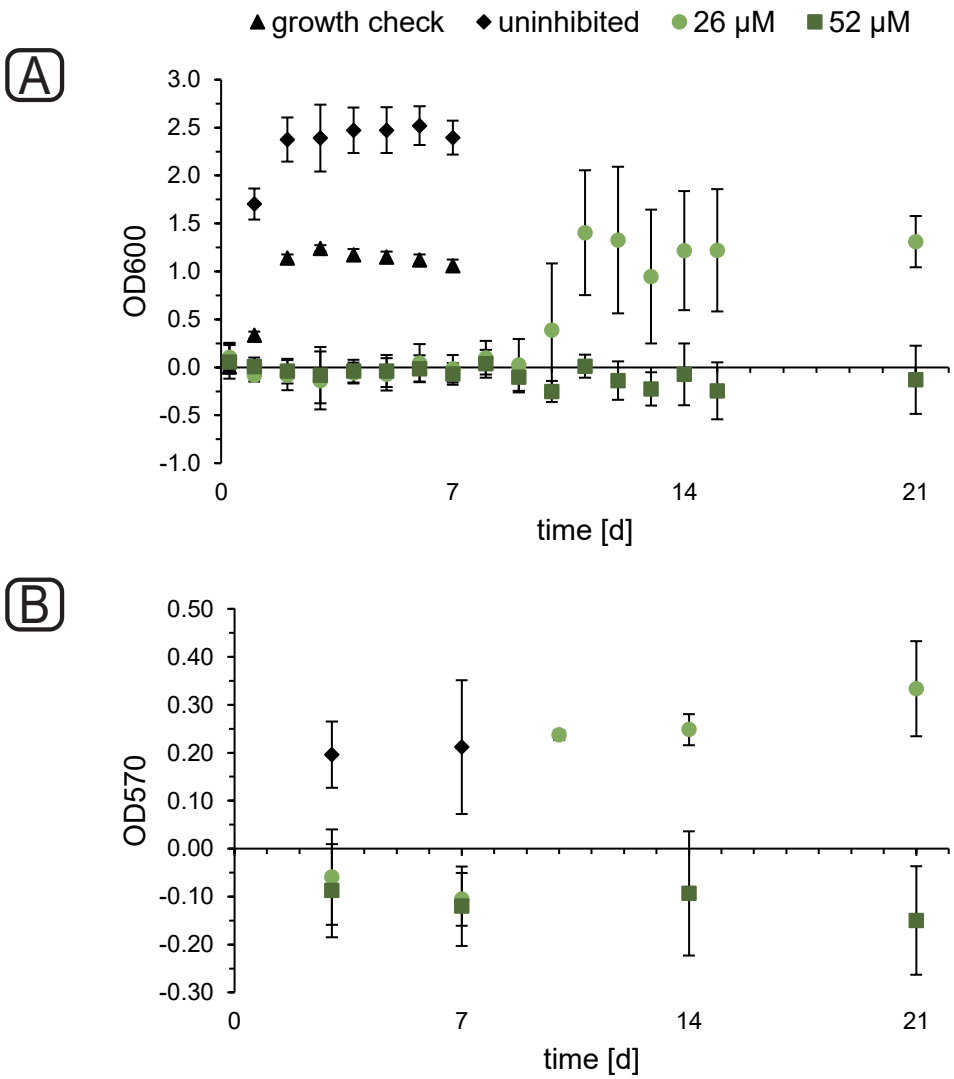

Figure 5.3.: Long-term analysis for the peptide P3 against the SRB *D. vulgaris* DSM 644: (A) OD600 and (B) OD570 over time (in days) for uninhibited bacteria culture (black diamonds), growth check without rivets (black triangles), and inhibited bacteria culture with a peptide concentration of 26 µM (green dots) and 52 µM (dark green squares); values are shown as means with standard deviation by error propagation; adapted from [134] CC-by 4.0

breakthrough at single MBIC compared to double MBIC could explain a long-term stability problem of the peptide. Since stability studies have been performed for seven days (see chapter 3.3.3), but not for a longer period, stability of this peptide was analyzed over 21 days. The results are shown in figure 5.4.

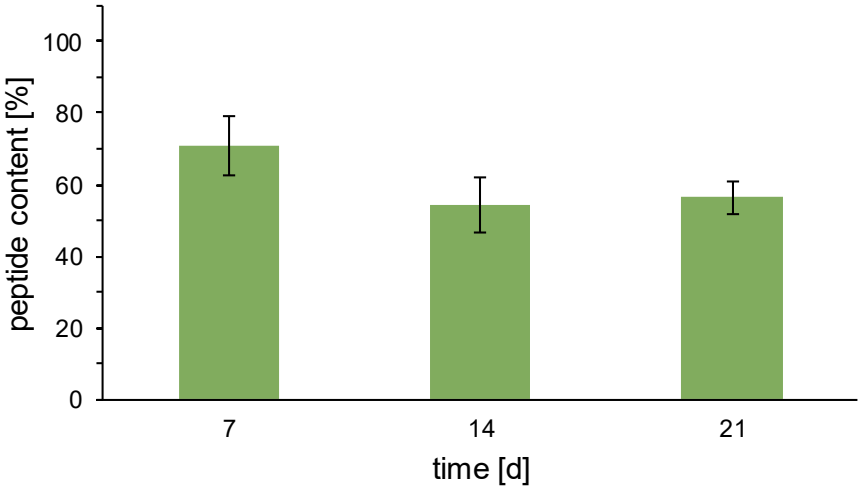

Figure 5.4.: Long-term stability for the peptide P3 in the supernatant of a seven-old culture of *D. vulgaris* DSM 644 (green) over 21 d, analyzed with RP-HPLC; 100 % peptide content corresponds with peptide content at 0 h; values are shown as means with standard deviation error propagation, n=; adapted from [134] CC-by 4.0

For the measurements in the culture supernatant (figure 5.4), only a peptide content of 70 % could be detected after 7 days. The decrease proceeded in the following days, resulting in a peptide content of only 56 % after 21 days. Peptide in the medium was almost stable until day 14 and showed a slightly decrease up to less than 80 % over the time, see figure A.5.

The stability data verifies the previous theory, where the peptide concentration was halved at day 10. The available concentration is thus only 50 % MBIC, which was found to be too low to inhibit the biofilm in the previous experiment. In contrast, the doubled concentration (56 µM) was effective over the entire period of 21 days. Halving the peptide concentration over time resulted in a concentration of around 26 µM, which is equivalent to MBIC and thus still effective. To achieve the longest possible biofilm inhibition, it is necessary to either apply a higher concentration than the real MBIC to buffer peptide degradation or to avoid peptide degradation by using D-amino acids, non-natural amino acids, or cyclization while maintaining peptide activity [150, 151]. However, a longer observation was not further possible with this setup due to starting evaporations. Instead, a long-term study above 21 days should be performed in a closed system.

5.3.4. Biofilm eradication

Similar to the MBIC-assay, the best four peptides as well as the best optimized version of the peptides P1-W4I6 and P2 were also tested for the determination of biofilm removal. The results are listed in table 5.3.

Table 5.3.: MBEC [µM] at 90 % (MBEC90) biofilm eradication for one SRB (*D. vulgaris* DSM 644) and one SFB (*P. aeruginosa* DSM 1117) for the four pre-selected peptide, analyzed of living bacteria within the biofilm via recovery plate (OD600) and of total biofilm matrix via crystal violet staining of the pegs (OD570); n=3; adapted from [134] CC-by 4.0 and completed

peptide number	*D. vulgaris*		*P. aeruginosa*	
	MBEC90 OD600	**MBEC90 OD570**	**MBEC90 OD600**	**MBEC90 OD570**
P1-W4I6	168	>674	>674	>674
P1-W4I6-2.2	615	>615	n.d.	n.d.
P2	75	>597	>597	>597
P2-2.3	555	>555	n.d.	n.d.
P3	311	>621	n.d.	n.d.
P4	336	336	n.d.	n.d.

P2 was detected as the most effective peptide in regard to OD600 with a MBEC90 of 75 µM against *D. vulgaris*. P1-W4I6 reached a MBEC90 of 168 µM and P3 reached a MBEC90 of 311 µM. P4 exhibited a 90 % reduction of OD600 up to 336 µM. For the removal of the total biofilm mass (OD570), only the peptide P4 was able to contribute (MBEC90 of 336 µM). The modified versions P1-W4I6-2.2 and P2-2.3 had a MBEC90 of 615 µM and 555 µM for OD600, while no activity could be detected against the total biofilm matrix. For the removal of the biofilm of *P. aeruginosa*, the tested peptides P1-W4I6 and P2 had no activity against both, OD600 and OD570.

In the MBEC-assay, all peptides showed killing activity against *D. vulgaris* based on the OD600 measurement of the recovery plate, indicating that all peptides were able to penetrate through the biofilm to the bacteria. But nevertheless, the EPS represents a high diffusion barrier and much higher concentrations are required to kill the bacteria in the biofilm than in MBIC. This is especially obvious for the optimized peptides P1-W4I6-2.2 and P2-2.3. In contrast to the MBIC, where the optimized peptides showed an increased activity (table 5.2), the optimized peptides exhibited reduced activity compared to the original peptides for biofilm removal. This could be explained by their larger molecular structure, particularly through the attachment of the lipid, which limits diffusion through the EPS. As a result, the peptide

concentration in the biofilm is reduced, explaining the decrease in activity. Additionally, EPS components, typically anionic eDNA, could bind to cationic AMPs, resulting in a loss of activity. Since the amount of eDNA in the EPS of *D. vulgaris* is below 5 µg/µL [152] and consists mostly of proteins (>40 µg/µL) [152, 153], this interaction can be disregarded and the main decrease of activity can be explained by the diffusion barrier of the biofilm. For the reduction of the total biomass, only the peptide P4 was successful. The remarkably strong antibiofilm activity of P4, specifically in eradicating total biofilm mass, may be related to its structure. P4 has a cyclic form with a hydrophobic ring and a hydrophilic tail in an oxidative milieu due to the presence of two cysteines [40]. This characteristic structure enables surfactant-like interactions. Thereby, surface-active peptides promote the degradation and detachment of biofilms by reducing surface tension [154, 155]. As in the MBIC-assay, an enhancement of peptide activity through the combination of two peptides is not possible due to the absence of eradication effects of the peptide P5, see figure A.4 C and D.

There was no activity of the tested peptides against the removal of *P. aeruginosa* biofilm (equation 3.1). The problem in combating *P. aeruginosa* is the production of seven extracellular proteases [149]. Thereby, aeruginolysin [51] and pseudolysin [50] represent the most challenging ones, as these preferentially cut before and after amino acids with hydrophobic residues. Since membranolytic AMPs are composed of approximately half of amino acid with hydrophobic residues, which are necessary for the integration of the AMP into the hydrophobic lipid bilayer of the bacterial membrane, their stability is limited in the presence of *P. aeruginosa*. The peptides were added directly to the diluted bacteria culture at the beginning of MIC determination, so a low protease concentration can be assumed, explaining the good activity values. For the biofilm assays, the pre-incubation and the delay in peptide addition suggests a higher protease concentration. This explains the significant decrease in activity compared to the planktonic activity data, especially for the MBEC results, where the peptides were added after the complete biofilm formation. In contrast to the EPS of *D. vulgaris*, eDNA is a major component of the biofilm matrix of *P. aeruginosa* [156], resulting not only in the reduction of AMP activity by proteolytic degradation but also by electrostatic binding to anionic eDNA. Therefore, it is crucial to combat *P. aeruginosa* early before biofilm formation could occur to enable AMP application also against these bacteria.

5.4. Conclusion

In this chapter, inhibition and eradication effects of AMPs were analyzed against *P. aeruginosa*, a representative of SFB and a member of the medical relevant ESKAPE organisms. Therefore, a modified CBD with microcentrifuge tubes as growth surface according to Reiferth et al. [143] was used for the determination of antibiofilm activity of peptides. However, treating *P. aeruginosa* biofilms is challenging because they express a large number of proteases [149] and eDNA [156], making AMP treatment more difficult. Additionally, the biofilm effectiveness for both, inhibition and eradication, against *D. vulgaris*, a representative of SRB was determined. For this purpose, all critical steps were performed in an aerobic-chamber to achieve anaerobic conditions and the growth surface was adapted to polished steel rivets. The measured MBIC was more than double so high than the MIC, which can be explained by the time gap between the start of the assay and the addition of peptide, resulting in an increased amount of planktonic bacteria and thus a more complicated killing. However, the biofilm could still be inhibited for up to 21 days, although stability problems due to peptide loss must be compensated in the required concentration. The concentration to kill bacteria in the biofilm was again higher due to the biofilm structure, especially the immense diffusion barrier by the EPS matrix, resulting in a more than ten-fold increase in MBEC for the best of the tested peptides. Additionally, MBEC experiments demonstrate that AMPs not only have killing activity against bacteria but also provide other biofilm-eradicating effects, as could be shown by the removal of the biofilm matrix by peptide P4 due to its surfactant-like cyclic structure.

Although an increase in the required concentration is necessary, biofilms of *P. aeruginosa* and *D. vulgaris* can be inhibited by the peptides used in this thesis and at the same time, these peptide can also kill bacteria in the biofilm and disturb the biofilm matrix. Therefore, these peptides represent potential candidates against planktonic bacteria and also for biofilm control. Thereby, different peptides should be preferred for different issues: P3 for biofilm inhibition, P2 for killing bacteria within the biofilm and P4 for eradication of the complete biofilm matrix.

6. Comparison of AMP development to current biocide treatment

6.1. Introduction

The current treatment for combating biocorrosive bacteria include different strategies like mechanically, chemically, physically, and biologically approaches. Each strategy is discussed in chapter 1.3.2. The most common method is the chemical treatment using non-oxidizing biocides, which seems to be the most effective one. However, the number of suitable biocides is limited since the following criteria should be achieved: Biocides should be able to kill a broad spectrum of different microorganisms like planktonic bacteria (i) as well as to inhibit biofilm formation and even to destroy the biofilm structure (ii). They should be biodegradable to avoid environmentally damage (iii). Additionally, they should have a simple and safe application and storage (iv), while also being cost-effective (v) [157]. Based on these selection criteria, two biocides, GA and THPS, are established in the oil and gas industry as well as in water systems [96]. However, these two biocides cannot achieve all criteria, as they are hardly biodegradable [157]. Nevertheless, their activity can be inactivated by condensation reactions of GA with amines or the oxidation of THPS to trishydroxymethyl phophine oxide [96, 158]. Biocides are also considered toxic and cause immense damage to humans and animals [157]. For this reason, current research is focusing on additives such as D-amino acids [104], chelate [106, 107], or herbal substances like D-limonene [108] to increase the effectiveness and consequently reduce the required biocide concentration. However, these additives only have an effect in combination and not alone, so biocide application is still necessary. The development of a new treatment method that completely eliminates the usage of biocides while achieving as many as possible of the above criteria is reasonable. Therefore, a new strategy by AMPs in combating biocorrosive bacteria is developed in this thesis.

The comparison of the two methods, AMP and biocide treatment, based on the selection criteria and resistance formation is discussed in this chapter, including the evaluation of AMP development.

6.2. Material & methods

6.2.1. MIC-assay and cytotoxicity-assay

The description of the cultivation of *D. vulgaris* can be found in chapter 3.2.3 as well as for the method of the MIC-assay in chapter 3.2.3.1 and of the cytotoxicity-assay in chapter 3.2.4. Therefore, the procedure was analogous to the testing of AMPs. GA and THPS (both Merck KGaA Darmstadt, Germany) were first diluted in milliQ to 1 mg/mL (1:700), and afterwards the serial 1:2 dilutions were prepared.

6.2.2. Planktonic resistance study

The planktonic resistance study was performed via serial transfer method of the adaptive laboratory evolution, where the MIC-assay was carried out 20 times in a serial manner. After each run, the OD600 was measured using Stratus microplate reader (Cerillo Inc., Charlottesville, United States of America) in the anaerobic chamber (Coy Laboratory Products Inc., Grass Lake, United States of America) and the MIC was determined. The bacterial suspension from the wells with 50 % MIC was used for the next subcultivation (1:100), which was used for the next run. After 20 runs, a normal cultivation (without drug) was performed, followed with a further MIC determination (21st run).

6.3. Results & discussion

6.3.1. Bacterial activity and toxicity

The results of the planktonic bacterial killing (MIC) as well as of the cytotoxicity (IC and SIcyto.) and of the hemolysis (HC and SIery.) are listed in the next table.

Table 6.1.: MIC [µM] at 95 % (MIC95) for *D. vulgaris* DSM 644 (detailed MIC values for the P1-P4 could be found in table 3.3), IC [µM] at 50 % (IC50) against HaCaT cells with calculated SIcyto.=IC50/MIC95, HC [µM] at 50 % (HC50) and 20 % (HC20) against sheep RBC with calculated SIery.=HC50/MIC95 for the four favorite peptides P1-P4 and two biocides GA and THPS

peptide number	MIC95	IC50	SIcyto.	HC50	SIery.
P1	15	118	8	>118	>8
P2	6	50	8	50	8
P3	13	13	1	104	>8
P4	7	>449	>64	>112	>16
GA	208	<26	<0.125	>1665	>8
THPS	51	6	0.118	>410	>8

A MIC95 against *D. vulgaris* of 208 µM was measured for GA and of 51 µM for THPS while all four peptides had a MIC95 below 15 µM. The SIcyto. was below 0.125 for GA and 0.118 for THPS. All peptides showed a SIcyto. $\geq$1. The SIery. was for all drugs, including the four peptides and the two biocides, $\geq$8. No HC50 values were achieved for the two biocides within the concentration range investigated here. Detailed information about the four peptides can be found in table 3.3 and 3.4.

While all peptides can be classified as non-toxic due to a SI greater than 1 against HaCaT and RBC, the biocides GA and THPS are considered highly toxic due to their very low SIcyto. This could be explained by difference in mode of action between these two drug classes. The mechanism of AMPs is initially based on the electrostatic interaction between the cationic AMPs and anionic bacterial membranes. In contrast, eukaryotes have a higher content of zwitterionic lipids like phosphatidylcholine in their cell membrane, resulting in significantly reduced electrostatic forces [159] and therefore the limitation of AMP effectiveness to bacterial killing. In contrast, both biocides act through non-specific mode of action that are not limited to certain species: GA through condensation reaction between aldehyde groups of GA with amine groups of all possible substances like proteins or nucleic acids, and THPS through different ways like the integration into the membrane as well as the inhibition of energy cycle or proton flux and the denaturation of proteins

[97]. A SIery. greater than 8 was determined for both biocides, suggesting these are non-toxic. Since a high toxicity was determined against HaCaT cells, a toxic effect against RBCs can also be expected. Despite lysis of RBCs, further reaction seems to be possible due to the unspecific mechanism of biocides. The biocide GA can be used in a wide concentration range, 0.001 %-8 % [160, 161], to fix cells within shortest time, typically <1 h, allowing for better shape analysis by microscopic methods due to their attachment to cell proteins. The fixation of RBCs prevents the deformability of the cells, which is important for the maintenance of their function, as RBCs must adapt their shape to appropriate flow conditions to minimize their destruction due to flow, as well as to thin blood capillaries to supply all areas of the body, allowing the transport of O_2 and CO_2 [161, 162]. Since the concentrations used in this assay are within the above-mentioned fixation range, fixation of RBCs can be assumed, indication an activity-limiting effect of GA on erythrocytes despite the results of the hemolysis-assay. No comparative data on the effect of THPS on RBC could be found, but due to its non-specific mode of action, similar further reactions can be assumed, whereby RBCs are damages, but without the release of hemoglobin.

6.3.2. Planktonic resistance

In figure 6.1, the results of the planktonic resistance study are shown.

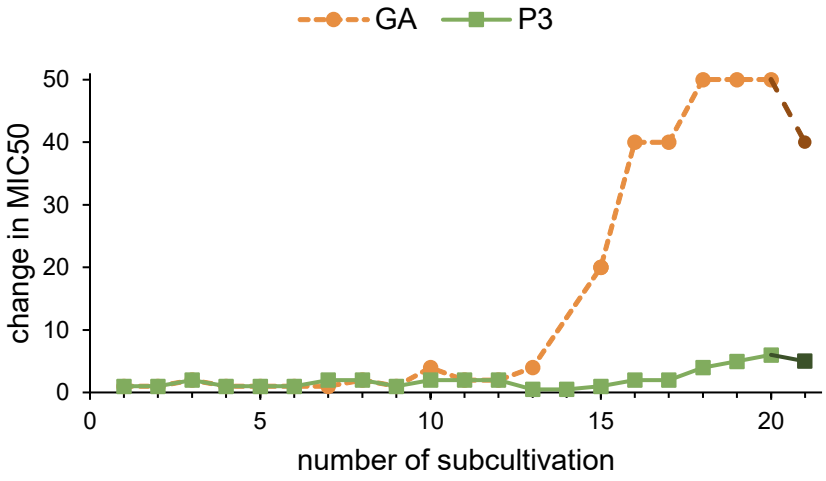

Figure 6.1.: Change in MIC at 50 % (MIC50) inhibition against *D. vulgaris* DSM 644 over 20 subcultivations for the biocide GA (orange dots, dotted line) and the AMP P3 (green squares, solid line), run 21 (dark color): cultivation without drug after 20 subcultivation with following MIC-testing; determined MIC of the first run represents the change in MIC50 value 1

Up to subcultivation 12, only minor changes in the MIC50 were observed for both

drugs. From the 13th subcultivation onward, an increase in MIC50 was detected for GA, resulting in a 50-fold increase compared to the initial run (figure 6.1: orange dots). In contrast, an increase in MIC50 of P3 was only detected from 18 subcultures, and after 20 subcultivations the MIC50 was six-fold higher than the initial MIC50 (figure 6.1: green squares). After a normal subcultivation, run 21 (figure 6.1: dark orange and dark green), a reduction of the required concentration to 40-fold (GA) and five-fold (P3), was detected.

The formation of resistance to AMP was almost ten-times lower after 20 subcultivations compared to GA. This can be explained by the interaction of AMPs with the bacterial membrane, as previously mentioned. A modification of the bacterial membrane, such as a change in the membrane charge, a change in the ratio of the different phospholipids, a change in the lipid arrangement or an increase in the membrane thickness by increasing the lipid length [159], represents an immense intervention in the bacterial morphology. Hence, this is much more difficult to enforce, but not completely impossible. Other measures, such as expressing proteases, encapsulation of the drug, or activating of efflux pumps to remove unwanted substances, are simpler and faster [42]. Antibiotics and biocides interact with specific points, such as GA with amino groups, making it easier to protect against drug attack. In the case of GA, overexpression of the enzyme aldehyde reductase was detected through upregulation of the yghD gene, allowing *E. coli* to survive even in the presence of GA [99]. The faster resistance development for GA can also be explained by the higher concentration required (table 6.1), resulting in contact with a larger number of biocide molecules and consequently initiate countermeasures more rapidly. In both cases (GA and AMP), resistance development is only partially reversible (run 21), and a significant amount of resistance remains. A genome analysis would provide detailed information on the change within the bacteria. This study here only assessed the change in MIC over the investigated period and did not analyze the impact on growth rate or biofilm formation. Further studies could explore these aspects. Furthermore, drug adaptation could be evaluated under flow conditions, e.g., by using a chemostat or a chip under realistic conditions such as those found in a pipeline [163, 164].

6.3.3. Advantages and disadvantages of each treatment method

In order to evaluate the developed AMP combating biocorrosion with the current biocide treatment, the advantages and disadvantages of each method are discussed in this chapter. A whole list of the comparison of different parameters can be found in table 6.2.

AMPs have strong advantages in terms of activity compared to the biocides GA and THPS (table 3.3 and 6.1), where the required AMP concentration is more than 13-fold lower for GA and 3-fold lower for THPS. Additionally, AMPs have inhibitory and eradication biofilm effects (table 5.2 and 5.3). Thereby, the AMP activity is

Table 6.2.: Comparison of different parameters between AMP treatment developed here and the current biocide treatment via GA and THPS

parameter	**AMP**	**biocide (GA and THPS)**
bacterial activity against *D. vulgaris*	bacterial killing activity: MIC95 >7 µM, MBIC90 >26 µM, MBEC90 >75 µM biofilm removal: MBEC90 >336 µM	bacterial killing activity: MIC95 >50 µM
toxicity	non-toxic: SI>1	toxic: SI<1
resistance rate	6-fold MIC increase	50-fold MIC increase[c]
modification	simple and fast (via SPPS)	not possible
biodegradability	degradable into single amino acids	only inactivation
stability	for short storage (few days to weeks) between 4 °C and room temperature for dry peptides, for long storage <-20 °C is preferred to protect peptides against chemical changes and proteolytic degradation [165]	<25 °C up to 12 months[c] [166]
costs	~ 1.30 €/L[a]	~ 0.01 €/L[b,c]
license	not yet an approved biocide (approval procedure required)	approved biocide

a calculated with synthesis costs for 1 kg peptide P1-W4I6-2.2 through SPPS (GenScript Biotech, Piscataway Township, United States of America) and MIC95 against *D. vulgaris*

b calculated with costs for 230 kg for Protectol® GA50 (BASF SE, Ludwigshafen, Germany) and MIC95 against *D. vulgaris*

c data only for GA

limited to the bacterial effect (table 3.4), whereas the biocide concentration required for bacterial activity is also toxic to other species like human cells and leads also to their damage (table 6.1). In addition, the effectiveness of AMPs remains constant over a longer period of time, with a resistance rate nearly ten-times lower than GA. Consequently, in addition to the lower initial concentration, a lower long-term concentration of the drug is required to combat biocorrosive bacteria (figure 6.1). Another advantage of AMPs is their modifiability, especially through chemical synthesis. This method allows simple and fast sequence changes, including modifications of the amino acid composition and peptide ends. As a result, a significant increase in activity can be achieved with minimal effort, and AMPs can be adapted to specific bacteria and their conditions, such as lipidation for improved activity in high salt environment for marine SRBs (table 4.4 and 4.6). Proteases can split AMPs into individual amino acids, leading to a high biodegradability [167]. In contrast, GA and THPS could only be inactivated and they are not degradable [96]. While storage for dry peptide powder at 4 °C to room temperature is still possible for a short period of time, longer storage, especially for aqueous peptide solution, requires temperatures below -20 °C to avoid chemical reactions of the amino acids and thus changes of the peptides, as well as proteolytic degradation [165]. In contrast, GA can be stored at temperatures below 25 °C for up to 12 months without any restrictions [166]. The production costs for 1 L active biocide solution are only a few cents, calculated by the price for 230 kg of Protectol® GA50 (BASF SE, Ludwigshafen, Germany). In contrast, the production costs for 1 L of active peptide solution are approximately 1.30 €, calculated on the production costs for 1 kg of peptide P1-W4I6-2.2 through SPPS (GenScript Biotech, Piscataway Township, United States of America). Biocorrosion treatment with AMPs represents a new process and therefore AMPs are not yet approved as biocides and are not allowed to be used. Nevertheless, due to the aforementioned advantages, their incorporation as biocide products is a logical conclusion, despite the inherent complexity of the process [168]. GA is approved as a biocide for disinfection (product class 2), veterinary (class 3), food and feed sector (class 4), as well as for protection during storage (class 6), for cleaning of cooling and processing systems (class 11) and for slime control (class 12) [169]. THPS is approved for the product classes 6, 11, and 12 [170]. The approval for the product class 2 is currently under review [171].

6.4. Conclusion

The current situation of AMP treatment against biocorrosive bacteria can generally be evaluated as very positive since many of the selection criteria for new combating drugs mentioned in this introduction could be achieved, especially the first three ones. The more potent AMPs offer advantages in bacterial activity compared to conventional biocides and are less resistant due to lower required concentration and a non-specific membranolytic mode of action, allowing the required concentration to be kept low over a longer period of time. The main advantages is the limitation of AMPs activity to the bacterial effect, resulting in non-toxicity to humans and environment, whereas biocides have a drastically damaging effect. As environmental compatibility has gained importance in recent years, for example through the addition of green drugs like herbal D-limonene for reducing biocide concentration [108], the higher cost of AMP treatment is still acceptable. For price calculation, it is important to consider that a smaller batch size was used for AMPs. However, a scale-up of the peptide production and switching to liquid phase peptide synthesis would enable large-scale production in higher kg to ton range and also require a lower amount of solvents, which could be reflected in lower synthesis costs [172]. As peptides can be easily modified, for example through lipidation or amidation as shown in this thesis, their bacterial activity can be further increased, resulting also in reduced costs. Nevertheless, there are still criteria that were not satisfied by AMPs and challenges to be overcome in establishing AMP treatment, in particular, the increase in AMP stability. While there are already numerous approaches to address this issue [173], it remains limited and renders the AMP application more difficult.

But all in all, the AMP method can be considered as a promising alternative treatment to the current biocide method in combating biocorrosive bacteria and their biofilms. Thereby, AMPs are advantageous compared to biocides in terms of the important criteria activity and biodegradability, although they may be less cost-efficient and have lower storage stability. But due to the immense viability of AMPs, their potential as a alternative anti-biocorrosion drug is not yet exhausted and can be increased by further experiments that hopefully all criteria, also cost-efficiency and stability, could be better achieved.

7. Concluding discussion and outlook

The selection of membranolytic AMPs based on the peptide characteristics charge, hydrophobicity, amphiphilicity, length, and simplicity from common peptide database seems to be a suitable method, as the majority of the selected peptides had MIC values in the single-digit or lowest two-digit µM-range (table 3.3 and A.1). Thereby, the selection was focused on membranolytic peptides, since these ones can kill a larger number of different bacteria species due to non-specific mode of action, based first on the electrostatic interaction between cationic AMP and anionic bacterial membrane, followed by the integration of the hydrophobic part into the lipid bilayer [27]. AMPs with a specific mode of action are not as efficient as these membranolytic ones, since the corresponding intracellular targets must be present in the same way in all different bacteria. As expected, their activity covered a wide range of different gram-negative bacteria, including the standard laboratory bacterium *E. coli*, different biocorrosive bacteria SRBs, SOB and SFB, as well as pathogenic *P. aeruginosa* species (table 3.3). Therefore, these membranolytic AMPs are suitable for combating biocorrosion, where a lot of different bacteria species are involved (chapter 1.3). An additional determination of the minimum bactericidal concentration (MBC) was dispensed due to the considerable additional effort compared to its supplementary information, in particular due to the preparation of anaerobic agar. The alanine-scan as a classical tool for detecting important amino acid positions was used in this thesis to determine the role of tryptophan at the amphiphilic interface within the peptide structure as a central component for peptide activity. The peptide is anchored to the bacterial membrane due to many possible interactions of its with both hydrophobic and hydrophilic components of the lipid bilayer, resulting in a high anchoring, a high stabilization and a right orientation [38], which is reflected in an increased activity. Optimizing the peptide sequence through common modification methods, such as C-terminus amidation and N-terminus lipidation, can be considered as highly effective due to low required MIC values up to 2 µM (table 4.4 and 4.6). The optimization has advantages beyond increasing activity as it also enhances peptide stability through endopeptidases by the modification of the peptide termini. Stability analysis of *D. vulgaris* supernatant enables fast and simple analysis without complex preparations of different protease mixtures, and at the same time analysis under identical conditions as bacterial growth. In each real environment, not only the proteases of the bacterium to be killed are present, but also numerous proteases from other bacteria may be present and complicate the

application. Therefore, the stability determined using this method does not have to indicate good stability in real applications, whereby this method can be regarded as a good tool for initial experiments, but further analyses are necessary for complete stability results. Lipidation and amidation are effective in improving activity and stability, but limit biodegradability due to non-possible degradation by endopeptidases. Biodegradability is a crucial advantage of AMPs against biocides, as these can be cleaved to harmless amino acids. However, it is important to find a balance between these three points, as high activity and stability are also essential for application. The second method for improving activity is the combination of AMPs, which can be considered as a failure due to the same mode of action of both AMPs used in this thesis and thus the same target in form of electrostatic and hydrophobic interaction with the bacterial membrane. In contrast, the combination with intracellular P5 resulted in halving of the required concentration compared to single dose (figure 4.4). However, intracellular AMPs have limited suitability for later application due to several reasons: (i) There are typically only effective against specific bacterial species and do not have universal bacterial activity like membranolytic ones. (ii) They have a higher resistance rate by an easy change of the target. (iii) The immobilization of AMPs, which is necessary due to open systems in industrial applications, while maintaining their activity is more difficult to achieve than for membranolytic AMPs, which already need the usage of linkers to ensure flexibility of the immobilized peptides to allow the contact with the bacterial membrane [66].

Additional to the planktonic activity of AMPs, also their antibiofilm activity against both, inhibiting and eradicating, is also important, as the majority of bacteria accumulate as a biofilm [7]. Therefore, the CBD is a tool for high-throughput screening of antibiofilm substances in a simple and fast way [142]. The modified version with microcentrifuge tubes as growth surface provides additional advantages due to flexibility in the analysis of individual pegs, as well as cost reduction through the usage of typical laboratory equipment in form of 96-well plates and microcentrifuge tubes [143]. This modified assay can be evaluated as suitable for evaluating antibiofilm-peptides against *P. aeruginosa.* Despite low MIC values $\leq$6 µM, these peptides showed almost no antibiofilm activity against *P. aeruginosa* (table 5.2 and 5.3), explaining by high amount of extracellular proteases and eDNA, which hinders peptide activity due to proteolytic degradation or electrostatic binding [149, 156]. This results in a limited usage of these AMPs against *P. aeruginosa* biofilms. Satisfactory results could only be achieved through early AMP treatment for the directly elimination of planktonic bacteria to avoid biofilm formation. However, this modified CBD could not be successfully transferred to *D. vulgaris* due to low and heterogeneous biofilm on microcentrifuge tubes (table 5.1), requiring an adaptation of the growth surface to polished steel rivets. Growth materials utilized for *D. vulgaris* primarily consist of metals, such as steel, titanium, and aluminum, which have also been pretreated to enhance roughness for an improved biofilm formation [98, 146]. Manually roughening of rivets by mechanical treatment with sandpaper provides a good growth surface for *D. vulgaris*, but limits high-throughput and reproducibil-

ity, requiring an automatized pretreatment procedure for the expansion of further screening experiments. The usage of rivets enables the analysis on realistic surface material and facilitates the investigation of biocorrosion on further relevant metals besides steel due to simply change of growth surface material by the usage of other rivets, as well as of other issues such as the antibiofilm activity of clinical bacteria like the ESKAPE organisms on medically relevant materials like stainless steel or titanium. Thus, this further modified assay was used for the determination of MBIC and MBEC against *D. vulgaris* (table 5.2 and 5.3), which are both increased in comparison to MIC due to challenges of biofilm structure, especially by the diffusion barrier against external substances of the EPS matrix. In addition to determining MBIC and MBEC, the identical assay can also be used to analyze long-term effects of biofilm inhibition, allowing the investigation of multiple issues with the same equipment. Although these AMPs provide biofilm activity against *D. vulgaris* compared to *P. aeruginosa*, an early treatment should also be investigated to reduce the required peptide concentration, resulting in reduced costs and especially a decreased environmental impact. Additionally, peptide mixtures can be useful to address all different stages in biologically and biofilm growth as microbiological assays indicates that different AMPs are preferred for different targets: P1 and its optimized versions against planktonic bacteria, P2 against bacteria within the biofilm, P3 for biofilm inhibition, and P4 for complete removal of the biofilm matrix. The assay adapted here to SFB and SRB can be considered as suitable for an initial screening-study of antibiofilm activity of new substances, but further experiments are necessary to get detailed information: Since the OD600 analysis is only a turbidity method and thus the measurement of bacterial fragments could also occurs, the more accurate method by determining the living cell number should be performed, which should be avoid in screenings due to complex procedure. Despite toxicity, end-point measurements and low reproducibility, staining with crystal violet has been established as a common method for general biofilm analysis in screenings due to simple indication. Due to the lack of differentiation between cells and other anionic biofilm components such as eDNA through non-specific electrostatic binding, further investigations should be performed for a complete biofilm analysis [141, 174].

Despite the antimicrobial activity of AMPs, further aspects like a low toxicity are crucial. Therefore, the calculated SI against HaCaT and RBC suggests non-toxic AMPs. For the industrial application envisaged here, the criteria do not need to be as strict as for biotherapeutics, because there is no in-vivo application of them. However, ensuring health safety is important to prevent any human damage during application. Especially a contact with the human skin could occur, but these contact time is limited for example, for the cleaning time of the plant, and therefore usually protection equipment like gloves are worn. Thus, the IC50 determined here with a contact time of 24 h represents the upper limit, and a lower cell damage can be assumed with a shorter incubation time, as further studies have already shown [175, 176]. In industrial applications, contact with RBC does not typically exist. However, it is still important to determine their harmlessness in the case of possible

injuries and therefore possible contact with them. Both methods, MTT-assay and hemolysis-assay, are common laboratory methods for analyzing the toxicity of substances [177]. The MTT-assay is based on the reduction of yellow MTT into purple formazan by mitochondrial dehydrogenases, requiring an active reductase system for this reaction, and so allowing a correlation between the amount of formazan with the number of living cells. But misinterpretations may occur due to indirect cell determination based on metabolic activity. Non-metabolically dependent assays like the neutral red uptake could be an alternative, since the accumulation in lysosomes could correlated with the number of living cells [178, 179]. The released amount of hemoglobin is measured in the hemolysis-assay, allowing a correlation to the amount of disrupted erythrocytes, which represents an ideal template for cheap, fast and effective experiments against human cells [177]. The MTT-assay is appropriate for determining the toxicity for both, AMPs and biocides. However, the hemolysis-assay is only suitable for AMPs since biocides do not seem to cause RBC lysis. Instead, biocides may cause unspecific interactions, resulting in loss of RBC deformability. This assay cannot provide this analysis, so further analyses such as ektacytometry are necessary [162]. Although HaCaT and RBC are commonly used for toxicity testing, especially in pharmaceutical development, they only represent a selection of possible cells. In industrial applications, particularly in the marine area, AMPs come into contact with a wide range of other species, such as fish, algae, or mollusks. Therefore, an extension of toxicological studies should be envisaged to ensure future applicability in this area as well.

The peptides P1-P4 used in this thesis demonstrated high planktonic activity against a wide range of gram-negative bacteria, as well as antibiofilm activity and low toxicity. Therefore, these AMPs are preferred to the current biocide treatment in some aspects, especially their higher activity. For a overview of comparison see table 6.2. Nevertheless, the results represent a first possibility study and further experiments should follow to overcome challenges in establishing AMP treatment and to simulate more realistic conditions.

The presented results indicate a significant decrease in activity with increasing biofilm maturation, resulting in not applicable MBEC values for combating bacteria within the biofilm. Nevertheless, it could be shown that AMPs, such as peptide P4, have further interaction points within the biofilm. Based on this fact, the development of further structure-dissolving peptides should be investigated to achieve synergistic effects with killing peptides like P2, which showed the lowest MBEC for bacterial killing, allowing also the suitable application of AMPs against existing biofilms. This requires more in-depth biofilm analyses, not only on the complete biofilm matrix through crystal violet staining as done here, but also analyzing each individual EPS-component and different biofilm relevant pathways. Although biofilm inhibition was successfully detected over 21 d (figure 5.3), AMPs mainly provide short-term protection rather than long-term effects due to their low stability. There are various design approaches to increase the stability of AMPs, in particular proteolytic protection, and thus enable better applicability: peptide

modifications like lipidation and amidation (as used in this thesis), glycosylation, addition of polyethylene glycol; peptide cyclization including disulfide bridges, peptide stapling and among other cyclization such as head to tail, head to side or side to tail; substitution with non-natural amino acids like D-amino acids and multimerization to peptide polymers [173]. With all the measures taken to increase stability, the maintenance of AMP activity and the resulting reduction of biodegradability, representing the main advantages compared to the current biocide treatment, should not be disregarded. As mentioned above, a balance between high activity and high stability but also degradability into single amino acids should be investigated. Since it is not possible to achieve all parameters simultaneously, it may be necessary to adjust the priorities based on specific application. In closed areas, such as storage tanks or pipelines, the focus should be on high stability to reduce AMP dosing. In contrast, biodegradability is more important in open systems like marine environments due to the presence of numerous other species and their protection. Despite further experiments for the improvement of activity and stability, experiments to determine the applicability of AMPs under more realistic conditions should be performed, too. Therefore, these experiments should include dynamic tests in addition to static MIC, MBIC, and MBEC-assay used in this thesis, which allow analysis under flow conditions and thus the simulation of for example a pipeline. Dynamic assays like drop flow reactors, rotary devices or microfluidic chips are characterized by a continuous nutrient flow [180], which may result in a change of drug efficiency caused by a higher variability in biofilm architecture and a continuous availability of fresh nutrients [181] or even in a complete change of peptide mechanisms like peptide GL13K, where cell lysis was only detected in experiments in drip flow reactor [182, 183]. Additionally, the assays in this thesis were performed under optimal growth conditions for each bacteria, including both nutrient availability and incubation parameters. For more realistic conditions, it is necessary to adjust the incubation temperature as well as the medium to simulate industrial conditions. An immobilization of AMPs is also necessary due to large and open systems to avoid drastic dilution effects and ensure the presence of AMPs at the required location. There are three strategies for this purpose, primary coating through binding sequence, secondary coating through functionalized surface and tertiary coating through matrix or gel formation [184], whereby the first two methods seem to be suitable for industrial applications. But there are still challenges to overcome for an effective AMP immobilization, such as maintaining AMP activity after immobilization by the usage of linkers to ensure flexibility [66].

However, this thesis provides a promising start for the usage of AMPs combating biocorrosive bacteria and their biofilm, resulting in a serious alternative to the current biocide treatment.

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A. Appendix

A.1. Appendix 1: Selection of AMPs against planktonic biocorrosive bacteria

Table A.1.: Overview about the further selected AMPs, with sequence, source and MIC [µM] at 100 % (MIC100), 95 % (MIC95) or 50 % (MIC50) inhibition for two biofilm-forming bacteria (*E. coli* DSM 5698 (K12), *P. fluorescens* DSM 289), one SRBs (*D. vulgaris* DSM 644), and one SRB (*T. intermedia*); additionally to table 3.3

peptide number	**sequence**	**source**	***E. coli***	***P. fluorescens***	***D. vulgaris***	***T. intermedia***
			MIC100	**MIC100**	**MIC95**	**MIC50**
P5	ILPWKWPWWPWRR-NH_2	[102]	22	87	11	5
P6	KKLFKKILKYL	AP02671 [15]	15	4	117	>117
P7	KLLKLLLKLLLKLLK	[185]	93	47	47	3
P8	VRLIVRIWRR	ID171 [71]	>138	>138	138	69
P9	RFKRVARVIW	ID172 [71]	>125	63	>125	125
P10	IGKEFKRIVQRIKDFLRNL	ID15 [71]	35	18	70	9
P11	IKKILSKIKKLLK	ID131 [71]	54	3	107	>107
P12	KRIVQRIKDFLR	ID22 [71], AP00608 [15]	106	27	>106	>106

Table A.2.: Geometric mean (GM) of all determined MIC [µM] (individual MIC values could be found in table 3.3 and 4.4), IC [µM] at 50 % (IC50) against HaCaT cells with calculated SIcyto.=IC50/MIC95, HC [µM] at 50 % (HC50) against sheep RBC with calculated SIery.=HC50/MIC95 for the four favorite AMPs

peptide number	GM MIC	IC50	SIcyto.	HC50	SIery.
P1	17	118	7	>118	>7
P2	12	50	4	50	4
P3	19	13	0.7	104	6
P4	21	>449	>21	>112	>5

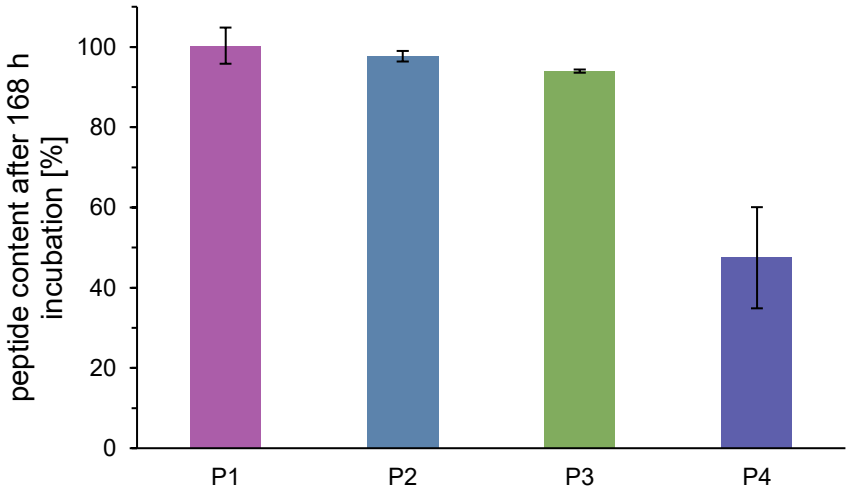

Figure A.1.: Stability in cultivation medium (Postgate C) for *D. vulgaris* of the four favorites peptides P1 (pink), P2 (blue), P3 (green), and P4 (purple); analyzed with RP-HPLC, 100 % peptide content corresponds with the peptide content at 0 h; values shown as means with standard deviation n=3; adapted from [103] CC-by 4.0

A.2. Appendix 2: Improvement of AMP activity through sequence optimization and combination

Table A.3.: Geometric mean (GM) of all determined MIC [µM] (individual MIC values could be found in table 3.3 and 4.4), IC [µM] at 50 % (IC50) against HaCaT cells with calculated SIcyto.=IC50/MIC95, HC [µM] at 50 % (HC50) against sheep RBC with calculated SIery.=HC50/MIC95 for the modified versions of peptide P1

peptide number	GM MIC	IC50	SIcyto.	HC50	SIery.
P1	17	118	7	>118	>7
P1-W4I6	>7	28	<4	56	<8
P1-W4I6-1.1	11	14	1	28	3
P1-W4I6-2.2	8	7	0.9	13	2

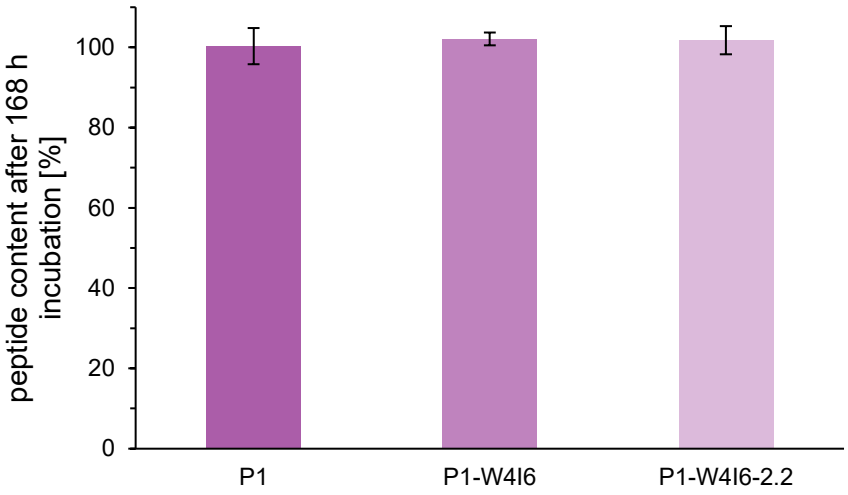

Figure A.2.: Stability in cultivation medium (Postgate C) for *D. vulgaris* of P1 (pinkt) and its best modifications P1-W4I6 (light pink) and P1-W4I6-2.2 (ultra light pink); analyzed with RP-HPLC, 100 % peptide content corresponds with the peptide content at 0 h; values shown as means with standard deviation n=3; adapted from [103] CC-by 4.0

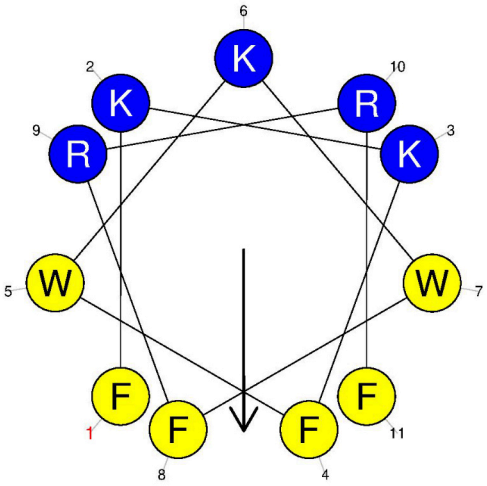

Figure A.3.: Helical structure of P2, the arrow symbolizes the hydrophobic moment, simulated with HeliQuest [114]

A.3. Appendix 3: Antibiofilm-assay for AMPs against biocorrosive bacteria

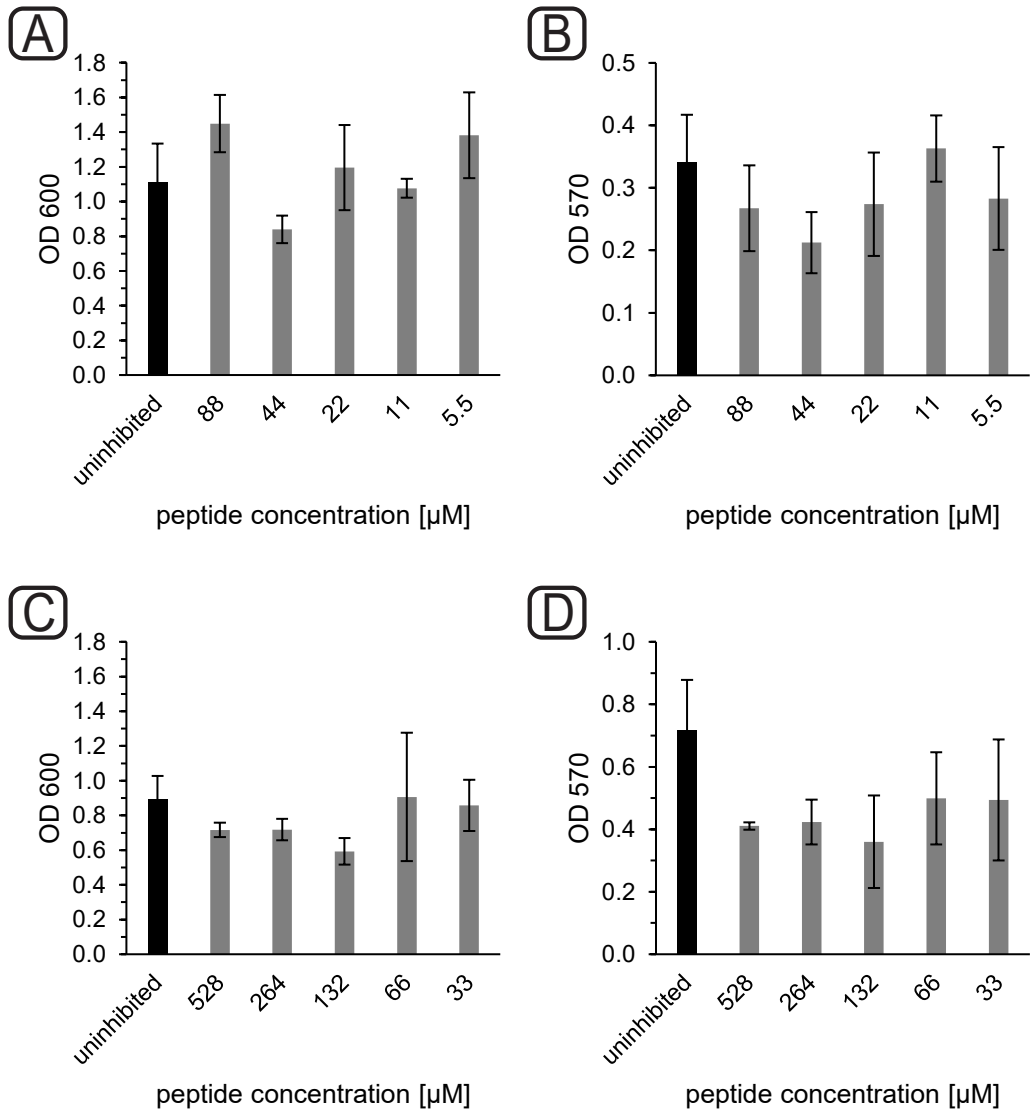

Figure A.4.: Antibiofilm-assay for peptide P5 against *D. vulgaris* DSM 644: MBIC for (A) OD600 and (B) OD570, MBEC for (C) OD600 and (D) OD570 for uninhibited sample (black) and five peptide concentrations (gray) in µM; values shown as means with standard deviation n=3

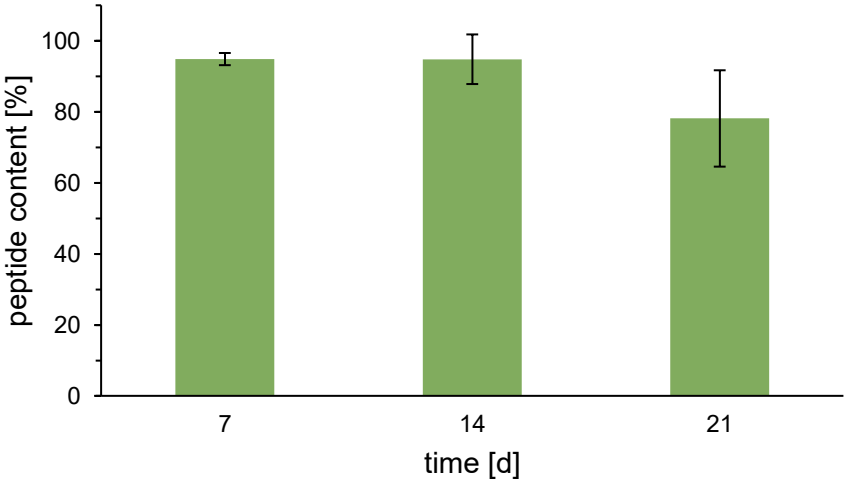

Figure A.5.: Stability in cultivation medium (Postgate C) for *D. vulgaris* of P3 (green); analyzed with RP-HPLC, 100 % peptide content corresponds with the peptide content at 0 h; values shown as means with standard deviation n=3; adapted from [134] CC-by 4.0

B. Publications from this work

B.1. Paper

- Stillger, L.; Viau, L.; Holtmann, D.; Müller, D. (2023): Optimization of antimicrobial peptides for the application against biocorrosive bacteria. In: Appl Microbiol Biotechnol. DOI: 10.1007/s00253-023-12562-9 [134]
- Stillger, L.; Viau, L.; Kamm, L.; Holtmann, D.; Müller, D. (2023): Antibiofilm-Assay for Antimicrobial Peptides combating the sulfate-reducing bacteria *Desulfovibrio vulgaris*. In: MicrobiologyOpen. doi:10.1002/mbo3.1376 [103]
- Stillger, L.; Müller, D. (2022): Peptide-coating combating antimicrobial contaminations: a review of covalent immobilization strategies for industrial applications. In: J Mater Sci 57 (24), S. 10863–10885. DOI: 10.1007/s10853-022-07266-w. [66]

B.2. Conference

- 08/2023: German Peptide Symposium, Jena; Talk: Stillger, L.; Viau, L.; Kamm, L.; Holtmann, D.; Müller, D.: Screening of Novel Antimicrobial Peptides as a New Strategy against Anaerobic Biocorrosive Bacteria and their Biofilm
- 05/2022: BIOFILM10 conference, Leipzig
- 04/2022: DECHEMA/GfKORR Group: “Mikrobielle Materialzerstörung und Materialschutz”, online; Talk: Stillger, L.; Mukoo, P.: Antimikrobielle Peptide gegen Biokorrosion

C. Supervised theses

- Sena Akkus: research & development project as part of master´s degree, completion: 01/2024: "Verstärkung der Antibiofilm-Aktivität von antimikrobiellen Peptiden"
- Jan Schäfer: bachelor thesis, completion 06/2023: "Untersuchung von synergistischen Effekten bei dem Einsatz von antimikrobiellen Peptiden gegen *Desulfovibrio vulgaris*"
- Lucile Viau: master thesis, completion 12/2022: "Etablierung und Validierung mikrobieller Assays for anaerobe Sulfat-reduzierende Bakterien zur Aktivitätsbestimmung von Antibiofilm-Peptiden"
- Lena Kamm: research & development project as part of master´s degree, completion: 11/2022: "Synthese und Stabilitätsuntersuchungen von antimikrobiellen Peptiden"

D. Curriculum vitae

Not part of the online version

Acknowledgments

This thesis was done between 2021 and 2024 at the institute of bioprocess engineering and biopharmaceutical technology at Technische Hochschule Mittelhessen. A large number of people supported me during this period, and I would like to thank all of them. Nevertheless, I would like to highlight a few in particular.

I would like to thank **Prof. Dr. Kai Thormann** for being my first supervisor as a matter of course and for the good scientific feedback during the whole time.

Special thanks goes to **Prof. Dr.-Ing. Dirk Holtmann** for his supervision, the numerous scientific discussions, the many tips and advice, and especially for his help and support at all times.

I would like to thank **Dr. Daniela Müller** for giving me the opportunity to work on this topic and for introducing me to it as well as **Prof. Dr. Franz Cemič** for taking over the project management after its change.

I extend gratitude to **Prof. Dr. Stephanie Gokorsch, Johanna Rühl-Teichner** (institute of hygiene and infectious diseases of animals, Justus Liebig University Giessen) and **Dr. Peggy Schlupp** for the shared use of their laboratories as well as **Gerhild Donnevert** and **Angelika Müller-Green** for the opportunity of LC-MS measurements.

I am also thankful to my students **Sena Akkus**, **Lena Kamm**, **Jan Schäfer** and **Lucile Viau**, whom I could supervise them as part of their research and development projects or thesis, for their support in the laboratory.

Additionally, I express my gratitude to all members of the whole working group Holtmann - Intensification of bioprocesses. I would like to extend special thanks to my PhD colleagues **Lucas Becker**, **Sera Bolat**, **André Gemünde**, **Jakub Gizewski**, **Alexander Langsdorf**, **Marc Pfitzer**, **Björn Sabel-Becker**, **Lisa van der Sande**, **Niklas Teetz** and **Hannah Wohlers** for their natural acceptance into the group and the great hours spent together, both in lab and during lunch breaks and after work.

Special thanks to **Carolin Anna Lappöhn** and **Lisa-Marie Sittek**: Thank you for the "AMP self-care group" and the immense support, not only on scientific level, but also for all kinds of problems and worries.

I thank my family, my parents **Martin** and **Susanne** and my brother **Lukas**, for their support on all my steps of life. Very special thanks to **Tim** - you are my home!