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Optimization of the removal efficiency of antimicrobial resistance (AMR) by micro- and ultrafiltration treating WWTP effluents

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„Probleme kann man niemals mit derselben Denkweise lösen, durch die sie entstanden sind“.

Albert Einstein

ABSTRACT

The European Centre for Disease Prevention and Control (ECDC) reports a continuous increase from 11.6 % in 2016 to 16.8 % in 2020 of vancomycin resistant *Enterococcus faecium* in the European Union (WHO and ECDC, 2022). At the same time, there is a decreasing number of newly introduced antibiotics worldwide. While 31 antibiotics have been introduced for clinical applications against priority pathogens in 2017, this number decreased to 27 antibiotics in 2021 (WHO, 2022(1)). Thus, antibiotic resistance is a rising problem, because unfortunately more and more antibiotics are inefficient against bacterial infections.

Antibiotic resistance has become not only a threat to human health in clinical facilities, but also an environmental challenge with regard to the spread of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) across the aquatic environment (Hembach et al., 2017). Worldwide, antibiotics are introduced to treat bacterial infections in human and in veterinary therapy. Since humans and animals cannot completely metabolize antibiotics, large amounts of antibiotics and antibiotic resistant bacteria emitted by feces find their way into the so-called urban water cycle (representing the impact of wastewater/ biosolids/ manure on surface water and drinking water).

Bacterial adaption to antibiotics in the water cycle can be divided into two main promoters that can be described as anthropogenically-derived and background antibiotic resistance. The prevalence of anthropogenically-derived antibiotic resistance is a result of antibiotics emissions. Gullberg et al. (2011) demonstrated in a laboratory study investigating the growth of antibiotic susceptible bacteria in the presence of different antibiotic concentrations that bacterial adaption to antibiotic resistance can occur already at extremely low concentrations of antibiotics. Further studies in the aquatic environment confirm that higher antibiotic concentrations do result in higher ARB and ARGs' abundances (Yang et al., 2008; Sigala, Unc, 2012; Rizzo et al., 2013; Li et al., 2015; Um et al., 2016).

Bacterial adaption to antibiotic resistance without anthropogenic influence is the second promoter of antibiotic resistance. Antibiotic resistance is an ancient process and a natural phenomenon. Bacteria can distribute antibiotic resistance genes by vertical and horizontal gene transfer. During vertical gene transfer, bacteria have cell divisions and the next-generation bacteria have the same DNA. Mutations in cell genes like chromosomal mutations can lead to an antibiotic resistant bacterium. Cell divisions of ARB promote antibiotic resistance. Horizontal gene transfer describes a different mechanism of antibiotic resistance spread. ARGs can be transferred from bacterium to another bacterium by plasmids and transposons (conjugation). Furthermore, antibiotic resistance genes can be incorporated in the plasmids of bacteria from free plasmids of dead cells (transformation) and gene transfer occurs by bacteriophages and integrons (transduction)

(Giedraitiene et al., 2011). The mechanisms of horizontal gene transfer demonstrate that prevention of the spread of antibiotic resistance in the aquatic environment should not be limited to the removal of antibiotic resistant bacteria. Since bacteriophages and free antibiotic resistance genes like free plasmids after bacterial cell lysis, integrons and transposons also promote the spread of antibiotic resistance, they have to be reduced as well in order to prevent the spread of antibiotic resistance in the aquatic environment.

However, preventing the spread of antibiotic resistance by WWTP discharges comes with a number of challenges. Since antibiotic resistance is known as a global health risk due to the increase of more and more ineffective antibiotics, antibiotic consumption is regulated by law (Regulation EU 2019/6) with the aim of reducing antibiotic consumption in particular in the veterinary sector. In human medicine, the 2011 law amending the Infection Protection Act and other laws in Germany tightened hygiene requirements in healthcare facilities and improved outbreak control options. However, AMR increase and spread in the aquatic environment was studied only in basic research studies. Present directives for wastewater treatment in Europe do not require removal of antibiotic resistant bacteria during wastewater treatment. Antibiotic resistance is even not part of statutory provisions in the water treatment. Therefore, basic research is needed to assess any potential elevated risk for human health or the environment.

For a comprehensive assessment, a literature review study is needed to highlight AMR abundance of low impacted surface waters with background antibiotic resistance as well as urban surface waters with impacts of WWTPs discharges. Furthermore, advanced treatment processes should be investigated enabling a higher AMR removal efficiency in WWTP effluents. The membrane filtration process should be compared to other advanced treatment processes as appropriate technology to achieve a high AMR removal efficiency.

To address these shortcomings, an initial literature review study was conducted as part of this dissertation on reported antibiotic microbial resistance (AMR) removal efficiencies by conventional and advanced treatment processes. Based on a comprehensive literature study, the occurrence and transport of antibiotic microbial resistance in the urban water cycle revealed that discharges from conventional wastewater treatment facilities increase antibiotic microbial resistance in downstream surface waters.

The presence of antibiotic resistance in low impacted surface water could be considered as a proxy of background antibiotic resistance level, which might serve as a reference for treatment targets in the absence of health-based threshold levels. Different biological, physical and disinfection/oxidation processes employed in wastewater treatment and their efficacy regarding their removal of antibiotic resistant bacteria and antibiotic resistance genes (ARGs) were also evaluated. These included membrane filtration, ozonation, UV-irradiation or chlorination capable of achieving ARG levels typically observed in urban surface water or low impacted surface water.

Since several studies of AMR removal efficiencies of WWTPs employing mechanical and biological treatment showing inefficient AMR retention (Alexander et al. 2015; Hembach et al. 2017), membrane filtration processes turned out to be the most promising process of the examined advanced treatment processes exhibiting very high AMR removal efficiencies. Membrane filtration technology is applied in Germany predominantly for drinking water treatment, municipal wastewater treatment (e.g., membrane bioreactor in case of less catchment area of the WWTP) and industrial wastewater treatment (Dechema 2022).

Therefore, membrane filtration pilot-scale studies should provide the basis of an economic assessment of this advanced treatment for AMR removal. Furthermore, specific research questions should be addressed: Where is the best position for the implementation of a membrane filtration process in an existing conventional treatment train? What kind of pore size of the membrane is needed to assure a high AMR removal efficiency under economic conditions? What operational conditions are required to guarantee a high AMR removal efficiency while maintaining economic conditions? What strategies are needed to prevent AMR associated regrowth?

The described research questions of the literature review study were elaborated into hypotheses that were tested in MF and UF pilot-scale studies. The key hypotheses are as follows:

- ARGs removal efficiency of micro- and ultrafiltration of WWTP effluent is higher than 90 percent during standard filtration mode.
- Chemical enhanced backwash results in significant higher ARGs levels in the UF filtrate than during regular backwash.
- AMR associated regrowth in UF filtrate can be prevented by activated carbon pretreatment of feed water.
- Presence of antibiotics in secondary effluent can cause bacterial adaption to antibiotic resistance in UF filtrate.
- AMR regrowth in the UF filtrate of ultrafiltered secondary effluent can be prevented by continuous chlorine dosing.

The pilot-scale studies were conducted at the WWTP Steinhäule in order to quantify AMR removal efficiencies, operational requirements, and to determine design values during pilot-scale membrane filtration applications. Low-pressure membrane filtration was investigated at pilot scale with regard to its capability to remove ARGs using conventional secondary treated wastewater plant effluents during standard filtration mode. While operating microfiltration (MF) and ultrafiltration (UF) membranes, key operational parameters and key factors influencing AMR removal efficiencies were examined. These studies revealed that the main factor determining AMR removal is the pore size of the membrane. The fouling layer forming on capillary membranes had only a small additive effect on ARG removal. Using feed waters with different ARGs abundances revealed that higher ARG feed concentrations resulted in higher ARG abundance in the filtrate. Live-dead cell counting in UF filtrate showed intact bacteria breaking through the UF membrane. Surprisingly, strong correlations were revealed

between *16SrRNA* gene (as a surrogate for quantifying total bacteria) and the *sul1* gene in UF filtrate indicated ARB likely breaking through the UF membranes.

In addition, experiments were conducted to investigate AMR removal efficiency directly after backwash and chemical enhanced backwash. Findings revealed that the abundance of the total biomass measured as surrogate parameter (high nucleic acid count HNAC, total cell count TCC and the ribosomal RNA gene *16SrRNA*), analyzed in the samples taken directly after backwash and CEB within the first minute, were more than 1 log unit higher compared to the samples taken within 5 and 55 minutes of standard filtration mode. While *ermB* and *vanA* genes exhibited no increase after backwash and CEB, *sul1* gene increased significantly within the first minute. However, further investigations revealed that the increase in biomass and *sul1* gene in UF filtrate was caused by microbial regrowth in the backwash tank contaminating the backwash water. This has practical implications for filter-to-waste protocols and properly maintaining backwash water quality to prevent regrowth and horizontal gene transfer where a high microbial filtrate water quality is desired for downstream use.

While AMR removal efficiencies were quantified during standard filtration mode and after backwash and CEB, ARG associated regrowth effects at the UF filtrate side were further investigated. During UF pilot-scale studies ARG associated regrowth effect in UF effluents were examined using secondary effluent, activated carbon treated secondary effluent and tertiary effluent of the WWTP Steinhäule as feeds. The study results revealed that typical antibiotic concentrations of sulfamethoxazole and erythromycin, measured in secondary effluent, had no ARG associated regrowth effects at the filtrate side. In contrast, about 1 log unit increase of *16SrRNA* gene and *sul1* gene were analyzed using secondary effluent as feed and a hydraulic retention time of 3 hours in UF filtrate tank operated in continuous flow mode. The highest ARG associated regrowth was detected in UF filtrate using secondary effluent as feed affected by temporarily stagnant UF filtrate in the bypass filtrate tank. In this case, *16SrRNA* gene and *sul1* gene significantly increased by about 3 and 4 log units. Measures to prevent ARG associated regrowth was also examined. Pretreatment of the feed secondary effluent by activated carbon treatment and sand filtration is recommended as measure to prevent ARG associated regrowth at UF filtrate side. Alternatively, ARG associated regrowth was inhibited in ultrafiltered secondary effluent by a continuous dose of 0.5 mg/L of sodium hypochlorite at the UF filtrate side. The study results of ARG associated regrowth have to be considered in the planning of advanced treatment processes in the wastewater treatment, when WWTP effluents are provided for water reuse purposes.

For the planning and implementation of the ultrafiltration process in conventional WWTPs to prevent the spread of AMR recommendations to design values of the membrane filtration technology for full-scale applications were elaborated. A full-scale ultrafiltration plant was designed for the application at the WWTP Steinhäule in Neu-Ulm.

The discharge of pathogenic bacteria carrying antibiotic resistance into surface waters might pose a potential threat to human health, especially if receiving rivers are subject to downstream use, for instance for recreational purposes or drinking water abstraction. However, the acute and long-term risk resulting from the release of resistance genes into the aquatic environment but also the presence of naturally-occurring resistance background, require further studies. Further research is needed to better understand potential pathways and threat of water-borne resistance genes to human health, even if they are not linked to living pathogenic bacteria. This information is needed to inform a risk-based determination of removal targets for wastewater treatment.

ZUSAMMENFASSUNG

Das Europäische Zentrum für Prävention und Kontrolle von Krankheiten (ECDC) berichtet über einen kontinuierlichen Anstieg von 11,6 % im Jahr 2016 auf 16,8 % im Jahr 2020 an Vancomycin-resistenten *Enterococcus faecium* in der Europäischen Union (WHO and ECDC, 2022). Gleichzeitig nimmt die Zahl der neu eingeführten Antibiotika weltweit ab. Während im Jahr 2017 31 Antibiotika zur klinischen Entwicklung gegen prioritäre Krankheitserreger im Fokus standen, nahm die Anzahl dieser Antibiotika im Jahr 2021 auf 27 Antibiotika ab (WHO, 2022(1)). Antibiotikaresistenzen sind ein wachsendes Problem, da immer mehr Antibiotika gegen bakterielle Infektionen unwirksam werden.

Die Antibiotikaresistenz ist nicht nur eine Bedrohung für die menschliche Gesundheit in klinischen Einrichtungen geworden, sondern auch eine Herausforderung für die Umwelt im Hinblick auf die Ausbreitung von antibiotikaresistenten Bakterien (ARB) und Antibiotikaresistenzgenen (ARG) in der aquatischen Umwelt (Hembach et al., 2017). Weltweit werden Antibiotika zur Behandlung bakterieller Infektionen beim Menschen und in der Tiermedizin eingesetzt. Da Menschen und Tiere Antibiotika nicht vollständig verstoffwechseln können, gelangen große Mengen an Antibiotika und antibiotikaresistente Bakterien über Fäkalien in den sogenannten urbanen Wasserkreislauf (der die Auswirkungen von Abwasser/Biofeststoffen/Dünger auf Oberflächengewässer und Trinkwasser darstellt).

Die bakterielle Selektion durch die Wirkung von Antibiotika im Wasserkreislauf kann in zwei Hauptpromotoren unterteilt werden, die als anthropogen bedingte und als Grundresistenz gegen Antibiotika beschrieben werden können. Die Prävalenz der anthropogen bedingten Antibiotikaresistenz ist eine Folge der Antibiotikaemissionen. Gullberg et al. (2011) haben in einer Laborstudie, in der das Wachstum antibiotikaempfindlicher Bakterien in Gegenwart unterschiedlicher Antibiotikakonzentrationen untersucht wurde, gezeigt, dass eine bakterielle Selektion

durch eine Antibiotikaresistenzentwicklung bereits bei extrem niedrigen Antibiotikakonzentrationen auftreten kann. Weitere Studien bestätigen, dass höhere anthropogen emittierte Antibiotikakonzentrationen zu höheren Abundanzen von ARB und ARGs führen können (Yang et al., 2008; Sigala, Unc, 2012; Rizzo et al., 2013; Li et al., 2015; Um et al., 2016).

Die Antibiotikaresistenzentwicklung der Bakterien ohne anthropogenen Einfluss ist der zweite Promotor der Antibiotikaresistenz. Antibiotikaresistenz ist ein uralter Prozess und ein natürliches Phänomen. Bakterien können die antibiotikaresistenten Gene durch vertikalen und horizontalen Gentransfer verbreiten. Beim vertikalen Gentransfer kommt es zu Zellteilungen der Bakterien. Die neuen Bakterien haben die gleiche DNA. Mutationen in Zellgenen wie Chromosomenmutationen können zu einem antibiotikaresistenten Bakterium führen. Zellteilungen von ARB fördern die Antibiotikaresistenz. Der horizontale Gentransfer beschreibt weitere Mechanismen der Verbreitung von Antibiotikaresistenzen. ARGs können durch Plasmide und Transposons von einem Bakterium auf ein anderes Bakterium übertragen werden (Konjugation). Außerdem können Antibiotikaresistenzgene aus freien Plasmiden abgestorbener Zellen in die Plasmide von Bakterien eingebaut werden (Transformation), und der Gentransfer kann durch Bakteriophagen und Integrons erfolgen (Transduktion) (Giedraitiene et al., 2011). Die Mechanismen des horizontalen Gentransfers zeigen, dass sich die Verhinderung der Ausbreitung der Antibiotikaresistenz in der aquatischen Umwelt nicht auf die Entfernung antibiotikaresistenter Bakterien beschränken kann. Bakteriophagen und freie Antibiotikaresistenzgene wie freie Plasmide nach der Lyse von Bakterienzellen, Integrons und Transposons fördern die Ausbreitung der Antibiotikaresistenz und müssen daher ebenfalls reduziert werden, um die Ausbreitung der Antibiotikaresistenz zu verhindern.

Die Verhinderung der Ausbreitung von Antibiotikaresistenzen in Kläranlageneinleitungen ist jedoch mit einer Reihe von Herausforderungen verbunden: Da die Antibiotikaresistenzen von pathogenen Bakterien aufgrund der Zunahme von immer mehr unwirksamen Antibiotika als globales Gesundheitsrisiko bekannt sind, wurde der Antibiotikaverbrauch gesetzlich reguliert (Verordnung EU 2019/6) mit dem Ziel der Antibiotikareduktion im Veterinärmedizinbereich. In der Humanmedizin wurden 2011 mit dem Gesetz zur Änderung des Infektionsschutzgesetzes und weiterer Gesetze in Deutschland die Anforderungen an die Hygiene in den Einrichtungen des Gesundheitswesens verschärft und die Möglichkeiten der Ausbruchsbekämpfung verbessert. Die Zunahme und Ausbreitung von Antibiotikaresistenzen in der aquatischen Umwelt wurde jedoch nach wie vor nur im Rahmen von Grundlagenforschungsstudien untersucht. Des Weiteren enthalten die derzeitigen Wasserrichtlinien für die

Abwasserbehandlung in Europa keine Anforderungen an die Entfernung von antibiotikaresistenten Bakterien bei der Abwasserbehandlung. Die Antibiotikaresistenz ist nicht einmal Teil der gesetzlichen Bestimmungen für die Wasseraufbereitung. Deshalb ist in diesem Bereich Grundlagenforschung zunächst erforderlich, um auf diesen Grundlagen ein Gefahrenrisiko für den humanen oder veterinären Bereich zu definieren.

Um die Abundanzen der antimikrobiellen Resistenzen (AMR) von Oberflächengewässern mit geringer Belastung und Hintergrund-Antibiotikaresistenzen sowie von urbanen Oberflächengewässern mit Einfluss von Kläranlageneinleitungen darzustellen, ist eine Literaturrecherche erforderlich. Darüber hinaus sollten weitergehende Behandlungsverfahren untersucht werden, die eine hohe Effizienz in der AMR-Entfernung in Kläranlagenabläufen aufweisen. Der Membranfiltrationsprozess sollte mit anderen weitergehenden Behandlungsverfahren verglichen werden, um eine geeignete Technologie mit hoher AMR-Entfernungseffizienz zu finden.

Um diese Unzulänglichkeiten anzugehen, wurde im Rahmen dieser Dissertation eine erste Literaturrecherche über die Wirksamkeit der Entfernung von antimikrobiellen Resistenzen (AMR) durch konventionelle und fortschrittliche Behandlungsverfahren durchgeführt. Die umfassende Literaturstudie über das Vorkommen und den Transport von AMR Abundanzen im urbanen Wasserkreislauf ergab, dass Einleitungen aus konventionellen Abwasserbehandlungsanlagen die AMR Abundanzen flussabwärts der Oberflächengewässer erhöhen. Das Vorhandensein von Antibiotikaresistenzen in gering belasteten Oberflächengewässern könnte als Vertreter für das Hintergrundniveau an Antibiotikaresistenzen angesehen werden, das als Referenz für Behandlungsziele dienen könnte, wenn keine gesundheitsbezogenen Schwellenwerte vorliegen. Außerdem wurden verschiedene biologische, physikalische Verfahren und Desinfektions-/Oxidationsverfahren, die bei der Abwasserbehandlung eingesetzt werden, auf ihre Wirksamkeit hinsichtlich der Entfernung antibiotikaresistenter Bakterien (ARB) und Antibiotikaresistenzgene (ARGs) bewertet. Dies beinhaltete die Membranfiltration, die Ozonierung, die UV-Bestrahlung und die Chlorung, mit denen ARG-Werte erreicht werden können, die typischerweise in urbanen Oberflächengewässern oder gering belasteten Oberflächengewässern beobachtet werden.

Da mehrere Studien zur Wirksamkeit der Antibiotikaresistenz-Entfernung in Kläranlagen mit mechanischer und biologischer Behandlung gezeigt haben, dass die Antibiotikaresistenz-Entfernung nicht ausreichend ist (Alexander et al. 2015; Hembach et al. 2017), erwies sich die Membranfiltration als das vielversprechendste Verfahren unter den untersuchten weitergehenden Behandlungsverfahren, das sehr hohe AMR-Entfernungseffizienzen aufwies. Die Membranfiltrationstechnologie wird in Deutschland überwiegend für die Wasseraufbereitung, kommunale Abwasserbehandlung (z. B.

Membranbelebungsanlagen im Fall einer gering vorhandenen Erweiterungsfläche der Kläranlagen) und die Behandlung von Industrieabwässer eingesetzt (Dechema 2022).

Daher sollten Pilotstudien zur Membranfiltration die Grundlage für eine wirtschaftliche Bewertung dieser weitergehenden Behandlung zur AMR-Entfernung bilden. Des Weiteren wurden spezifische Forschungsfragen gestellt: Wo ist die beste Position für die Implementierung eines Membranfiltrationsprozesses in eine bestehende konventionelle Abwasserbehandlungsanlage? Welche Porengröße der Membran ist erforderlich, um unter wirtschaftlichen Bedingungen eine hohe AMR-Entfernungseffizienz zu erreichen? Welche Betriebsbedingungen sind erforderlich, um eine hohe AMR-Entfernungseffizienz bei gleichzeitiger Aufrechterhaltung wirtschaftlicher Bedingungen sicherzustellen? Welche Strategien sind erforderlich, um eine AMR-bedingte Wiederverkeimung zu verhindern?

Die beschriebenen Forschungsfragen der Literaturstudie wurden zu Hypothesen ausgearbeitet, die in MF- und UF-Pilotstudien getestet wurden. Die Kernhypothesen lauten wie folgt:

- Die ARG-Entfernungseffizienz der Mikro- und Ultrafiltration vom Kläranlagenablauf liegt im Standardfiltrationsmodus bei über 90 Prozent.

- Die chemische Rückspülung führt zu deutlich höheren ARG-Abundanzen im UF-Filtrat als bei einer Rückspülung mit Filtratwasser.

- Die AMR-bedingte Wiederverkeimung im UF-Filtrat kann durch eine Aktivkohle-Vorbehandlung des Zulaufwassers verhindert werden.

- Das Vorhandensein von Antibiotika im mechanisch und biologisch gereinigtem Abwasser kann zu einer bakteriellen Anpassung an Antibiotikaresistenzen im UF-Filtrat führen.

- Die AMR-bedingte Wiederverkeimung im UF-Filtrat des mechanisch und biologisch gereinigten Abwassers kann durch eine kontinuierliche Chlordosierung verhindert werden.

Die Studien im Pilotmaßstab wurden in der Kläranlage Steinhäule durchgeführt, um die Effizienz der AMR-Entfernung und die betrieblichen Anforderungen zu quantifizieren und Auslegungswerte bei Membranfiltrationsanwendungen im Pilotmaßstab zu bestimmen.

Die Niederdruck-Membranfiltration wurde im Pilotmaßstab hinsichtlich ihrer Fähigkeit zur Entfernung von ARGs unter Verwendung von mechanisch und biologisch

gereinigtem Abwasser im Standardfiltrationsmodus untersucht. Beim Betrieb von Mikrofiltrations- (MF) und Ultrafiltrationsmembranen (UF) wurden wichtige Betriebsparameter und Schlüsselfaktoren untersucht, die die Effizienz der AMR-Entfernung beeinflussen. Diese Studien ergaben, dass der Hauptfaktor für die AMR-Entfernung die Porengröße der Membran ist. Der sich auf Kapillarmembranen bildende Filterkuchen hatte nur einen geringen zusätzlichen Effekt auf die ARG-Entfernung. Die Verwendung von Abwässern mit unterschiedlichen ARG-Abundanzen ergab, dass höhere ARG-Zulaufkonzentrationen zu einer höheren ARG-Abundanz im Filtrat führten. Die Zählung lebender/toter Zellen im UF-Filtrat zeigte, dass intakte Bakterien die UF-Membran durchbrachen. Überraschenderweise wurden hohe Korrelationen zwischen dem *16SrRNA*-Gen (als Ersatz für die Quantifizierung der Gesamtbakterien) und dem *sul1* Gen im UF-Filtrat festgestellt, was darauf hindeutet, dass ARB wahrscheinlich die UF-Membranen durchbrechen.

Außerdem wurden Untersuchungen der Entfernungsleistung der Ultrafiltration durchgeführt, in denen die AMR-Entfernungseffizienz des Ultrafiltrationsprozesses direkt nach dem Wasserrückspülmodus oder dem chemischen Rückspülmodus analysiert wurde. Die Ergebnisse zeigten, dass die Abundanzen der Biomasse gemessen mit Ersatzparametern (Zellen mit hohem Nukleinsäuregehalt HNAC, Gesamtzellzahl TCC und das ribosomale RNA Gen *16SrRNA*) in Proben, die direkt nach dem Rückspülen mit Wasser und der CEB innerhalb der ersten Minute genommen wurden, um mehr als eine Log-Einheit höher waren als in den Proben nach 5 und 55 Minuten im Standardfiltrationsmodus. Während die Gene *ermB* und *vanA* nach Wasserrückspülung und CEB keinen Anstieg zeigten, stieg das Antibiotikaresistenzgen *sul1* innerhalb der ersten Minute deutlich an. Weitere Untersuchungen ergaben jedoch, dass der Anstieg der Biomasse und des *sul1*-Gens im UF-Filtrat durch einen mikrobiellen Aufwuchs im Rückspültank zurückzuführen war, das das Rückspülwasser kontaminierte. Dieses Ergebnis hat praktische Auswirkungen auf ‘Filter-to-Waste‘ Protokolle und auf die ordnungsgemäße Qualitätssicherung des Rückspülwassers, um eine Wiederverkeimung und einen horizontalen Gentransfer zu verhindern, wenn eine hohe mikrobielle Filtratwasserqualität für die nachgeschaltete Verwendung gewünscht wird.

Während die Wirksamkeit der Antibiotikaresistenz-Entfernung bei der Standardfiltration und nach der Wasserrückspülung sowie bei der chemischen Rückspülung quantifiziert wurde, wurde nun die ARG assoziierte Wiederverkeimung auf der UF-Filtratseite untersucht. Dafür wurde die ARG-assoziierte Wiederverkeimung in Ultrafiltrationsabwässern unter Verwendung des Ablaufwassers der Nachklärung, des mit Aktivkohle behandelten Ablaufwassers der Nachklärung und des Ablaufwassers der Sandfilteranlage der Kläranlage Steinhäule untersucht. Die Studienergebnisse zeigten, dass die typischen Antibiotikakonzentrationen von Sulfamethoxazol und Erythromycin,

die im Ablaufwasser der Nachklärung gemessen wurden, keine Auswirkungen auf die ARG-assoziierte Wiederverkeimung auf der Filtratseite hatten. Im Gegensatz dazu wurde ein Anstieg des *16SrRNA*-Gens und des *sul1*-Gens um etwa eine log-Einheit bei Verwendung von Ablaufwasser der Nachklärung als Zulauf und einer hydraulischen Verweilzeit von 3 Stunden im UF-Filtratbehälter im kontinuierlichen Durchflussmodus analysiert. Die höchste ARG-assoziierte Wiederverkeimung wurde im UF-Filtrat bei Verwendung von Ablaufwasser der Nachklärung festgestellt, das durch temporär stagniertes UF-Filtratwasser im Bypass-Filtratbehälter hervorgerufen wurde. In diesem Fall stiegen das *16SrRNA*-Gen und das *sul1*-Gen signifikant um etwa 3 und 4 log-Einheiten an. Im Rahmen der UF-Studien wurden auch Maßnahmen zur Verhinderung der ARG-assoziierten Wiederverkeimung untersucht. Die Behandlung des Ablaufwassers der Nachklärung durch eine Aktivkohlebehandlung und Sandfiltration wird als Maßnahme zur Verhinderung der ARG-assoziierten Wiederverkeimung auf der UF-Filtratseite empfohlen. In diesem Fall können Bakterien, die die Membran passieren, bereits durch diese Vorbehandlungsschritte reduziert werden. Alternativ wurde die ARG-assoziierte Wiederverkeimung im ultrafiltrierten Ablaufwasser der Nachklärung durch eine kontinuierliche 0,5 mg/L-Dosierung von Natriumhypochlorit auf der UF-Filtratseite verhindert. Die Ergebnisse zur ARG-assoziierten Wiederverkeimung sind wichtig bei der Auslegung von weitergehenden Behandlungsverfahren in der Abwasserreinigung zu berücksichtigen, wenn der Kläranlagenablauf für die Wasserwiederverwendung eingesetzt werden soll.

Für die Planung und Umsetzung des Ultrafiltrationsverfahrens in konventionellen Kläranlagen zur Verhinderung der Ausbreitung von Antibiotikaresistenzen wurden Auslegungswerte der Membranfiltrationstechnologie für großtechnische Anwendungen ausgearbeitet. Für den Einsatz auf der Kläranlage Steinhäule in Neu-Ulm wurde der Entwurf einer großtechnischen Ultrafiltrationsanlage konzipiert.

Der Eintrag von pathogenen Bakterien, die Antibiotikaresistenzen tragen, kann in Oberflächengewässer eine potentielle Gefahr für die menschliche Gesundheit darstellen, insbesondere wenn die Flüsse flussabwärts, z. B. für Erholungszwecke oder zur Trinkwassergewinnung verwendet werden. Das akute und langfristige Risiko, das sich aus der Freisetzung von Resistenzgenen in die aquatische Umwelt ergibt, aber auch das Vorhandensein eines natürlich vorkommenden Resistenzhintergrunds, erfordert jedoch weitere Untersuchungen. Weitere Forschungsarbeiten sind erforderlich, um die potenziellen Übertragungswege und die Bedrohung der menschlichen Gesundheit durch Resistenzgene im Wasser besser zu verstehen, auch wenn sie nicht mit lebenden pathogenen Bakterien in Verbindung gebracht werden. Diese Informationen werden

benötigt, um eine risikobasierte Festlegung von Entfernungszielen für die Abwasserbehandlung zu ermöglichen.

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ABBREVIATIONS AND ACRONYMS

AMR	Antimicrobial resistance
AOP	Advanced oxidation processes
ARB	Antibiotic resistant bacteria
ARG	Antibiotic resistance gene
BMBF	German Federal Ministry of Education and Research
CAS	Conventional activated sludge treatment
CEB	Chemical enhanced backwash
COD	Chemical oxygen demand
DIN	Deutsches Institut für Normung
DNA	Deoxyribonucleic acid
DOC	Dissolved organic carbon
dPCR	digital polymerase chain reaction
dsDNA	double stranded DNA
eARG	Extracellular ARG
ECDC	European Centre for Disease Prevention and Control
EPS	Extracellular polymeric substances
EU	European Union
EU WRRL	Europäische Wasserrahmenrichtlinie
GAC	Granulated activated carbon
H_2SO_4	Sulphuric acid
HCl	Hydrochloric acid
HGT	Horizontal gene transfer
HNAC	High nucleic acid count
iARG	Intracellular ARG
ICE	Integrative and conjugative elements
ID	Identification number
ISO	International Organization for Standardization
kDa	Kilo Dalton
LC–MS/MS	Liquid chromatography coupled with tandem mass spectrometry
LMH	Liters per square meter per hour
LNAC	Low nucleic acid count
LOD	Limit of detection
LOQ	Limit of quantification
Max	Maximum
MBR	Membrane bioreactor
MDa	Mega Dalton
MF	Microfiltration
MGE	Mobile genetic element
Min	Minimum
MWCO	Molecular weight cut-off
n	Number of observations/measurements
NaOCl	Sodium hypochlorite
NaOH	Sodium hydroxide
NF	Nanofiltration
NOM	Natural organic matter
O_3	Ozone
PAC	Powdered activated carbon
PACl	Polyaluminium chloride
PCR	Polymerase chain reaction
PES	Polyethersulfone
ppm	Parts per million
PTFE	Polytetrafluoroethylene
PVDF	Polyvinylidene fluoride
qPCR	real-time quantitative digital polymerase chain reaction
Re	Reynolds number

RNA	Ribonucleic acid
RO	Reverse osmosis
rRNA	Ribosomal ribonucleic acid
SE	Secondary effluent (after conventional wastewater treatment)
SF	Sand filtration
std	Standard deviation
TCC	Total cell count
TE	Tertiary effluent (secondary effluent after PAC adsorption and sand filtration)
TMP	Transmembrane pressure
TOC	Total organic carbon
UBA	Umweltbundesamt
UF	Ultrafiltration
UN	United Nations
UV	Ultraviolet
UV_{254}	Ultraviolet absorbance at 254 nm
v %	volume percent
VGT	Vertical gene transfer
WFD	Water Framework Directive
WHO	World Health Organisation
WWTP	Wastewater treatment plant

1 INTRODUCTION

In human and veterinary medicine, diseases caused by bacteria are commonly treated by antibiotics. However, antibiotics are also applied in agriculture and aquaculture to combat pathogenic bacteria. Although antibiotics may no longer be used prophylactically or to promote growth in industrial agriculture throughout the EU since 2006, antibiotic consumption in agriculture has rarely decreased.

Humans and animals metabolize only about 30 to 90 % of the antibiotics they take. As a result, several tons of antibiotics contribute to the influent of wastewater treatment plants each year via feces and urine (Gao et al., 2012). This comes with unintended consequences since even low concentrations of antibiotics in wastewater can lead to bacterial adaption leading to antibiotic resistant bacteria (Gullberg et al., 2011). The bacterial concentration between the influent and effluent of a wastewater treatment plant can be reduced with current mechanical and biological treatment stages by several orders of magnitude. However, a sufficient removal of pathogenic antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) is not possible while employing conventional mechanical, biological and adsorptive treatment stages. Pathogenic antibiotic resistant bacteria including multi-antibiotic resistant bacteria as well as antibiotic resistance genes are thus emitted into the urban water cycle through point sources such as wastewater treatment plants (Yang et al., 2008; Sigala, Unc, 2012; Rizzo et al., 2013; Li et al., 2015; Um et al., 2016).

The consequence of an ever-increasing antibiotic consumption is an increase in the abundance of antibiotic resistant bacteria and antibiotic resistance genes in the environment, food, and drinking water. The increase of antibiotic resistance in the environment is accompanied with inefficient antibiotics that cannot be applied anymore against bacterial infections in human and veterinary medicine. For the therapy of bacterial infections in humans and animals, the pharmaceutical industry is constantly developing new antibiotics, whereby bacterial adaption to newly developed antibiotics occurs within an even shorter period of time. Therefore, the use of antibiotics in human and veterinary medicine should be reduced to the necessary level.

Given these challenges, it is important to examine the role of conventional and advanced wastewater treatment plants (WWTPs) regarding an effective removal of antibiotic resistance in the aquatic environment. So far, the spread of antibiotic microbial resistance (AMR) in the aquatic environment has been studied only during basic research studies. Current water directives for wastewater treatment in Europe do not impose requirements regarding the removal of antibiotic resistant bacteria. Antibiotic resistance

is even not part of statutory provisions in water treatment. Therefore, basic research is needed to define a hazard risk for the human or veterinary environment.

This dissertation project aims to review the current knowledge of the spread of antibiotic resistance caused by treated wastewater discharges of WWTPs in the urban water cycle. Subsequently, systematic investigations on the reduction of bacteria and ARGs by membrane filtration of effluents were conducted using a pilot-scale ultrafiltration plant at the WWTP Steinhäule. The use of capillary membranes with regard to the removal of bacteria and ARGs under different operating conditions in the effluent of a conventional secondary clarification, after a powdered activated carbon plant and in the effluent of a powdered activated carbon plant with a sand filtration was investigated.

2 STATE-OF-THE-ART

2.1 Antibiotic Resistance – The Global Threat to Human Health

In 1928, Sir Alexander Fleming discovered penicillin for medical use (Ventola, 2015) and a new age of medical care began saving millions of lives. The increase of antibiotics consumption intensified the selection pressure to bacteria in the environment. However, in 1940 the first penicillin resistant *Staphylococcus* was identified. Penicillin resistance was tried to medicate with a new antibiotic. Introducing tetracycline in 1950, the first tetracycline resistant *Shigella* was identified in 1959. Methicillin was successfully established in 1960 and two years later the first methicillin resistant Staphylococcus was identified. Methicillin resistance was treated with vancomycin in 1972 ending with vancomycin resistant *Enterococcus* in 1988 (Ventola, 2015). Today, antibiotics are the first choice also in aquaculture and livestock farms for veterinary therapy. In 2010, 63,151 tons of antibiotics (Van Boeckel et al., 2015) were produced, consumed and metabolized incompletely in humans and animals. According to Du and Liu (2011) 30 to 90 percent of the ingested antibiotic was emitted by urine and feces. Not metabolized antibiotics find their way into the urban water cycle via wastewater, manure and biosolids. Multi-resistant bacteria to several antibiotics were detected. The water cycle is one of the most important means of the distribution of antibiotic resistance (Li et al., 2015; Rizzo et al., 2013; Sigala, Unc, 2012). Within the urban water cycle, raw water is purified for drinking water. Pathogens can be transmitted when bathing in contaminated water or drinking untreated water.

Antibiotic resistance and the global threat to human health was quantified for the year 2019 in an article (‘Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis’) of the Lancet published in 2022. It was reported that infections with antibiotic-resistant bacteria are among the most common causes of death worldwide. 4.95 million people lost their lives in 2019 due to diseases associated with AMR. 1.3 million died directly caused by infections with antibiotic resistant bacteria – this means that more people died due to antibiotic resistant bacterial infections than from AIDS or malaria. Informations of a literature study, from hospitals and further sources were evaluated whereas 470 million data sets were summarized in this article. The study results revealed that the six deadliest bacterial pathogens were responsible for almost three quarter of all deaths attributed to antibiotic resistance. Approximately 200,000 people died in 2019 as a result of infection with antibiotic resistant *Escherichia coli (E. coli)* alone. Hundreds of thousands of people died contracting *Staphylococcus aureus, Klebsiella pneumoniae, Streptococcus pneumoniae, Acinetobacter baumannii* or *Pseudomonas aeruginosa*. The number of deaths from a specific pathogen bacterium significantly differ by region. Half

of those who died from AMR were infected by *Staphylococcus aureus* and *Escherichia coli* detected in countries with high-income. In contrast, in African countries people predominantly died due to *Streptococcus pneumoniae* and *Klebsiella pneumoniae*. However, all mentioned pathogenic bacteria are water-borne microorganisms.

2.2 Water quality challenges concerning the prevention of the spread of antibiotic resistance in the urban water cycle

2.2.1 Pathogens

Pathogens can cause infections in human and animal body. Many pathogens can grow in the aquatic environment and adhere to suspended solids in water so that infective dose measured by the average water concentration in water samples cannot be predicted. The disease caused by pathogens depends on the infective dose, invasiveness and virulence of the pathogen, and the individual state of health of a human or animal. The World Health Organization has summarized pathogenic microorganisms (indicator organism) like bacteria, viruses, protozoa and helminths that are shown in Table 2-1.

Table 2-1: (Indicator) microorganisms that can be present in drinking water and are pathogenic (WHO, 2022(2)).

Pathogen	Type species/ genus/group[b]	Health significance[c]	Persistence in water supplies[d]	Resistance to chlorine[e]	Relative infectivity[f]	Important animal source
Bacteria						
Burkholderia	*B. pseudomallei*	High	May multiply	Low	Low	No
Campylobacter	*C. coli* *C. jejuni*	High	Moderate	Low	Moderate	Yes
Escherichia coli – Diarrhoeagenic[g]		High	Moderate	Low	Low	Yes
E. coli – Enterohaemorrhagic	*E. coli* O157	High	Moderate	Low	High	Yes
Francisella	*F. tularensis*	High	Long	Moderate	High	Yes
Legionella	*L. pneumophila*	High	May multiply	Low	Moderate	No
Mycobacteria (non-tuberculous)	*Mycobacterium avium* complex	Low	May multiply	High	Low	No
Salmonella typhi		High	Moderate	Low	Low	No
Other salmonellae	*S. enterica* *S. bongori*	High	May multiply	Low	Low	Yes
Shigella	*S. dysenteriae*	High	Short	Low	High	No
Vibrio	*V. cholerae* O1 and O139	High	Short to long[h]	Low	Low	No
Viruses						
Adenoviridae	Adenoviruses	Moderate	Long	Moderate	High	No
Astroviridae	Astroviruses	Moderate	Long	Moderate	High	No
Caliciviridae	Noroviruses, Sapoviruses	High	Long	Moderate	High	Potentially
Hepeviridae	Hepatitis E virus	High	Long	Moderate	High	Potentially
Picornaviridae	Enteroviruses, Parechoviruses, Hepatitis A virus	High	Long	Moderate	High	No
Reoviridae	Rotaviruses	High	Long	Moderate	High	No

Protozoa						
Acanthamoeba	*A. culbertsoni*	High	May multiply	High	High	No
Cryptosporidium	*C. hominis/parvum*	High	Long	High	High	Yes
Cyclospora	*C. cayetanensis*	High	Long	High	High	No
Entamoeba	*E. histolytica*	High	Moderate	High	High	No
Giardia	*G. intestinalis*	High	Moderate	High	High	Yes
Naegleria	*N. fowleri*	High	May multiply	Low	Moderate	No
Helminths						
Dracunculus	*D. medinensis*	High	Moderate	Moderate	High	No

[a] This table contains pathogens for which there is some evidence of health significance related to their occurrence in drinking-water supplies. More information on these and other pathogens is presented in chapter 11.
[b] The type species listed (e.g. *L. pneumophila*) are those most commonly linked to waterborne transmission but other species may also cause disease.
[c] Health significance relates to the incidence and severity of disease, including association with outbreaks.
[d] Detection period for infective stage in water at 20 °C: short, up to 1 week; moderate, 1 week to 1 month; long, over 1 month.
[e] Within pathogen species and groups, there are likely to be variations in resistance, which could be further impacted by characteristics of the water supply and operating conditions. Resistance is based on 99% inactivation at 20 °C where, generally, low represents a Ct_{99} of < 1 min.mg/L, moderate 1–30 min.mg/L and high > 30 min.mg/L (where C = the concentration of free chlorine in mg/L and t = contact time in minutes) under the following conditions: the infective stage is freely suspended in water treated at conventional doses and contact times, and the pH is between 7 and 8. It should be noted that organisms that survive and grow in biofilms, such as *Legionella* and mycobacteria, will be protected from chlorination.
[f] From experiments with human volunteers, from epidemiological evidence and from experimental animal studies. High means infective doses can be 1–10^2 organisms or particles, moderate 10^2–10^4 and low > 10^4.
[g] Includes enteropathogenic, enterotoxigenic, enteroinvasive, diffusely adherent and enteroaggregative.
[h] *Vibrio cholerae* may persist for long periods in association with copepods and other aquatic organisms.

These pathogens are commonly present in municipal wastewater (Shannon al., 2007). Shannon et al. (2007) quantified the following pathogens in wastewater influents and effluents: *E. coli*, *K. pneumoniae*, *C. perfringens*, and *E. faecalis.* The pathogen removal efficiencies were 3.52–3.98, 4.23–4.33, 3.15–3.39, and 3.24 orders of magnitude respectively, between raw wastewater and the final effluent. However, remaining pathogens in WWTP effluents are emitted into surface waters whereas many of the microorganisms can survive and potentially impact downstream drinking water intakes.

Pathogens can be reduced and maintained at a lower level in water by applying chemical agents. Chlorination (Rose et al., 1996), ozonation and physical UV-irradiation (Gomes et al., 2019) are common disinfection technologies to prevent the increase of pathogens in a water purification plant. However, as listed in Table 2-1, specific microorganisms can be more resistant to chemical agents and might persist in water supplies.

2.2.2 Driving forces of the spread of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs)

Charles Darwin described in "*On the Origin of Species"* (1864) the mechanism of natural selection, better known as "survival of the fittest." The Darwinian evolutionary theory asserted that species perfectly adapted to the local environment are more likely to survive and to reproduce than those with less helpful adaptions. Neo-Darwinian evolutionists thought that natural evolution and genetic mutations together are the evolutionary promoters. 'Failures' in DNA replication are called mutations. Mutations cause the genetic change and maintain evolution (Thompson, 1994). However, does this evolutionary theory also apply to antibiotic resistance?

Due to high number of bacteria of microbial populations in tight spaces, bacteria live in competition. Bacteria discovered self-produced antibiotics to inhibit the increase of

other bacteria populations in order to gain a population advantage. For example, the bacterium mold *Penicillium chrysogenum* produces penicillin (Thompson, 1994). Surviving against bacteria producing antibiotics, other bacteria discovered antibiotic resistance due to selection pressure in order to survive in that environment. This process Lopez experienced with *Staphylococcus aureus* bacteria that have not been antibiotic resistant. The bacteria were hold under typical biofilm conditions in tight space and under limited nutrient supply. Mutations of *Staphylococcus aureus* occurred beside antibiotic production. Antibiotic producing bacteria were more successful in reproduction. After five days there were three bacteria groups at the biofilm: the harmless vaccinated bacteria, the antibiotic producing bacteria, and the antibiotic resistant bacteria (Lopez, 2014).

There are several resistance mechanisms bacteria discovered to fight against antibiotics. Antibiotics can be inactivated by building a double cell wall (e.g., ß-lactams and glycopeptide), inhibiting the protein production (e.g., macrolides and tetracyclines), operating with nucleic acid production (e.g., fluoroquinolones and rifampin), changing the membrane structure (e.g. aminoglycosides), discovering new transport ways through the membrane (e.g. chloramphenicol), and inhibiting the metabolic process (e.g., trimethoprim-sulfamethoxazole) (Giedraitiene et al., 2011).

This evolution process is not a new one. Antibiotic resistance is an ancient process and a phenomenon naturally happening. D'Costa et al. (2011) extracted samples from about 30,000-year-old Late Pleistocene permafrost sediments analyzing ancient DNA. They identified antibiotic resistance genes like the ribosomal protection protein *tetM* that refer to tetracycline antibiotic resistance; a penicillin-inactivating ß-lactamase *bla* was similar between 53 % and 84 % to already known determinants. Permutation tests showed that ancient *vanHA* clones have been similar to modern *vanH* and *vanA* genes and ancient *vanAX* clones have been similar to modern *vanA* and *vanX* genes (D'Costa et al., 2011).

Bacteria distribute the antibiotic resistance genes by vertical and horizontal gene transfer. In vertical gene transfer, bacteria have cell divisions. The new bacteria have the same DNA. Mutations in cell genes like chromosomal mutations can lead to an antibiotic producing bacterium. Cell divisions could maintain to dissemination of antibiotic resistance. The spread of antibiotic resistance can also be exhibited by the so-called horizontal gene transfer (Figure 2-1).

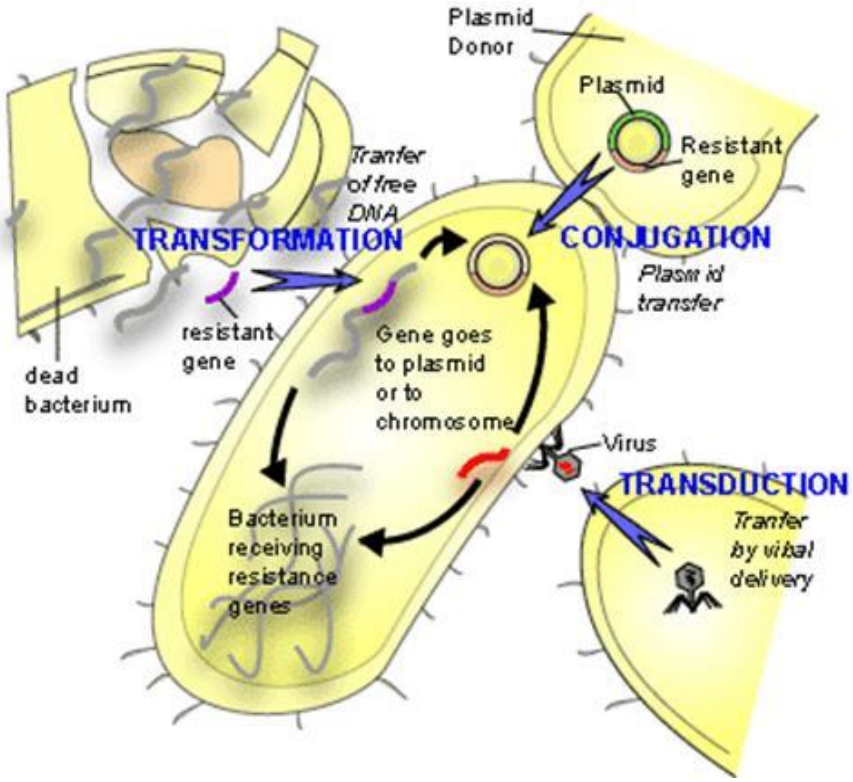


Figure 2-1: horizontal gene transfer occurs due to conjugation, transformation and transduction (Kim, 2016)

The horizontal gene transfer describes the gene transfer from bacterium to bacterium by plasmids and transposons (conjugation). Antibiotic resistance genes in plasmids can be incorporated from dead cells (transformation) and gene transfer occurs by bacteriophages and integrons (transduction) (Giedraitiene et al., 2011).

The widely and excessive consumption of antibiotics as well as incomplete metabolism of drugs result in the ubiquitous occurrence of these compounds in the environment at the nanogram to low microgram per liter concentration range. Studies published in the recent past suggest that even such low concentrations may impose a constant selection pressure on bacteria in natural ecosystems leading to formation of antibiotic resistance strains (Salcedo et al., 2015). Even the presence of very low antibiotic concentrations below the minimum inhibitory concentrations (MIC) might already result a spread of antibiotic resistant bacteria (ARB) (Gullberg et al., 2011; Chow et al., 2015).

To summarize, driving forces concerning bacterial adaption to antibiotic resistance (biotic and abiotic factors) are predominantly the biofilm composition and the nutrient supply, vertical and horizontal gene transfer, chromosomal mutations and antibiotic concentrations.

2.3 Analytical techniques for bacteria and AMR analyses

The predominant methods to analyze antibiotic resistance in water samples are cultivation- and molecular-based methods. Flow cytometry is another analytical method to quantify bacterial and viral cells in water samples.

2.3.1 Cultivation

Living bacteria from wastewater samples grow on selective culturing media using the cultivation-based method. Through the addition of antibiotics on selective culturing media it can be determined whether the growing bacteria are resistant or receptive to antibiotics. Antibiotic resistant bacteria can be qualified and quantified.

The advantages of the method are the standardized procedure and the results are easy to compare. Furthermore, this method is cheap for ARB determination and multi-antibiotic resistant bacteria are easy to determine. The disadvantage of the method is the fact that only limited predefined bacterial strains can be quantified in water samples. Due to the visible but not cultivable state (VNBC state) the cultivation method is not so accurate than molecular based methods. Furthermore, laboratory infections on the culture media can appear so that other living bacteria grow on the culture media. Finally, this method is time consuming (Rezaei, 2022).

2.3.2 Quantitative polymerase chain reaction (qPCR)

The prevalent molecular based method to identify microorganism is called the polymerase chain reaction technique on the basis of nucleic acid amplification. The PCR technique is illustrated in Figure 2-2.

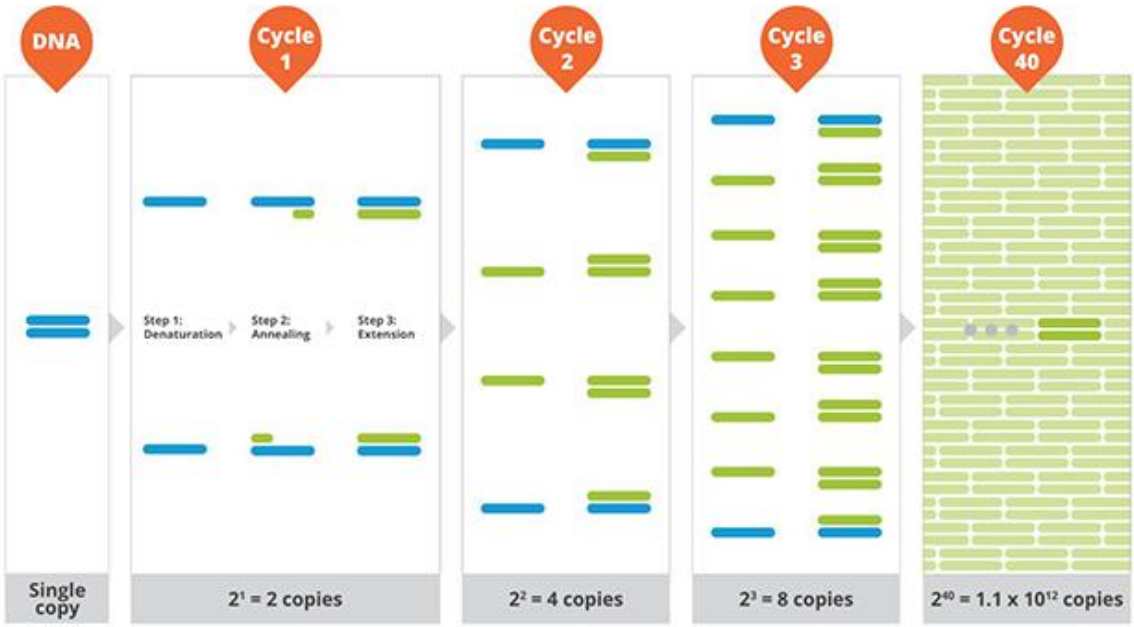


Figure 2-2: The basic molecular method is the quantitative polymerase chain reaction (qPCR) whereas DNA sequences were denaturated, annealed and extensioned to quantive genes in water samples (Rezaei, 2022)

The PCR enable to qualify and quantify antibiotic resistant bacteria and antibiotic resistance genes. The first step is the denaturation stage whereas the sample is heated and the double-stranded DNA (**d**eoxyribo**n**ucleic **a**cid**)** is separated. During the annealing stage, the temperature is decreased. Here, primers bind to regions of the target DNA sequence so that a photocopied or amplified copy can arise. In the extension phase, the temperature is increased whereas a new strand of DNA is produced. This described DNA amplification process is repeated in about 40 thermal cycles until all reaction components are consumed. The qPCR cycles are summarized in an amplification curve that can be described with a beginning phase, an exponential phase and a plateau phase. The time of the DNA amplification of the 40 thermal cycles correlates with the amount of target genes that have been in the water sample (Stewart, 2021).

The advantage of the molecular based method is the fact that the qPCR technique has a high specificity and a low measurement error of about 10 percent (Kitchen et al., 2010). ARGs can be determined across all bacterial strains and bacteriophages, mobile DNA fragments like plasmids, transposons and integrons as well as “free” environmental DNA (Quirós et al., 2014; Xu et al., 2016; Muziasari et al., 2014) and the examination is rapid within 24 hours. On the other hand, the qPCR technique is an expensive analyzing method for ARGs determination. Furthermore, several sample preparation protocols are applied. However, comparable results with different sample preparation protocols are not always achieved. In addition, the analyses of a specific gene give limited information about living antibiotic resistant bacteria due to the fact that the target gene was detected intra- or extracellular. This could be important aspect to consider for future AMR regulations.

2.3.3 Flow Cytometry

Flow cytometry enables to quantify the total cell counts (TCC) of a water sample. Furthermore, the flow cytometry can distinguish between small cells with low nucleic acid counts (LNAC) and larger cells with high nucleic acid counts (HNAC). The sum of LNAC and HNAC is the TCC. The relation of LNAC and HNAC sample describes the microbiological fingerprint of a water sample. Santos et al. (2019) investigated flow cytometry at different sampling sites of a river. Bacteria community analysis exhibited high HNAC density sampling downstream of the WWTP discharge. A higher LNAC density was analyzed sampling river headwater characterized by an oligotrophic environment. That is the reason why wastewater samples in this study were analyzed by flow cytometry whereas the HNAC value served as surrogate for total bacteria count. Even disruptions in the microbiological system can be observed analyzing LNAC and HNAC (Kötzsch, Sinreich 2014.). The principles of flow cytometry measurements are illustrated in Figure 2-3.

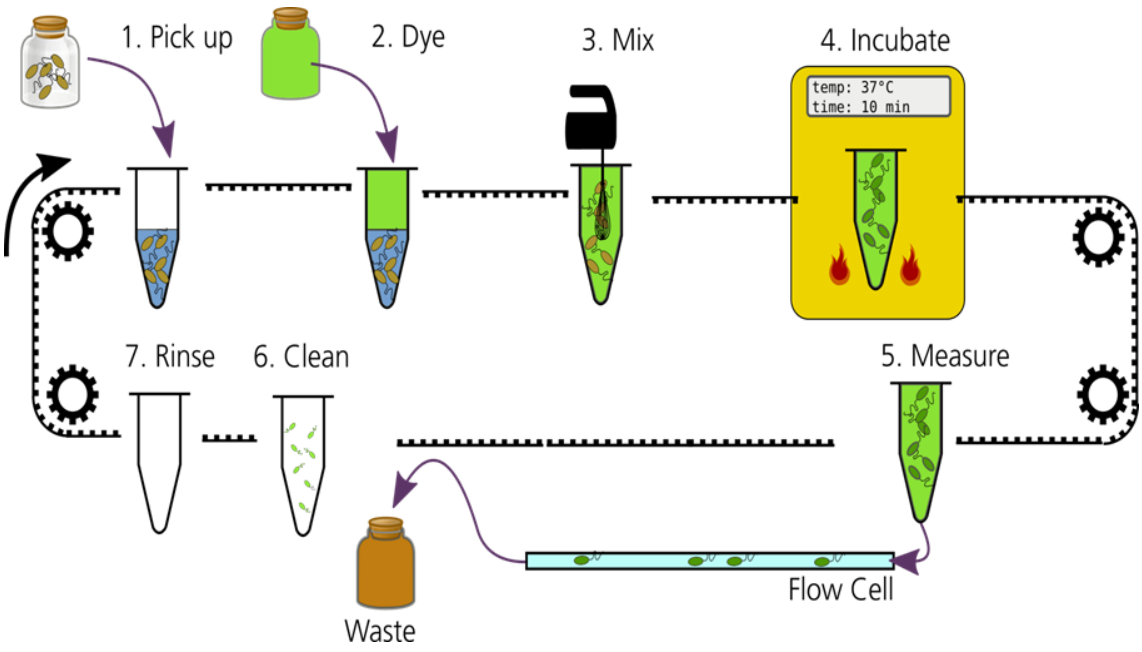


Figure 2-3: A: Flow cytometry analysis protocol with the steps sampling, dye dosing, mixing, incubating, measuring via flow cell, cleaning and rinsing (Sigrist, 2018).

Water samples were stained and mixed for example with the fluorescent dye SYBR® Green. After incubation of the water sample with the dye for 10 minutes at 37°C, the sample is sent to the flow cell. The fluorescent dye SYBR® Green binds to double stranded DNA (dsDNA). During measurement of the cells in the flow cell, a laser beam with 488 nm is continuously conducted to the flow cell. The cell measuring is based on the emission of optical signals from the cell when it passes the laser beam. Here, the given SYBR® Green dye let the cells emit fluorescent light. Each cell passing through the laser beam leads to light scattering and fluorescence light emission that depends on the applied

fluorescent dye. The signal is passed via filters to detectors, where the signal is recorded. The analysis software of the flow cytometer enables to assign each individual particle or cell a scattered light and fluorescence signal. The TCC, HNAC and LNAC values were analyzed using main fluorescent channels between 525 and 545 nm (FL1) and low pass fluorescent channels of more than 715 nm (FL2) (Sigrist, 2018). The signals of FL1 and FL2 of every particle or cell are summarized in a graph with x- and y-axes. In this graph, the measured particles or cells are fixed with a gating to quantify LNAC, HNAC and background signals using recommended values provided by the manufacturer (Kötzsch et al., 2012).

The advantage of the flow cytometry technique is a fast detection (20 minutes) of the total cell count of a water sample. The measurement accuracy is in the range of the molecular based methods (Sigrist, 2018). To distinguish between live and dead cells in water samples different dyes can be applied: For example, SYTO 9 nucleic acid stain showed intact cell membranes and fluoresces bright green. The applied fluorescent channel was 525 nm (FITC-H). For example, propidiumiodide indicates damaged membrane cells and fluoresces red. This application was executed in studies using LIVE/DEAD™ BacLight™ Bacterial Viability and Counting Kit, Thermo Fisher with an applied fluorescent channel of 690 nm (PC5.5-H). Furthermore, cell analyses can be differentiated with the application of sub-micron particles. The Sub-micron Particle Size Reference Kit (Thermo Fisher) with 0.2 µm beads was used. The application of defined sub-micron particles enables to quantify living and dead bacteria, whereas the gating can be adjusted to cells that are larger than 0.2 µm of the given sub-micron particles. The disadvantage of the flow cytometry technique is the fact that this measurement is expensive compared to cultivation-based methods. Only total bacteria and not specific bacterial strains can be quantified.

2.4 Membrane filtration process as advanced treatment process for AMR retention in WWTP effluents

2.4.1 Properties of MF and UF membrane modules

The pore sizes of MF membranes are commonly between 0.1 µm and 10 µm. UF membranes have pore sizes between 0.1 and 0.01 µm (Baker, 2012). There are different possibilities how pore sizes of membrane modules can be determined, but in all cases a given pore size always represents a pore size distribution. For example, the pore size or the molecular weight cutoff (MWCO) can be described as a molecule of a certain size that can be removed by 90 %. Therefore, a small amount of larger molecules or particles could theoretically migrate through the membrane. The so-called zeta potential of membrane surfaces and the arising electrostatic interactions is an important factor for

membrane fouling (e.g., salt concentrations). This fouling effect occurs when the membrane surface and solutes are oppositely charged (Breite et al., 2016). Another important factor for membrane fouling is the hydrophobicity of the membrane material commonly measured as contact angle. The smaller the contact angle is between the wetting liquid and the wetted solid surface, the higher is the wettability of the wetted solid surface. If the contact angle is between 0 and 90°, the wetted solid surface is hydrophilic. If the contact angle is more than 90°, the wetted solid surface is hydrophobic. While hydrophilic materials have a low fouling potential, hydrophobic membranes foul much faster in surface water or municipal wastewater (Melin and Rautenbach, 2007). Further aspects of the membrane properties are the roughness and the porosity of the membrane. In addition, the structure of the membrane (symmetric or asymmetric) is an important parameter for the membrane. While the symmetric membrane is often built up with uniform pores, the asymmetric membrane is produced with a thin, active layer out of small pores followed by a thick porous structure with larger pores. Furthermore, membranes can have a double-asymmetric structure to improve a particle flushing from the feed to the filtrate side. If symmetric and asymmetric membranes with the same pore sizes are compared to flux rates, asymmetric membranes have a remarkably higher flux rate (Ulbricht, 2015).

UF membranes are produced of ceramic and polymeric materials. Typical ceramic membranes are built up of various metal oxides such as aluminum oxide (Al_2O_3), zirconium oxide (ZrO_2), or titanium dioxide (TiO_2). Polymeric membrane materials are for example polysulfone, polyvinylidene fluoride, polyethersulfone or polypropylene. Ceramic membranes are more stable according to chemicals, high temperature or mechanical stress compared to polymeric membranes (Goswami and Pugazhenthi, 2020). Furthermore, ceramic membranes achieve a longer lifetime and integrity than polymeric membranes (Wang et al., 2019). Ceramic membranes can be operated with higher fluxes than polymeric membranes and the ceramic membrane are more hydrophilic than polymeric membranes (Jeong et al., 2018; Miehe et al., 2013). However, ceramic membranes are more expensive than polymeric membranes (Miehe et al., 2013).

2.4.2 Key factors of MF and UF removal efficiency

The removal efficiency of MF and UF membranes depends on the membrane properties as well as chemical and physical characteristics of the feed water. The membrane properties were described in chapter 2.4.1. Important biological, chemical and physical characteristics of the feed water are organic matter, water temperature, particle concentration, particle weight and geometry, particle charge and particle hydrophobicity, pH-value, ionic strength, and hardness of the water.

For the retention of particles like bacteria and virus, the interaction of the aforementioned parameter has to be considered. The size exclusion, cake layer formation, adsorption effects on the membrane surface according to opposite or identical electrical charge effects, and hydrophobic effects between membrane and particle are the predominant effects for particle or bacteria removal efficiencies (Goswami and Pugazhenthi, 2020).

However, factors affecting AMR removal during membrane filtration processes are still not fully understood and are the focus of this dissertation. Previous studies examined the removal of bacteria and viruses and concluded that different mechanisms exist. Elhadidy et al. (2013) studied the pore size distribution of ultrafiltration membranes with nominal pore size of 40 nm by atomic force microscopy and reported pores up to 90 nm. Therefore, a small number of larger molecules or particles could theoretically migrate through the membrane. Additional factors for ARB and ARGs breakthrough are membrane materials (Liu et al., 2019) and the increase of flux and TMP. Liu et al. (2019) reported rejection of bacteria by membranes of similar pore size (0.1 µm) but four different materials. While Hylemonella bacteria could pass the pores of polyvinylidene fluoride and polyethersulfone filters, no transmission was detected using polycarbonate and mixed cellulose esters filters. The increase of flux and TMP resulted in plasmids (Arkhangelsky et al., 2011), viruses (Arkhangelsky and Gitis, 2008), and bacteria (Suchecka et al., 2003) breakthrough. The breakthrough effect caused by higher flux and TMP was the result of membrane pore enlargement (Arkhangelsky and Gitis, 2008) and of bacterial cell deformation (Suchecka et al., 2003). The bacterial cell deformation seems to be strongly depended on the cell-wall structure of the bacteria. Lebleu et al. (2009) while investigating MF membranes concluded that the bacteria removal depends on the kind of bacteria. While gram-positive bacteria have a thicker peptidoglycan layer and thus are less formable and better retainable by MF, gram-negative bacteria with their thin peptidoglycan layer enable their better deformation and transmission through MF pores. In addition, Slipko et al. (2019) investigated extracellular DNA breakthrough during membrane filtration. They concluded that both size exclusion and surface charge of the membrane were important for extracellular DNA retention. Hence, negatively charged membranes exhibited lower free DNA retention than neutral charged membranes. In addition, extracellular DNA like plasmids are ARG carriers and can pass membranes by elongation in the converging and accelerating flow fields, which usually occurs above the openings of the membrane pores. This transport of small particles like free DNA or plasmids through the membrane has been reported by Arkhangelsky et al. (2011), Latulippe et al. (2011), and Schwaller et al. (2022).

3 RESEARCH OBJECTIVES, HYPOTHESES AND DISSERTATION STRUCTURE

This chapter represents the research objectives with associated research hypotheses as well as the general structure of this dissertation.

3.1 Research objective #1

State-of-the-art – Antibiotic resistance in the aquatic environment and WWTP discharges

A comprehensive literature study regarding AMR occurrence, spread and prevention in the aquatic environment and by WWTP discharges was performed. Several studies reporting total ARB and ARGs' abundances in the aquatic environment suggest an anthropogenic contribution of antibiotic resistance worldwide (Alexander et al., 2016; Munir and Xagoraraki, 2011b; Allen et al., 2010; Kristiansson et al., 2011). Thus, peer-reviewed literature was also reviewed concerning 'pristine' or 'natural' antibiotic resistance in aquatic environments in order to provide an assessment of possible differences in comparing anthropogenic affected surface waters and pristine surface waters. Actually, no scientific definition of natural antibiotic resistance exists and the levels of naturally-occurring resistance can vary widely (Rothrock et al., 2016). Monitoring of fate and transport of AMR was focused on different antibiotic classes.

In order to compare removal efficiencies of different advanced wastewater treatment processes and different aquatic environments, indicator ARB and ARGs have been determined as a point of reference. Resistance to broad-spectrum antibiotics can be used as an indicator to evaluate removal efficiencies during different treatment processes, whereas AMR to antibiotics of last resort are more relevant to assess associated health risks. Thus, study results revealed that the resistance genes of broad-spectrum antibiotics like *sul* genes, *erm* genes and *tet* genes with high detected ARGs abundances were suggested to monitor for AMR. Antibiotic resistance gene of antibiotic of last resort *vanA* is evaluated according to its important medical application.

However, the number of studies reporting total ARGs abundances in low impacted surface waters is insufficient to carry out any statistical analysis. Thus, results of relative ARGs abundances (normalized by 16S gene copies) in conventional WWTP effluents, urban rivers (anthropogenically-affected by upstream WWTP discharges), and low impacted rivers (no WWTP discharges) were compiled. On the basis of this analysis, a decreasing relative ARG abundance was observed from conventional WWTP effluents, to urban rivers and very low detected relative ARG abundances in low impacted rivers. In some studies, however, the ARG analyses revealed unexpectedly higher total ARG

abundances in urban rivers compared to WWTP effluents (Böckelmann et al., 2009; Makowska et al., 2016). This might be a result of contributions by combined sewer overflow (CSO) or other sources upstream of WWTPs since urban rivers are not characterized as low impacted waters in many settings.

In order to assess whether conventional WWTP discharges directly influence AMR abundances of surface waters, the review also focused on AMR studies that have collected corresponding samples upstream and downstream of conventional WWTP discharges. The study results revealed that the majority of all corresponding samples exhibited an increase of AMR in surface waters after discharge of conventional WWTP effluents with the downstream samples representing a mixture of AMR abundances from upstream surface water and WWTP effluent.

Advanced treatment processes were investigated in order to assess individual AMR removal efficiencies resulting in AMR levels similar to low impacted surface water levels. The removal of ARGs by oxidation and disinfection processes is closely related to the mode of action of the respective oxidants. Disinfection processes provide an efficient barrier for pathogenic bacteria independent whether they carry AMR. However, it is noteworthy that ozonation of wastewater sometimes is designed for the removal of trace organic chemicals at which dose it cannot be considered a reliable disinfection process. The review results of advanced treatment processes illustrate that the most efficient ARGs removal was achieved by membrane filtration technologies. Therefore, in this dissertation low-pressure membrane filtration technology is evaluated for further in-depth examinations. However, nanofiltration and reverse osmosis are not investigated further due to the known drawback of brine generation for which limited options exist to safely and economically dispose concentrate streams.

The outcome of the literature studies addressing research objective #1 is summarized in paper #I published in the journal '*Science of the Total Environment*.' The review paper entitled '**Antibiotic microbial resistance (AMR) removal efficiencies by conventional and advanced treatment processes: A review**' is presented in chapter 4.

3.2 Research objective #2

Investigation of key factors influencing antimicrobial resistance (AMR) removal efficiency of MF/ UF technology during standard filtration mode

Already published membrane filtration studies focused on the reduction of ARB and ARGs during standard filtration mode. However, key operational parameters for the operation of the membrane filtration are often not reported suitably. Knowledge gaps about key factors influencing AMR removal efficiency during membrane filtration of

wastewater exist. Therefore, the aspects of the bacteria and ARGs loads in the feed water and the consequences to filtrate quality, the pore size of membranes, and the possible effect of an increasing fouling layer with progressive filtration time on ARG removal efficiencies are the focus of research objective #2.

ARG abundance can be divided into intrachromosomal and extrachromosomal ARGs. Beside of incorporated ARGs in the DNA of bacteria, ARGs can be part of the DNA in bacteriophages or part of 'free DNA' for example in free plasmids, transposons or integrons. Hence, ARGs can be present either intrachromosomal or extrachromosomal in wastewater effluents. Intrachromosomal ARGs should be restrained by ultrafiltration due to larger size of bacteria while extrachromosmal ARGs, the "free DNA" is a long, flexible and thin molecule which could theoretically filtrate length-wise through the membrane and could pass pores in this way (Breazeal et al., 2013). Removal of extrachromosomal ARGs of 'free DNA' by membrane filtration processes has not been previously examined in scientific studies but is addressed in this dissertation.

To address this research objective, core hypothesis I '*ARGs removal efficiency of micro- and ultrafiltration of WWTP effluent is higher than 90 percent during standard filtration mode*' is proposed and divided into three sub-hypotheses:

> ***Research hypothesis #2.1:*** *The increase of a fouling layer with progressive filtration time will lead to a higher AMR removal efficiency.*
>
> ***Research hypothesis #2.2:*** *Higher AMR feed abundance results in significantly higher AMR abundance in UF filtrate.*
>
> ***Research hypothesis #2.3:*** *Comparing AMR removal efficiencies of membranes with large pore size differences (UF = 20 nm vs. MF = 450 nm) will result in significantly different AMR removal.*

Research hypotheses #2.1, #2.2 and **#2.3** are addressed in **Chapter 5**: Pilot-scale membrane filtration studies were conducted. The study results revealed that the key factor for AMR removal is the pore size of the membrane. Furthermore, it was concluded that higher ARG abundance in the feed resulted in higher ARG abundance in the filtrate. The built-up of a fouling layer with progressive filtration time exhibited a decrease of predominant intra- and extrachromosomal *vanA* gene. However, no additional removal effect was observed by *sul1* and *ermB* genes. Surprisingly analyzing living and dead bacteria in UF filtrate showed intact bacteria breaking through the UF membrane. In addition, the penetrated bacteria in UF filtrate correlated with *sul1* gene.
Examinations of the research objective #2 lead to paper II published in the journal *'Science of the Total Environment'* entitled 'Removal of antibiotic microbial resistance by micro- and ultrafiltration of secondary wastewater effluents at pilot scale' presented in chapter 5.

3.3 Research objective #3

Investigation of key factors influencing antimicrobial resistance (AMR) removal efficiency of UF technology directly after backwash and CEB mode

Many membrane filtration studies elaborating on the reduction of ARB and ARGs during standard filtration mode. However, the AMR removal efficiency of the membrane filtration process was not studied directly after backwash and CEB mode so far. Thus, investigations of research objective #3 focused on backwash and CEB modes, whereas samples were taken immediately after the backwash and the chemical enhanced backwash within the first minute, after 5 minutes, and after 55 minutes during UF standard filtration mode. The core hypothesis II '*Chemical enhanced backwash results in significant higher ARGs levels in the UF filtrate than during regular backwash*' is examined on the basis of four sub-hypotheses to address research objective #3:

> ***Research hypothesis #3.1:*** *CEB will result in higher total biomass and ARGs abundance in UF filtrate within the first minute of standard filtration mode than backwash.*
>
> ***Research hypothesis #3.2:*** *Lower cake layer after backwash and CEB mode will result in higher AMR abundances in UF filtrate.*
>
> ***Research hypothesis #3.3:*** *Backwash mode and CEB mode will result in higher AMR abundance in UF filtrate due to contaminated filtrate from the filtrate tank.*
>
> ***Research hypothesis #3.4:*** *CEB event using sodium hypochlorite instead of sodium hydroxide will result in significantly lower biomass and ARG abundances in UF filtrate.*

Research hypotheses #3.1, #3.2, #3.3 and #3.4 are tested in **Chapter 5**: Findings revealed that the total biomass, detected as HNAC, TCC and *16S rRNA* gene and measured directly after backwash and CEB within the first minute were up to one order of magnitude higher compared to 5 and 55 minutes of standard filtration mode. While *ermB* and *vanA* genes exhibited no increase after backwash and CEB, *sul1* gene increased significantly within the first minute. However, further investigations revealed that the increase in biomass and *sul1* gene was not caused by less efficient membrane retention in the absence of a cake layer immediately after backwash and CEB, but by microbial regrowth in the backwash tank contaminating the backwash water. The comparison of TCC and HNAC abundances after CEB mode of sodium hydroxide (train #1) and sodium hypochlorite (train #2) showed high TCC and HNAC abundances that only appeared during CEB with sodium hydroxide. It was concluded that CEB with sodium hydroxide had both a low disinfection effect on TCC and HNAC abundance. In contrast, the CEB event using sodium hypochlorite resulted in significantly lower biomass abundances in

UF filtrate. However, an ARG reduction accompanied by total biomass reduction, using CEB with sodium hypochlorite, was not observed.

Studies addressing research objective #3 resulted in paper III published in the journal 'Membranes' entitled 'Impact of backwash and CEB modes on retention of biomass and antibiotic resistance genes during ultrafiltration of WWTP effluents' presented in chapter 6.

3.4 Research objective #4

Investigation of pretreatment and post-treatment steps to prevent an ARG associated regrowth at UF effluent.

Since AMR removal during standard filtration mode was examined in previous UF studies in depth, this UF study is the first to report lower total AMR removal efficiency due to ARG associated regrowth effects. Published studies reported of ARGs abundance in UF filtrate above the limit of detection. It was hypothesized that further extrinsic factors like temporarily stagnant water, antibiotic concentrations and the hydraulic retention time could affect ARG associated regrowth. In addition, no mitigation measures were studied so far that prevent ARG associated regrowth at UF filtrate side under economic conditions. This research objective #4 is addressed by three core hypotheses: Hypothesis III '*AMR associated regrowth in UF filtrate can be prevented by activated carbon pretreatment of feed water*,' and hypothesis IV '*Presence of antibiotics in secondary effluent can cause bacterial adaption to antibiotic resistance in UF filtrate*', and hypothesis V '*AMR regrowth in the UF filtrate of ultrafiltered secondary effluent can be prevented by continuous chlorine dosing*'. To test these hypotheses, the following sub hypotheses were proposed:

> ***Research hypothesis #4.1:*** *AMR associated regrowth in UF filtrate can occur using secondary effluent as feed.*
>
> ***Research hypothesis #4.2:*** *Advanced wastewater treatment of the feed wastewater prior to ultrafiltration will result in no ARG associated regrowth in the filtrate water in a bypass filtrate tank.*
>
> ***Research hypothesis #4.3:*** *Typical erythromycin and sulfamethoxazole concentrations of secondary effluent will not result in antibiotic resistant bacteria at the UF filtrate side.*
>
> ***Research hypothesis #4.4:*** *The filtrate tank in continuous flow mode can reduce ARG associated regrowth effect compared to bypass mode operation.*

Research hypothesis #4.5: *Continuously dosing 0.5 mg/L sodium hypochlorite in UF filtrate can successfully prevent ARG associated regrowth in UF filtrate tank during continuous flow mode using secondary effluent as feed.*

Research hypotheses #4 are tested within **Chapter 7**: The biomass and ARG analyses in UF filtrate and biofilm samples revealed that *sul1* gene, *intl1* gene and bacteria measured as *16SrRNA* gene can break through the UF membrane module. It seems that the increase of this penetrated bacteria was accompanied with an increase of *sul1* and *intl1* genes at the filtrate side with progressive filtration time. Furthermore, the temporarily stagnant water, the hydraulic retention time and antibiotic concentrations were examined as extrinsic factors for ARG associated regrowth. Typical antibiotic concentrations measured in secondary effluent and in the range of PNEC values relevant to human health revealed no selection pressure to antibiotic resistant bacteria at the filtrate side. While three hours of hydraulic retention time in filtrate tank in continuous flow mode resulted in about 1 log unit higher *sul1* gene resistant bacteria, temporarily stagnant filtrate water in filtrate tank in bypass mode strongly promoted *sul1* gene resistant bacteria to increase by more than 3 log units. Since analyzed ARGs like *ermB* gene and *vanA* gene did not increase at the filtrate side, other not detected ARGs like the *sul1* gene could also increase.

In order to prevent ARG associated regrowth and to maintain a high ARG removal efficiency, pretreatment of the feed wastewater by advanced treatment processes was examined as a potentially successful measure. Activated carbon treatment and sand filtration of the feed wastewater could significantly reduce ARG and bacteria abundances in the feed so that lower ARG and bacteria abundance at the filtrate side occurred resulting in no ARG associated regrowth. Continuously dosing 0.5 mg/L sodium hypochlorite at the UF filtrate side is an alternative option to successfully prevent ARG associated regrowth using secondary effluent as feed.

ARG associated regrowth studies of the UF process addressing research objective #4 are presented in paper IV entitled ‘Factors affecting antibiotic resistance gene associated regrowth in WWTP effluents after membrane filtration’. This paper is published in the journal ‘Water Research’ and presented in chapter 7.

3.5 Research objective #5

Investigations of UF pilot-scale examinations as a basis for upscaling to a UF full-scale facility.

The use of capillary membranes with regard to the removal of bacteria and ARGs under different operating conditions in the effluent of a conventional secondary effluent,

after dosing powdered activated carbon, and after dosing powdered activated carbon plant followed by sand filtration was investigated as part of research objective #5. While the capillary membrane filtration technology is the state-of-the-art in water treatment in Germany, membrane filtration has so far been used in wastewater treatment predominantly as membrane bioreactor process (MBR) mainly with microfiltration membranes. The MBR process is operated with a high dry matter content, low flux, high membrane pressure, high pumping costs and low backwash intervals compared to capillary membranes. The pilot-scale membrane filtration plant with capillary membranes is operated with the aforementioned effluents at the WWTP Steinhäule, whereby these effluents have low turbidity of < 1-5 FNU, which would favor economical operation of the capillary membrane filtration technology. However, for full-scale implementation the following research questions were addressed: Where is the best position for the implementation of the membrane filtration process within the wastewater treatment process at WWTP Steinhäule? What are the operational conditions to achieve a high AMR removal efficiency while maintaining economic conditions? What strategies are needed to prevent AMR associated regrowth?

Design considerations of the UF pilot-scale studies are being discussed in **Chapter 8**. The study results demonstrated that the tertiary effluent is the best position for the implementation of the UF process within the wastewater treatment process at the WWTP Steinhäule. The comparison of secondary and tertiary effluents showed a *sul1* gene resistant bacteria breakthrough in the UF while treating secondary effluent. This bacteria breakthrough effect resulted in a *sul1* gene associated regrowth at the UF filtrate side. In contrast, no *sul1* gene resistant bacteria breakthrough effect and no *sul1* gene associated regrowth at UF filtrate side was observed using tertiary effluent as feed. Furthermore, the UF pilot-scale studies revealed that optimal operational conditions using tertiary effluent occurred at a sustainable flux of 70 LMH. This UF operation showed the demand of 2 mg/L polyaluminum chloride (PAC) as continuous coagulation dosage so that the cake layer formation during dead-end operation could be removed sustainably during backwash mode. The backwash mode was executed at a flux of 230 LMH. The CEB was performed once a day, using 150 ppm of sulfuric acid and 150 ppm of sodium hydroxide. The application of tertiary effluent as feed resulted in no ARG associated regrowth effects in the bypass filtrate tank. Thus, in this case no additional advanced treatment technology (e.g., continuous chlorination) is necessary to prevent a possible ARG associated regrowth at the UF filtrate side. A UF full-scale plant for the WWTP Steinhäule is planned for a treatment capacity of a maximum flow of 3,965 L/s. Planning documents and a cost calculation are presented in chapter 8.

3.6 Dissertation structure

This dissertation consists of a cumulative collection of four peer-reviewed research papers, whereas every paper represents an individual chapter of this dissertation. **Chapter 4** represents **Paper I**, whereas research objective #1 (section 3.1) is addressing the state-of-the-art of antibiotic resistance in the aquatic environment and in WWTP discharges.

In **Chapter 5**, **Paper II** addresses the research objective #2 (section 3.2). MF/UF pilot-scale studies during standard filtration mode were conducted to quantify the total biomass and ARGs removal efficiencies and define factors affecting the total biomass and ARGs removal efficiencies.

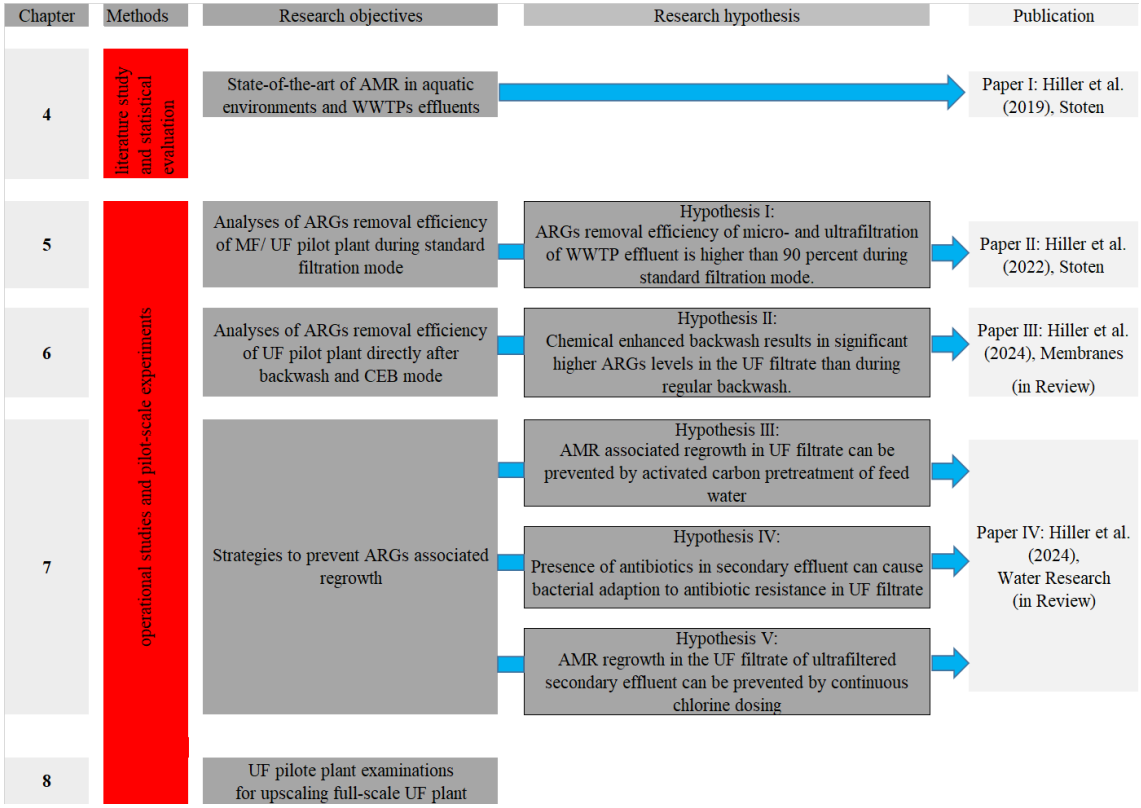
The **Paper III** is presented in **Chapters 6** focusing on research objective #3 (section 3.3). This chapter addresses the total biomass and ARGs removal efficiencies of the UF pilot-scale studies conducted directly after backwash and CEB mode.

The research objective #4 (section 3.4) was investigated in **Chapter 7** and published as **Paper IV**. These UF pilot-scale studies reveal the impact of feed quality and the consequences to filtrate quality inclusive ARG associated regrowth. Extrinsic factors affecting ARG associated regrowth at UF filtrate like temporarily stagnant water, hydraulic retention time and antibiotic concentrations are tested and are evaluated.

In **Chapter 8**, research objective #5 (section 3.5) was further investigated. Here, design values of the UF pilot-scale studies were considered for upscaling to a full-scale UF facility.

The structure of the cumulative dissertation with the chapter, the applied methods, all research objectives, research hypotheses as well as publications are summarized in Table 3-1.

Table 3-1: Dissertation structure with research objectives, hypotheses and publications. Former Hypothesis V of the Research Proposal with the topic 'Typical chlorine dosages of CEB and CIP present in UF filtrate can result in bacterial selection and a significant increase of erythromycin & sulfonamide resistant bacteria downstream of ultrafiltration' was part of the examination in research objective #3 with the research hypothesis #3.4 entitled 'CEB event using sodium hypochlorite instead of sodium hydroxide will result in significantly lower biomass and ARG abundances in UF filtrate.' Therefore, the former Hypothesis VI of the Research Proposal is now the Hypothesis V.



4 ANTIBIOTIC MICROBIAL RESISTANCE (AMR) REMOVAL EFFICIENCIES BY CONVENTIONAL AND ADVANCED WASTEWATER TREATMENT PROCESS: A REVIEW

Research hypothesis #1 is tested in chapter 4. This chapter is a comprehensive review about the state-of-the-art of antibiotic resistance in the aquatic environment. The anthropogenic impact of WWTP discharges on surface waters are illustrated and compared to low impacted surface waters. Conventional and advanced treatment processes are compared regarding their AMR removal efficiencies. AMR analyses methods are presented whereas advantages and disadvantages of the methods are shown.

This chapter has been published as follows:

Hiller, Christian X.; Hübner, Uwe; Fajnorova, S.; Schwartz, T.; Drewes, Jörg E. (2021): Antibiotic microbial resistance (AMR) removal efficiencies by conventional and advanced wastewater treatment processes. A review. Science of the Total Environment, **685**, 596-608. *DOI:* https://doi.org/10.1016/j.scitotenv.2019.05.315

Author contributions: Christian X. Hiller and Jörg E. Drewes developed the research objective. Christian Hiller, Uwe Hübner and Sona Fajnorova elaborated the content of the review paper. Thomas Schwartz and Jörg E. Drewes supervised the study and reviewed the manuscript. All authors approved the final version of the manuscript.

4.1 Abstract

The World Health Organization (WHO) has identified the spread of antibiotic resistance as one of the major risks to global public health. An important transfer route into the aquatic environment is the urban water cycle. In this paper the occurrence and transport of antibiotic microbial resistance in the urban water cycle are critically reviewed. The presence of antibiotic resistance in low impacted surface water is being discussed to determine background antibiotic resistance levels, which might serve as a reference for treatment targets in the absence of health-based threshold levels. Different biological, physical and disinfection/oxidation processes employed in wastewater treatment and their efficacy regarding their removal of antibiotic resistant bacteria and antibiotic resistance genes (ARGs) were evaluated. A more efficient removal of antibiotic microbial resistance abundances from wastewater effluents can be achieved by advanced treatment processes, including membrane filtration, ozonation, UV-irradiation or chlorination, to levels typically observed in urban surface water or low impacted surface water.

Keywords: Advanced water treatment, Antibiotic resistant bacteria, Antibiotic resistance genes, Background antibiotic microbial resistance, Low impacted surface water, Wastewater treatment

4.2 Introduction

In the 20th century, several antibiotics were developed to combat bacterial infections. As a consequence, bacteria responded to antibiotic resistance by adapting to increasing levels of antibiotics in the environment. Today, mankind is faced with a rapidly increasing number of antibiotic-resistant strains (Jenkins and Cooper, 2012; Lee, 2015; Morrill et al., 2015; Gillespie, 2002). The use of antibiotics is the first choice in human and veterinary therapy, aquaculture, as well as life-stock farming. Klein et al. (2018) analyzed the antibiotic consumption expressed in defined daily doses (DDD), in 76 countries from 2000 to 2015 and reported an increase of 65% (21–34.8 billion DDDs). Without major policy changes, global antibiotic consumption is likely going to increase (Klein et al., 2018). However, consumed antibiotics are not completely incorporated but partially metabolized by humans and animals. Between 30 and 90% of the ingested antibiotics to humans and animals are excreted by urine and feces (Du and Liu, 2011). The widely and excessive consumption of antibiotics, the incomplete metabolism of drugs and the partial removal in wastewater treatment plants (WWTPs) are resulting in the ubiquitous occurrence of some of these compounds in the aquatic environment at the nanogram to low microgram per liter concentration range (Alexander et al., 2015; Chow et al., 2015; Kümmerer, 2004). Studies published in the recent past suggest that even low concentrations of these antimicrobial drugs may pose a constant selection pressure on bacterial populations. In order to survive against antibiotics, bacteria generate antibiotic resistance by mutations or by horizontal gene transfer (Salcedo et al., 2015; Gullberg et al., 2011; Chow et al., 2015). Antibiotic microbial resistance (AMR) is carried by antibiotic resistant bacteria (ARB) and expressed through antibiotic resistance genes (ARGs). ARB and ARGs may find their way into the urban water cycle via wastewater discharge or applications of manure and biosolids and associated unintended run-off from agricultural land. Thus, the urban water cycle is considered an important pathway for distributing antibiotic resistance (Chow et al., 2015). Several recent reviews have highlighted key aspects related to the increasing spread of AMR in the aqueous environment. The reviews of Bouki et al. (2013) and Rizzo et al. (2013) summarized commonly applied methods to determine AMR in wastewater samples and quantify ARB and ARG emissions of conventional WWTP discharges into the aquatic environments. Bouki et al. (2013) described AMR removal efficiencies of different oxidation processes. Furthermore, Rizzo et al. (2013) discussed the effect of advanced treatment processes like sand filtration, adsorption, membrane filtration and advanced oxidation processes. Recent reviews by Umar et al. (2019), Michael-Kordatou et al. (2018) and Yan et al. (2019) also examined the removal of AMR in specific advanced treatment processes such

as UV irradiation, chemical advanced oxidation processes (AOP), and bioelectrochemical systems (BES) in wastewater. Reviews by Nnadozie et al. (2017), Krzeminski et al. (2019) and Barancheshme and Munir (2018) discussed the fate of ARB and ARGs by both, conventional and advanced treatment processes. Research studies addressing AMR removal efficiencies of conventional and advanced treatment processes are illustrated according to individually examined ARB and ARGs. The aim of this review paper is to complement existing literature on specific treatment processes with a broader perspective on challenges using analytical techniques, occurrence in different aquatic compartments (WWTP effluents, urban and low impacted surface waters), and a discussion on suitable indicator ARB and ARGs. Firstly, the challenges and limitations of commonly applied methods to determine AMR are presented and discussed as a prerequisite for the assessment of the AMR threat. The different classes of antibiotics and their consumptions (based on data in Germany) are illustrated in order to identify suitable indicator antibiotics and respective ARB and ARGs to assess the risk of AMR. Indicator ARB and ARGs are suggested for AMR monitoring in wastewater treatment and in surface waters. Furthermore, AMR removal efficiencies of conventional wastewater treatment plants (WWTPs) and advanced treatment processes are critically reviewed to identify knowledge gaps and potential strategies for AMR mitigation. Furthermore, the impact of AMR affecting surface water qualities are presented based on studies analyzing ARB and ARGs abundances downstream and upstream of discharge points. Subsequently, occurrence of AMR is illustrated for different water bodies.

4.3 Determination of antibiotic resistance in water samples

In order to quantify AMR in water samples, it is necessary to be familiar with the pros and cons of commonly applied culture-based and molecular-based detection methods. For the separation of ARB and ARGs from water samples, membrane filtration with pore sizes of 0.2 or 0.45 μm is commonly applied (EPA, 2002), followed by culture-based methods or molecular-based DNA extractions. Using culture-based methods, bacteria recovered on membrane filters are transferred to selective culture media and quantified applying standard procedures (Rizzo et al., 2013). Minimum inhibitory concentration (MIC) values of antibiotics to specific bacteria are defined in order to determine whether the analyzed sample contains antibiotic susceptible or antibiotic resistant bacteria (Rodloff et al., 2008). The MIC values are introduced for defined bacteria species (e.g., E. coli), but are not qualified for environmental bacteria since they can rarely grow on culture media. Hence, these methods do not provide any information on ARGs, which might be incorporated by other bacteria. Standardization of culture-based methods

enables a simple and cost-effective comparison of results, especially in nutrient poor habitats like drinking water matrices. However, typical disadvantages of culture-based methods include the need for immediate sample processing (typically within 24 h) (Rizzo et al., 2013) and the limitation to predefined bacteria strains and cultivable bacteria on synthetic media (Staley and Konopka, 1985). They also exclude bacteria in a viable but non-culturable (VBNC) state (Exner and Schwartz, 2015) or non-target bacteria that carry the ARG but that do not grow on selective media; both of which can lead to false-negative results. The prevalent molecular-based method for detection of antibiotic resistance genes is the quantitative polymerase chain reaction (qPCR) technique on the basis of the nucleic acid amplification. In this method, the bacterial DNA is recovered from total DNA samples and antibiotic resistance genes are amplified according to standardized and commercially available protocols. Predefined primers are used to determine specific bacterial genes. The advantage of the qPCR method is the high specificity and the rapid examination within 24 h and low measurement error of about 10% (Kitchen et al., 2010). The qPCR enables to detect the total abundance of (targeted) ARGs across all bacterial strains, bacteriophages (Quirós et al., 2014), mobile DNA fragments like plasmids, transposons and integrons (Xu et al., 2016; Muziasari et al., 2014) as well as “free” environmental DNA (eDNA). Moreover, the pretreatment of samples with propidium monoazide (PMA) principally enables qPCR analyses to differentiate DNA from living cells versus DNA derived from dead cells, phages, or for instance eDNA fragments (Nocker et al., 2010; Jäger et al., 2018a, 2018b). Thus, potential antibiotic resistance donors of ARGs can be quantified. However, PCR detection alone does not identify the bacteria carrying the specific resistance genes and their relevance to human health.

Table 4-1: Classes of antibiotics and applied antibiotics in human and animal therapy in Germany.

antibiotic groups	class of antibiotics drugs	antibiotics use in veterinary medicine (t/a) a)	antibiotics use in ambulant human therapy, (Mio. DDD) b)	examples for antibiotics	resistance gene
broad-spectrum antibiotics	broad-spectrum Penicillin d)	450 e)	90.6 e)	Ampicillin	ampC
	Tetracycline	342	66.3	Tetracyclin	tet
	Sulphonamide	121	not specified	Sulfamethoxazole	sul
	Phenicole	5.3	not specified	Chlorampehnicol	cml
	Aminoglycoside	38	not specified	Gentamicin	aac
	first and second generation Cephalosporin d)	not specified	not specified	Cefaclor	blaROB-1
antibiotics of last resort and antibiotics of emergency	Macrolide	109	52.5 c)	Erythromycin	erm
	Quinolone and fluoroquinolone	12.3	37.5	Ciprofloxacin	qnr
	3rd, 4th and 5th generation Cephalosporin d)	5.8 f)	not specified	Cefotaxim	blaCTX
	Polymyxin	107	not specified	Colistin	mcr - 1
	Antipseudomonal penicillin + beta-lactamase inhibitor d)	not specified	not specified	Piperacillin-tazobactam	-
	Oxazolidinones	not specified	not specified	Linezolid	-
	Glycylcycline	not specified	not specified	Tigecycline	-
	Cyclopeptide	not specified	not specified	Daptomycin	-
	Carbapenem and Penems d)	not specified	not specified	Imipenem	blaVIM
	Glycopeptide	not specified	not specified	Vancomycin	van
	Monobactame d)	not specified	not specified	Aztreonam	-
narrow spectrum antibiotics	Nitrofurane	not specified	11.6	Nitrofurantoin	nfsA
	Folic acid antagonists antibiotics	19	15.5	Trimethoprim	dfr
	Linconsamide	15	6.6	Clindamycin	lnu
	Pleuromutilins	13	not specified	Tiamulin	rplC
	narrow-spectrum Penicillin d)	not specified	not specified	Oxacillin	oxa1
	Nitroimidazole	not specified	not specified	Metronidazol	rdxA

a) literature source of Germany (2014): Bundesamt für Verbraucherschutz und Lebensmittelsicherheit (BVL)
b) literature source from statutory health insurance of Germany (2011): WIdO, GKV-Arzneimittelindex
c) old and new macrolide combined
d) penicillin, cephalosporin, carbapenem and monobactam belong to the betalactam group of antibiotics that have a betalactam ring in common. The betalactam resistance genes are called **bla genes**
e) antibiotic consumption values included broad-spectrum and narrow-spectrum penicillins
f) first, second, third, fourth and fifth generation cephalosporins combined

Furthermore, the method is limited to known genes, which renders it difficult to conclude on the overall resistance to a specific antibiotic, if e.g. the resistance can be transferred by alternative resistance genes (e.g., *vanA* and *vanB* gene can both independent of each other have resistance against vancomycin). Therefore, the determination of a single resistance gene might give limited information regarding the real resistance to the specific antibiotic in the analyzed water sample. Here, highly parallel qPCR assays, ChipSeq, microarrays or next generation sequencing surpasses the dilemma but often require considerable expertise and financial resources.

Currently, reporting the presence of AMR in water samples is not consistent in the literature. ARB and ARGs are usually reported as total abundance as CFU/mL (ARB) and gene copies/mL (ARGs), but results are often shown as relative abundance. The relative abundance is normalized to either the bioburden measured as all cultivable bacteria, to the measured *16S rRNA* gene copies, or to the total amount of DNA (Alexander et al., 2015). Thus, these relative abundances imply errors leading to considerable deviations. For instance, *16S* gene copies depend largely on the *16S* gene copy numbers in the individual genome (ranging from 1 to 15); the total amount of DNA depends largely on the extraction efficiencies, commercial kit components, impurities, and the amount of eukaryotic and eDNA; cultivatable bacteria are largely biased by the uncultured bacteria and by the VBNCs. Therefore, care must be taken when linking relative abundances to regulatory removal requirements.

4.4 Occurrence of antibiotic resistance

4.4.1 Antibiotics and antibiotic resistances of interest

After the introduction of the first antibiotic, an ever-increasing number of antibiotics for medical use has been manufactured. The most applied antibiotics and antibiotic classes and their consumptions in veterinary and ambulant human therapy in Germany are summarized in Table 4-1. The antibiotic consumption in veterinary medicine is presented in tons per annum. The antibiotic consumption in ambulant human therapy is expressed in defined daily doses (DDD). These antibiotics are categorized into narrow and broad spectrum antibiotics, applied against a limited number of specific bacteria and a large group of bacterial infections. The antibiotics of last resort and antibiotics of emergency consider priority antibiotics so that certain drugs can be reserved for human therapy. The World Health Organization (WHO) implemented the antibiotic categorizations “Access” for first and second choice antibiotics and “Watch” and “Reserve” antibiotics for antibiotics of last resort as well as for antibiotics of emergency (WHO, 2017). Broad-spectrum antibiotics are classified as all-purpose

antibiotics with high application rates in Germany for veterinary and for human ambulant therapy (Table 4-1), which can also be detected in WWTPs' influents. For example, Alexander et al. (2015) reported sulfamethoxazole (sulphonamide) concentrations up to 2.8 µg/L in the influent of a WWTP, a compound class that has received a lot of attention in AMR research. Antibiotics of last resort and emergency antibiotics are used in lower quantities and applied basically for exceptional cases when other antibiotics were not effective in therapy. They are predominantly used in clinical human and in veterinary therapy (BVL, 2015). Galvin et al. (2010) demonstrated that hospital wastewater had higher abundance of cephalosporin and quinolone resistance compared to municipal wastewater without hospital wastewater contributions. Rizzo et al. (2013) analyzed imipenem resistance measured as blaVIM genes in wastewater samples of residential areas, hospital, and WWTPs and reported highest abundances in hospital wastewater. Narrow spectrum antibiotics are used in clinical and ambulant human therapy as well as in veterinary therapy (BVL, 2015; DGI, 2013). Since these antibiotics are selected to treat a narrow spectrum of bacterial infections, their consumption and measured antibiotic concentrations are lower compared to broad-spectrum antibiotics (Table 4-1). For example, Jiang et al. (2014) measured several antibiotics in river water as well as in groundwater and reported that broad spectrum antibiotics like oxytetracycline were abundant in higher concentrations than narrow spectrum antibiotics like trimethoprim. The German Society of Infectiology recommends the prevalent use of narrow-spectrum antibiotics instead of broad-spectrum antibiotics in order to keep the AMR development at a lower level (DGI, 2013). The graphical overview of total ARGs abundances analyzed in the influent and in the effluent of conventional WWTPs is illustrated in Figure 4-1. The articles, considered in Figure 4-1 were selected based on resistance genes to specific antibiotics. Table 4-1 provides information regarding different consumption of antibiotics applied in veterinary and ambulant human therapy. The antibiotics with the highest consumptions (e.g., penicillin, tetracycline, sulfonamide, macrolide, beta-lactam antibiotics) as well as one antibiotic with lower consumption that is typical used for clinical therapy (glycopeptide) are evaluated for further investigations. These antibiotics have been the focus of this study in order to quantify if long term antibiotic application and higher consumptions correlate with higher antibiotic resistance abundances in the influent and effluent of conventional WWTPs. In addition, evaluated antibiotics typical used for clinical therapy with lower consumptions should result in lower antibiotic resistance abundances analyzed in influents and effluents of conventional WWTPs. In these papers, different ARGs were analyzed. The following *tet* genes were considered in Figure 4-1: *tetA, tetB,*

tetM, tetC, tetO, tetQ and tetW. The *sul* gene analyses targeted *sul1* and *sul2* genes; *ermB* and *ermF* genes were summarized in *erm* gene analyses. *blaTEM, blaVIM-1, blaCTX-M-32, blaSHV-34* and *blaOXA-58* genes were considered in the bla gene group in Figure 4-1. All ARGs analyses were performed using qPCR. The selection of articles was performed using Google Scholar whereas the following keywords were applied: 'antibiotic resistance', 'antibiotic resistance genes', 'antibiotic resistant bacteria' as well as 'wastewater treatment', and 'conventional wastewater treatment plant'. Papers published between 2005 and 2019 have been considered.

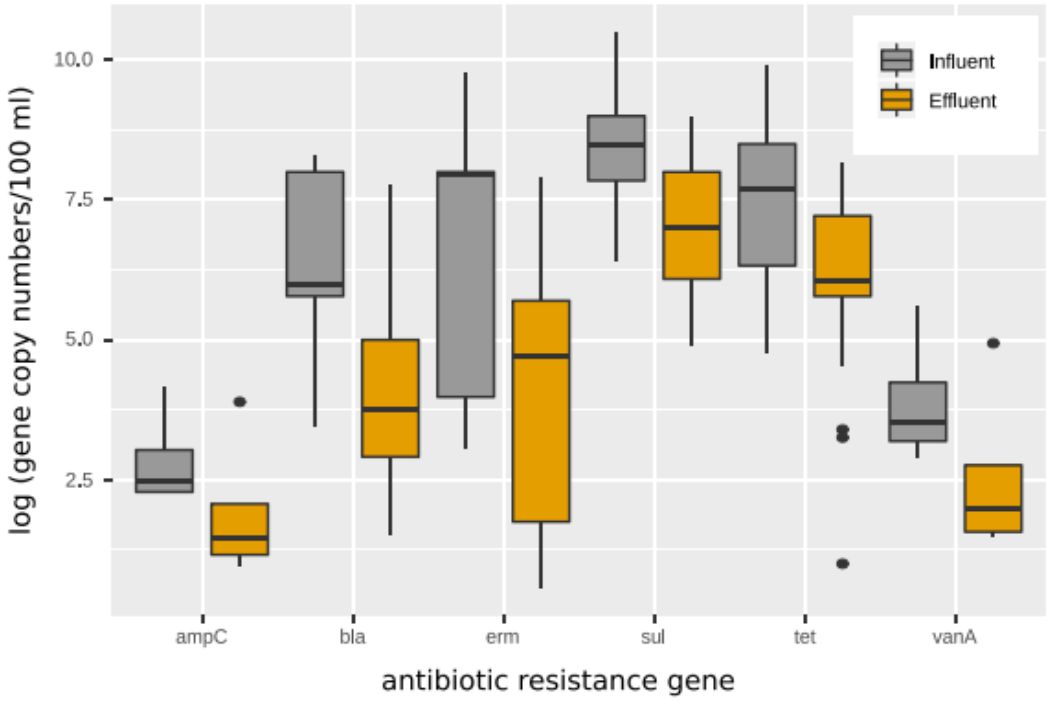


Figure 4-1: Total abundance of ARGs (ampC, tet, sul, ermB, vanA and bla genes) detected in influent and effluent samples of conventional WWTPs shown as Tukey-boxplots (McGill et al., 1978). Data from following studies were included: Laht et al., 2014; Salcedo et al., 2015; Du et al., 2015; Alexander et al., 2016; Böckelmann et al., 2009; Czekalski et al., 2012; Koczura et al., 2016; Makowska et al., 2016; Rodriguez-Mozaz et al., 2015; Negreanu et al., 2012; Michael et al., 2013; Mao et al., 2015; Zhang et al., 2009; Lachmayr et al., 2009; Al-Jassim et al., 2015; Quach-Cu et al., 2018; Narciso-da-Rocha et al., 2018; Proia et al., 2018

Data analyses of Figure 4-1 confirm consistently highest resistances to broad-spectrum antibiotics tetracycline or sulphonamide antibiotics while others were only detected at much lower abundances, e.g., resistance to the antibiotic of last resort vancomycin but also the broad-spectrum antibiotic ampicillin. Ampicillin is a representative of the penicillin class. Lower analyzed *ampC* abundances could be a matter of low ampicillin application compared to other penicillin antibiotics. Furthermore, the beta-lactam group represents antibiotics belonging to broad-spectrum, narrow spectrum antibiotics as well as antibiotics of last resort and antibiotics of

emergency having lower concentrations analyzed in the WWTP influent and effluent compared to the broadspectrum antibiotics class of drugs sulfonamide or tetracycline. However, resistance genes for erythromycin, an antibiotic of last resort and emergency, are detected at low and high levels. These erm genes results do not correlate with the erythromycin consumption of Table 4-1. In contrast, the tet-genes and sul genes abundances corresponds to tetracycline or sulfonamide consumption in animal or human therapy (Table 4-1). Despite building sums of different tet genes and sul genes, highest ARGs abundances with relatively small standard deviations are recognizable. Moreover, sulfonamide and tetracycline antibiotics were introduced in medical use in 1935 and 1950, respectively providing a selection pressure for many decades and increasing opportunities for AMR formation in the aquatic environment. According to Figure 4-1, the statement can be made that in particular tetracycline and sulfonamide resistance genes are suitable for ARGs monitoring of wastewater treatment processes. These genes had a relatively small standard deviation. Research studies of different countries resulted similar ARGs abundances measured in the influent and effluent of conventional WWTPs. Furthermore, this review revealed that the largest data set on AMR is available for broad-spectrum antibiotics providing the most comprehensive information for the evaluation of AMR in the urban water cycle. However, antibiotics of last resort and emergency antibiotics should also be considered to assess risks associated to the spread of AMR as well, since these antibiotics are often the last alternative to fight pathogenic bacteria in human therapy.

4.4.2 Natural occurrence and transfer of antibiotic microbial resistance

Findings of several studies confirm that AMR in the aquatic environment is prevalent worldwide (Munir and Xagoraraki, 2011; Alexander et al., 2015; Allen et al., 2010; Kristiansson et al., 2011). However, it is important to note that AMR is a natural phenomenon, which is often referred to as background or baseline AMR. D'Costa et al. (2011) extracted samples from approximately 30,000-year-old Late Pleistocene permafrost sediments and determined ancient DNA. They identified ARGs like the ribosomal protection protein *tetM* that refers to tetracycline resistance genes. Permutation tests showed that ancient *vanHA* clones were similar to modern *vanH* and *vanA* genes and ancient *vanAX* clones were similar to modern *vanA* and *vanX* genes (D'Costa et al., 2011). Bacteria develop self-produced antibiotics to inhibit the increase of other bacteria populations in order to have an evolutionary advantage. For example, the bacterium Penicillium chrysogenum produces penicillin (Thompson, 1994). To compete against bacteria which produce antibiotics, other bacteria

generate antibiotic resistances. Koch et al. (2014) observed this adaptation with Staphylococcus aureus bacteria that were not resistant to antibiotics. Under typical biofilm conditions in a confined space with limited nutrient supply, mutations of Staphylococcus aureus bacteria resulted in antibiotic production. After 5 days, three bacteria groups were detected, the susceptible bacteria, antibiotic producing bacteria, and antibiotic resistant bacteria (Koch et al., 2014). Furthermore, resistance formation can be divided into intrinsic and acquired resistance. While intrinsic resistance in bacteria is a stabile genetic property encoding chromosomally and found in all bacteria of a bacterial strain, acquired resistance genes are incorporated via horizontal gene transfer (HGT) by plasmids, transposons or integrons and transmissible genetic elements (Pak et al., 2016). Horizontal gene transfer is occurring naturally, independent of wastewater impact, not only across similar bacterial strains, but also between gram-negative and gram-positive bacteria as well as between pathogenic and nonpathogenic bacteria (Courvalin, 1994). For example, *tetK* and *tetL* genes were detected both in natural soil bacteria Bacillus spp. and in pathogenic Staphylococcus spp. (Salyers and Amábile-cuevas, 1997).

4.4.3 Sources of anthropogenically-derived AMR

Different researchers (Sigala and Unc, 2012; Li et al., 2015; Rizzo et al., 2013) analyzed AMR in raw wastewater of residential areas, hospitals, and from the influent and the effluent of municipal WWTPs. The results of these three studies revealed that AMR did not only occur in hospital wastewater but also in residential wastewater and consequently in municipal WWTPs. Elevated AMR abundances were also detected in effluents from a slaughterhouse (Um et al., 2016; Moura et al., 2007) and antibiotic manufacturing facilities in China (Yang et al., 2008) and India (Larsson et al., 2007). Besides WWTPs, combined sewer overflows (CSOs) can result in elevated discharge of AMR into receiving streams during heavy rain events. Alexander et al. (2015) compared wastewater samples of four different CSO basins with samples from four different WWTPs in Germany. The total abundance of *ampC*, *vanA*, *blaVIM*, and *ermB* genes (1.23–4.05 log gene copies/100 mL) in the effluent samples of the CSO basins was lower compared to influent samples of the WWTPs but higher than effluent samples of the WWTPs. Scheurer et al. (2015) studied the retention of ARB E. coli, Enterococci, and Staphylococci in soil retention filters reporting removal efficiencies of about 3, 2 and 2 log units measured as CFU/100 mL, respectively. Another pathway of anthropogenically derived resistances into surface waters is agricultural run-off that has been in contact with manure and land-applied biosolids, which is still being practiced in

Germany and worldwide (Munir and Xagoraraki, 2011). Knapp et al. (2010) analyzed ARGs from different classes of antibiotics in five long-term soil samples collected in the Netherlands between 1940 and 2008. Their results demonstrated that ARG from numerous classes of antibiotics increased significantly since 1940. Especially abundances of tetracycline ARG were 15 times higher in soil samples taken 2008 than from the 1970s. The facilities mentioned all contribute higher AMR abundances. These ARGs emissions can contribute to the increase of AMR in the aquatic environment. Future studies should also examine AMR emissions of these potential hot spots for the spread of antibiotic resistance. This review is focusing on AMR contributions from conventional WWTP discharges and on treatment strategies that are capable to reduce these AMR loads entering surface waters. Besides the direct release of ARB and ARGs from wastewater, manure and biosolids as potential antibiotic resistance factors into the environment, the constant exposure of bacteria to low concentrations of antibiotics might also constitute a continuous selection pressure on bacteria in aquatic systems. This can result in the adaption of susceptible strains becoming antibiotic resistant strains (Salcedo et al., 2015). Gullberg et al. (2011) demonstrated that adaption can already occur at antibiotic concentrations in the low µg/L range. The relative abundance of ARB increased at concentrations exceeding 1000 µg/L for streptomycin, 25 µg/L for tetracycline, and 0.46–2.88 µg/L for ciprofloxacin (Gullberg et al., 2011). The ratio of resistant to susceptible strains could be decoupled after 40 generations of growth using 2000 µg/L streptomycin, approximately 60 µg/L of tetracycline and 2.3 µg/L of ciprofloxacin. Comparable antibiotic concentrations were sporadically detected in water samples, e.g. Lien et al. (2016) reported between 0.6 and 40.2 µg/L of ciprofloxacin in urban hospital wastewater in Vietnam. Van den Hengel et al. (2015) measured ciprofloxacin concentrations in the range of about 600 µg/L in hospital and about 100 µg/L in household toilet wastewater in the Netherlands. Jiang et al. (2014) measured tetracycline concentrations up to 26 µg/L and ciprofloxacin concentrations up to 0.6 µg/L in the Wangyang River, China. However, other scientists, e.g. Kümmerer (2004) and Alexander et al. (2015), observed lower antibiotic concentrations in hospital wastewater, WWTP effluent as well as in surface water. Le-Minh et al. (2010) reported efficient removal of antibiotics by conventional wastewater treatment processes with effluent concentrations of antibiotics in the range of 10–500 ng/L. The question how the discharge of urban (waste-) water affects the aquatic environment is strongly linked to the characterization of low AMR impacted surface water. There have been several attempts to quantify AMR in low impacted (pristine) surface waters. Pei et al. (2006) quantified sulphonamide and tetracycline resistance genes in sediments of the mixed-landscape watershed of the Cache La Poudre River in northern Colorado, USA. Sampling points

were focused on pristine areas and both urban and agricultural affected areas. The relative abundances of resistance genes (normalized to the *16S* gene copy number) in pristine areas were significantly lower (*sul2* below detection limit and *sul1, tetW, tetO* about 2 log units gene copies/*16S rRNA* gene copies lower) for the pristine areas than for the anthropogenically affected areas, which was consistent with total ARB CFUs that were over an order of magnitude higher in the anthropogenic areas. The influence of wastewater inputs from WWTPs on four small streams in terms of ARGs was confirmed by Proia et al. (2016) by analyzing biofilm samples taken upstream and downstream of WWTPs. While low abundances of ARGs were detected at upstream sites (about 0.001–0.03 *sul1* and 0.001–0.01 (*ermB*) gene copies/*16S rRNA* copies), up to one order of magnitude of ARG copies/ *16S rRNA* copies higher relative abundances were observed at downstream sites (about 0.003–0.1 (sul1) and 0.01–0.1 (*ermB*) gene copies/*16S rRNA* copies). The presence of higher ARGs concentrations in downstream biofilms, water and sediment samples compared to upstream samples was even detectable in stream samples up to 1 km downstream of the point of discharge (Proia et al., 2016) and up to 20 km downstream of the point of discharge (Sabri et al., 2018). Further studies confirm the impact of conventional wastewater treatment plant discharges on AMR abundances in surface waters (Hembach et al., 2017; Jäger et al., 2018a, 2018b). The impact of discharge from conventional WWTPs regarding AMR abundances in surface waters is further examined in Chapter 4.4.4.

4.4.4 Fate and transport of AMR by WWTP discharges in the urban water cycle and AMR abundances of low and strong anthropogenically-impacted surface waters

Different studies reporting ARB and ARG concentrations in WWTP effluent and river samples collected upstream and downstream of wastewater discharge points were critically reviewed to assess the fate and transport of AMR. The review was limited to studies presenting results from all three environmental compartments (i.e., upstream and downstream of discharge and WWTP effluents). However, as specific information about river discharge, degree of wastewater effluent impact and sampling conditions (e.g., dry or wet weather, etc.) were often not reported, a more detailed data analysis could not be conducted. The investigated antibiotics (penicillin, tetracycline, sulfonamide and macrolides) with the highest consumptions (Table 4-1) and highest ARB and ARGs abundances analyzed in the influent and in the effluent of conventional WWTPs (expect of ampicillin concentrations) of Figure 4-1

were subsequently analyzed in Figure 4-2 for antibiotic resistant bacteria and antibiotic resistance gene abundances in Figure 4-2.

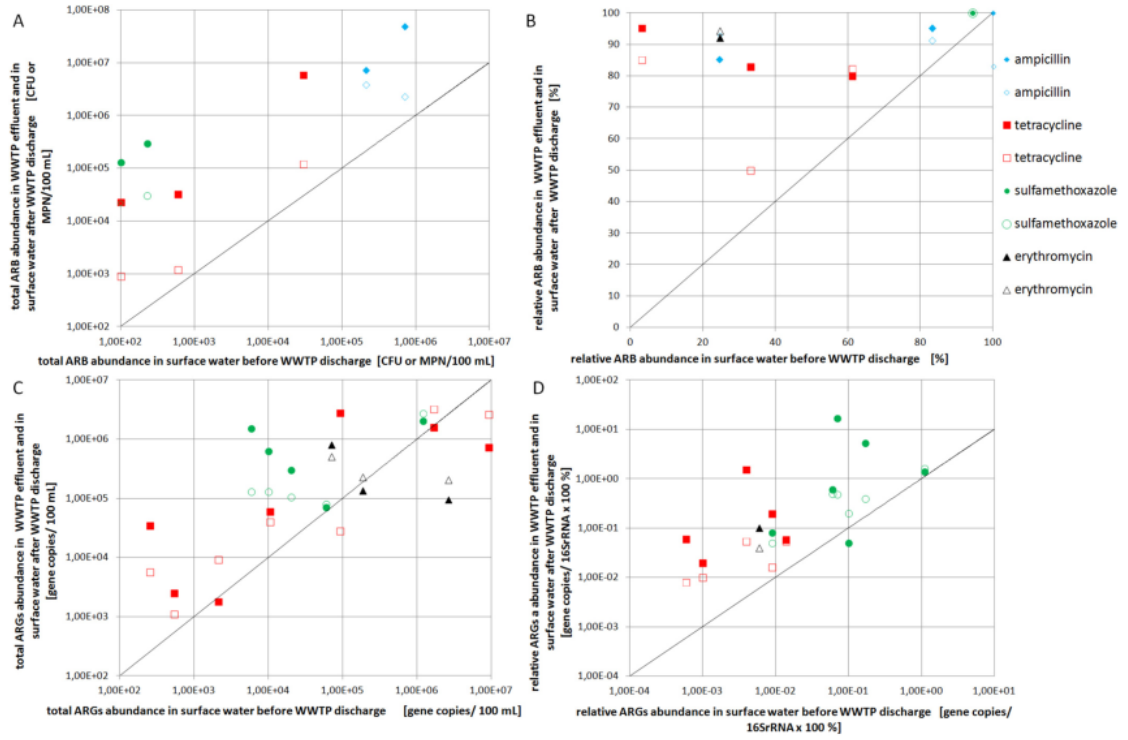


Figure 4-2: Summary of total and relative abundance of ARB (A, B) and ARGs (C, D) in WWTP effluent as well as receiving surface water illustrated in comparison to abundances in surface water before WWTP discharge (x axis). Shaded symbols represent data from WWTP effluents while empty symbols show abundance in receiving surface water after WWTP discharge (Young and Fellow, 2012; Makowska et al., 2016; Guyomard-Rabenirina et al., 2017; Li et al., 2010; Koczura et al., 2012; Böckelmann et al., 2009; Koczura et al., 2016; Rodriguez-Mozaz et al., 2015; Marti et al., 2013 To elucidate whether the illustrated WWTP release of AMR (Fig. 2) has a low or strong influence on AMR abundances in surface waters, different studies reporting ARB and ARGs measurements in low and strongly anthropogenically-affected surface waters were critically reviewed. For this examination, AMR analyses of municipal WWTP effluents are presented as 'WWTP effluent'. The term 'urban surface water' represents anthropogenically-impacted surface waters receiving WWTP effluent discharges, and 'low impacted rivers' are defined as low anthropogenically-affected surface waters with no direct WWTP discharge upstream. Furthermore, only studies reporting monitoring results from municipal WWTP discharges and corresponding (urban) rivers are considered. Other aspects characterizing different aquatic environments, e.g., percentage WWTP effluent compared to the amount of river water, wastewater sampling at dry or wet weather conditions, could not be considered due to a lack of information provided in these studies.

Furthermore, these antibiotics are evaluated due to long-term application in the medical sector providing a selection process of antibiotic resistance adaption in wastewater for many decades. The comparison of different studies reporting total abundance (Fig. 4-2 A, C) and relative abundance (Fig. 4-2 B, D) of ARB and ARGs in WWTP effluents and surface water prior to and after WWTP discharge clearly indicates that WWTPs are a significant contributor to the spread of bacterial resistance in receiving streams. Although the data revealed consistently elevated total ARB abundance (Figure 4-2 A)

and relative abundances of 80% of the bacteria in WWTP effluent with antibiotic resistance (Figure 4-2 B), results from river samples are highly variable indicating likely different origin and degrees of anthropogenic impact prior to WWTP discharge. In contrast, abundance of ARGs in the examined samples vary from 2 to 7 log units gene copies/100 mL most likely depending on the analyzed resistance gene and the investigated WWTP (Figure 4-2 C, D). However, the majority of all corresponding samples exhibits an increase of AMR in surface water after the discharge of WWTP with the downstream samples representing a mixture of abundances from upstream surface water and WWTP effluent. Data from tetracycline and sulfamethoxazole resistance genes are evaluated because these compounds are commonly used antibiotics worldwide for a long time. This might have allowed AMR adaption to these antibiotics which can be detected at higher abundances than resistances to antibiotics which have been introduced more recently or applied at lower amounts (Chapter 4.4.1). However, the number of studies reporting total ARGs abundances in low impacted surface waters is insufficient to carry out a statistical assessment. Thus, available results of relative ARGs abundances (normalized by *16S* gene copies) in WWTP effluents, urban (anthropogenically-affected) rivers, and low impacted rivers are summarized in Figure 4-3.

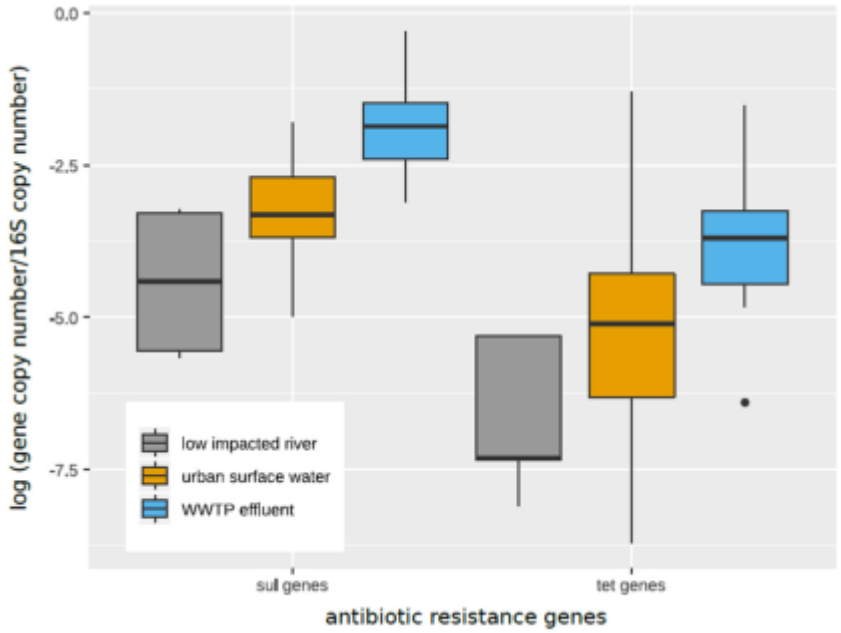


Figure 4-3: Relative ARG abundance (gene copies/*16S* gene copies) in low impacted river, urban surface water and WWTP effluent shown as Tukey-boxplots. Data from following studies were included: Koczura et al., 2016; Pei et al., 2006; Kristiansson et al., 2011; Makowska et al., 2016; Xu et al., 2014; Marti et al., 2013; Pruden et al., 2006; Berglund

et al., 2015 (individual studies can represent ARG measurements from several sampling campaigns)

On the basis of the study results summarized in Figure 4-3, a decreasing relative ARG abundance can be observed from WWTP effluents, to anthropogenically-affected urban surface water and very low detected relative ARG abundances in low impacted rivers. Further studies by Storteboom et al. (2010a, 2010b) on ARG abundances in WWTP effluents, urban and low impacted rivers confirmed the low abundances of ARGs in low impacted rivers. They investigated the La Poudre River and the South Platte River in Colorado, USA with a focus on the detection frequency of 11 *tet* genes and 2 *sul* genes. The average detection frequency of analyzed ARGs reached 56% in the WWTP effluent, 23% in urban rivers, and 8% in low impacted rivers in the first study (Storteboom et al., 2010a), and 70% in WWTP effluent, 29% in urban rivers, and 9% in low impacted rivers in a follow-up study (Storteboom et al., 2010b). In some studies, however, the ARG analyses revealed unexpectedly higher total ARG abundances in urban rivers compared to WWTP effluents (Böckelmann et al., 2009; Makowska et al., 2016). This might be a result of contributions by CSOs or other sources upstream of WWTPs since urban rivers cannot be considered low impacted waters in many settings. While a comparison of corresponding results from individual studies confirms the impact of treatment and discharge regarding ARG abundance, the findings also demonstrate that a comparison of data from different locations and studies is problematic (Figure 2). It has to be considered that obtained data are related to target ARB, ARGs and specific antibiotics and can vary as a function of target bacteria and type of antibiotic and resistance gene. Even measuring the same ARG in various WWTP effluents may result in differences of several orders of magnitude. Furthermore, different ARGs that trigger AMR to the same antibiotic (e.g., *tetO, tetW* genes), question a direct causal relationship between single ARGs and the corresponding antibiotics. In addition, abundances of ARGs in surface waters with a low anthropogenic impact show region and gene specific differences (Alexander et al., 2015; Koczura et al., 2016; Makowska et al., 2016). Based on this information, defining discharge standards or removal targets for wastewater treatment facilities will be a challenging task for regulators without taken into account the local background abundance levels.

4.5 AMR removal efficiencies in different wastewater treatment processes

Nine research studies of different wastewater treatment processes analyzing ARB abundances to tetracycline resistance have been selected

to quantify AMR removal efficiencies. In addition, 17 research studies reporting ARGs abundances, detecting sulfonamide, tetracycline and beta-lactam resistance, are presented. Agar diffusion and agar dilution studies were considered. *Fecal coliforms* (FC), *heterotrophic bacteria* (HB), and *enterococci* (ENT) were evaluated for ARB analyses of different wastewater treatment processes. Due to highest ARB and ARGs concentrations detected in WWTP influents and effluents (Figure 4-1), sulfonamide, tetracycline and beta-lactam resistances were evaluated for AMR using different advanced treatment processes. ARB and ARGs resistant to erythromycin were not investigated frequently enough to quantify AMR removal efficiencies during treatment processes. Removal efficiencies reported for different ARB and ARGs of different conventional and advanced water treatment processes are summarized in Figure 4-4 and reported as log removal of total abundance. Relative abundances, which were reported in some studies, are only sporadically discussed for reasons described in Section 4.4.3. Detailed results from individual studies (including relative abundance data) can be found in Tables 10-1 and 10-2 (Supplemental Information). All investigated research studies analyzing antibiotic resistant bacteria are applying primarily the agar dilution method whereas all ARGs research studies are using predominantly quantitative polymerase chain reaction (qPCR) for ARGs analyses. The presented results need to be evaluated carefully as they are often based on single or few individual studies. The limited number of available studies for some processes demonstrates that future research is needed to improve the understanding of AMR removal during various conventional and advanced treatment processes. The AMR removal efficiency of conventional wastewater treatment plants is studied sufficiently. However, most studies investigating AMR in WWTP collected samples from plant influent and effluent, frequently not considering individual stages of an entire treatment train and hydraulic residence times to establish proper synoptic sampling. Furthermore, treatment conditions as well as the type of wastewater treatment were often not specified. Hence, AMR removal efficiencies in WWTPs are presented without differentiation of mechanical and biological treatment steps. Removal efficiencies of physical processes (e.g., sand and GAC filtration, membrane filtration) as well as oxidation and disinfection processes (i.e., UV irradiation, ozonation, chlorination) are discussed as individual treatment processes. The different research studies of sand and GAC filtration, microfiltration and ultrafiltration as well as membrane bioreactor processes demonstrated comparable results for AMR removal efficiency concerning the individual treatment process. However, physical treatment processes were not studied very often justifying further studies in particular for processes like bank filtration, nanofiltration and reverse osmosis. Studies analyzing oxidation and disinfection processes (i. e., UV irradiation, ozonation, chlorination)

after conventional biological WWTP treatment represent the highest number of research studies.

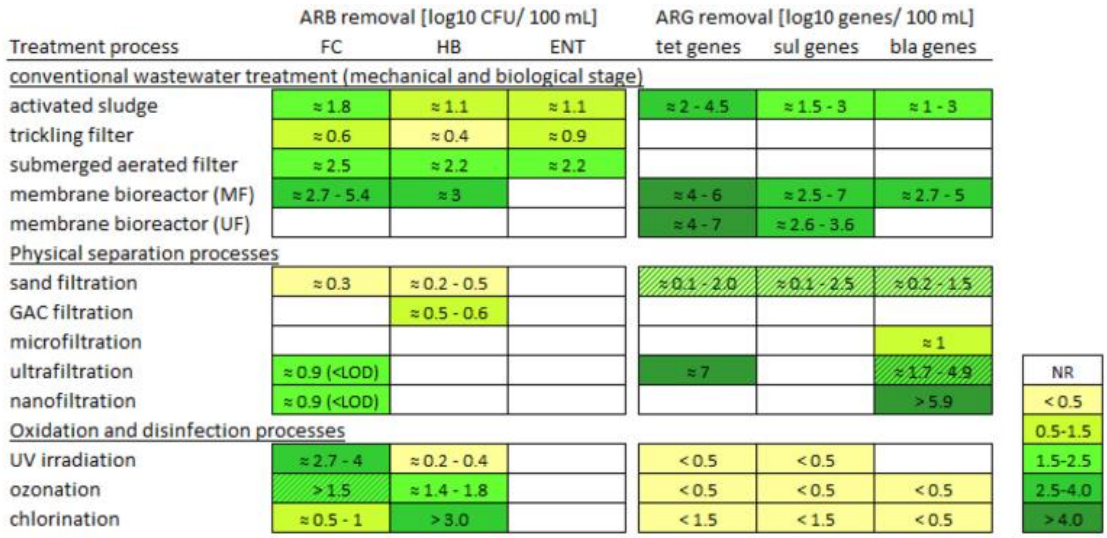


Figure 4-4: Summary of literature data on the removal of tetracycline resistant bacteria (FC: *fecal coliforms*, HB: *heterotrophic bacteria*, ENT: *enterococcus*) and ARGs during conventional and advanced treatment processes (hatched fields indicate different results from literature; NR: not reported)

4.5.1 Conventional biological wastewater treatment

Observed removal of *tet* genes in WWTPs can vary between 2 and 4.5 log units gene copies/100 mL. However, this efficiency was not confirmed by reported data from tetracycline resistant *E. coli*, *heterotrophic bacteria* (HB), and *Enterococci* (ENT) suggested a decrease between 1.1 and 1.8 log units (CFU/100 mL) in conventional biological wastewater treatment processes. In WWTPs with trickling filters and submerged aerated filters, total abundances of tetracycline resistant bacteria decreased by 1 log unit CFU/100 mL (Novo and Manaia, 2010) and 2 log units CFU/100 mL (Fry and Day, 1990; Galvin et al., 2010), respectively. Compared to tet genes, less efficient ARG removal during mechanical and activated sludge stages was reported for *sul* and *bla* genes (Figure 4-4). Tong et al. (2019) studied the fate of antibiotic resistance genes during six different full-scale municipal wastewater treatment processes and revealed that horizontal gene transfer mainly occurs in aerated tanks whereas anaerobic or anoxic tanks can reduce the occurrence of antibiotic resistance. However, the total abundances of ARGs in the WWTP effluents (activated sludge) varied between 10 and 2.33×10^6 gene copies/100 mL (Alexander et al., 2015; Munir et al., 2011; Gao et al., 2012) making a comparison of different studies difficult. Mahfouz et al. (2018) examined the pan-genome of *E. coli* in WWTPs and hospitals showing no difference between influent and effluent of WWTPs. This means that conventional WWTPs can reduce the amount

of bacteria into the environment, whereas the pathogenic potential for antibiotic resistance could not be reduced. Furthermore, the wastewater pan-genome was larger than a clinical pan-genome of similar size due to possible horizontal gene transfer in wastewater. Employing alternative processes for solids separation like membrane bioreactors seems to provide a viable alternative where improved removal for ARB and ARGs is desired. For example, efficient reduction by 5 log units gene copies/100 mL were measured for *tet* genes (*tetG, tetW, tetX*) and *sul1* genes in a MBR with membrane pore size of 0.1 to 0.4 µm (Du et al., 2015). The total ARG abundances in the effluent samples varied from 10^3 to 10^7 gene copies/100 mL. For an MBR with a membrane pore size of 0.04 µm, similar removal efficiency was reported for tet genes while the decline of sul genes was surprisingly lower (Figure 4-4) (Munir et al., 2011). Only 2.7 to 4 log ARGs (*tet*, *sul* and *bla* genes) removal by MBR technology with pore sizes of 100 kDa and 0,3 µm were detected by Kappell et al. (2018) and Cheng and Hong (2017). Munir et al. reported in the MBR study a 1–3 log units gene copies/100 mL higher ARGs removal efficiency compared to conventional wastewater treatment. In addition, 3 and 5 log abatement of tetracycline and sulfonamide resistant HB (CFU/100 mL), respectively, was reported. For non-resistant *E. coli* bacteria even higher removal efficiencies 6 log units are known for MBR treatment (Luca et al., 2013; Marti et al., 2011). Further studies are recommended to confirm the reported AMR removal efficiencies using MBR technology and to elucidate the underlying retention mechanisms.

4.5.2 Physical treatment processes

Several studies examined removal efficiencies of AMR employing physical treatment processes under various operational conditions including granular media filtration, adsorption onto GAC, and different membrane processes.

4.5.2.1 Granular media filtration and GAC adsorption

Studies investigating AMR in sand and GAC filtration were performed at water treatment plants (Xu et al., 2016; Zhang et al., 2016; El-Zanfaly, 2015), but also at wastewater treatment facilities, where filtration was used as a tertiary treatment process (Lüddeke et al., 2015) Figure 4-4. Summary of literature data on the removal of tetracycline resistant bacteria (FC: fecal coliforms, HB: heterotrophic bacteria, ENT: enterococcus) and ARGs during conventional and advanced treatment processes or as post-treatment after ozonation (Czekalski et al., 2016; Alexander et al., 2016; Lüddeke et al., 2015). Available studies reported limited AMR removal efficiencies varying between

inefficient up to 2 log units removal by sand filtration. Even an increase up to 1 log unit was reported for heterotrophic bacteria and ARGs in some studies (see Tables 10-1 and 10-2). Different results have been attributed to varying operational conditions and pre-exposures to antibiotic and AMR containing waters as most filters are biologically active (Czekalski et al., 2016). However, the current data do not allow a definite assessment of relevant mechanisms for the retention as well as transport of ARB and ARGs within sand filtration, e.g. horizontal gene transfer or biotic and abiotic factors that influence the observed AMR abundances. In most studies, operational conditions such as empty bed contact time, filter loading rate, filter media, grain size and backwash protocols were not specified. As expected from previous studies on non-resistant bacteria, few studies investigating AMR removal efficiencies by GAC filtration did not observe a strong removal enhancement compared to sand filtration (El-Zanfaly, 2015; Lüddeke et al., 2015). Thus, further investigations are needed to better understand the fate and transport of ARGs during granular media filtration as a function of operational conditions.

4.5.2.2 Membrane filtration

The rejection of suspended solids and total dissolved compounds during membrane treatment processes can be divided based on the membrane type into low-pressure membrane filtration (microfiltration, ultrafiltration) and high-pressure membrane filtration (nanofiltration, reverse osmosis). Microfiltration membranes are designed to reduce inorganic and organic particulate compounds with a size of 0.1–1 μm. By applying ultrafiltration (pore-size of 0.01 to 0.1 μm), a removal of bacteria and viruses can be achieved as well. Nanofiltration (NF, molecular weight cut-off 0.3–2 kDa) and reverse osmosis (RO, molecular weight cut-off b100 Da) are commonly applied water treatment processes where high water qualities are desired and are capable of removing impurities such as bacteria, viruses but also dissolved constituents including ions. Available data on AMR removal efficiencies during membrane treatment other than MBRs are limited. First studies confirm the high potential of membranes as a barrier for ARGs (Böckelmann et al., 2009; Breazeal et al., 2013). Breazeal et al. (2013) demonstrated a strong dependency of ARG reduction on the pore size or molecular weight cutoff of MF, UF and NF-membranes for the *blaTEM* gene (i.e., up to 1 log unit gene copies/100 mL with a pore size of 0.45 μm and 0.1 μm, 1.7 log units removal at 100 kDa, 4.7 log units removal at 10 kDa and 5.7 log units at 1 kDa). The authors of the study suggested an association of the removal of ARGs to DNA-colloid interactions. To the best of our knowledge, the impact of other relevant parameters (i.e., water quality, fouling propensities, flux) has not been

systematically studied yet. Further studies are recommended to understand underlying mechanisms and to confirm ARGs removal efficiencies as a function of key operational parameters.

4.5.3 Oxidation and disinfection processes

Removal of AMR by UV irradiation, ozonation and chlorination has been studied in more detail and researchers covered a wide range of oxidant dosages. Illustration of results in Figure 4-4 followed the approach to only include data relevant for environmental application. Data from experiments with extremely high or low dosages were excluded based on expert knowledge, detailed results from literature can be found in Tables 10-1 and 10-2.

4.5.3.1 UV irradiation

UV irradiation is a disinfection technology using ultraviolet (UV) light resulting in the damage of DNA nucleotides of pathogens. The German drinking water directive requires a UV fluence of 40 mJ/cm^2 for disinfection (TrinkwV, 2018). The National Water Research Institute (NRWI) in the USA recommends equivalent fluences 20 mJ/cm^2 for successful MS 2 phage reduction and about 180 mJ/cm^2 for successful Adenovirus inactivation in drinking water purification. In contrast, equivalent doses up to 100 mJ/cm^2 for MS 2 reduction are recommended by NRWI for UV disinfection in water reuse applications, depending on the specific pretreatment (e.g., granular media filtration, membrane filtration) (Emerick et al., 2012). Generally, applied UV fluences for WWTP effluent disinfection usually vary between 60 and 200 mJ/cm^2 (Bourrouet et al., 2001; Jolis et al., 2001). Total abundances of sulfamethoxazole and tetracycline resistant HB and tetracycline resistant FC significantly decreased at typical UV disinfection fluences (Meckes, 1982; Luczkiewicz et al., 2011; Guo et al., 2013 and Munir et al., 2011). However, despite effective abatement of tetracycline resistant fecal coliforms (CFU/100 mL dropped by 2.7 log units at UV fluence of 45 mJ/cm^2), the relative abundance of tetracycline resistant FC increased by 435% (Meckes, 1982). An increase in relative abundance of target tetracycline resistant bacteria was also confirmed in other studies (Guo et al., 2013; Luczkiewicz et al., 2011) indicating that bacteria carrying antibiotic resistance are also more tolerant to UV irradiation than non-resistant bacteria. Meckes (1982) suggested that specific proteins which are responsible for tetracycline resistance, may absorb UV light and protect the bacteria. However, the generally low ARB removal efficiencies of the presented UV irradiation studies could also be a matter of photo-reactivation (Hijnen et al., 2006; Jungfer et al., 2007; Guo et al., 2011; Guo et al., 2012) as well as other

factors like protection by colloids and particles in wastewater. Available literature data on ARB removal are not consistent and do not reflect inactivation efficiencies reported for non-resistant bacteria. Although driving mechanism of UV disinfection results in DNA damage, limited removal of ARGs was reported at UV fluences typically applied for disinfection (200 mJ/m^2; McKinney et al., 2012; Zhang et al., 2015). Compared to rapid inactivation of ARB, high fluences are needed to remove ARGs (Zhuang et al., 2015). It is suggested that this discrepancy might be an artifact of the applied qPCR analyses of ARGs using short amplicons often 200 base pairs whereas an increase in amplicon length would likely result in increased log removal of ARGs. This effect was previously reported for the analysis of virus removal by UV disinfection using qPCR methods (Ho et al., 2016). Results from a recent study confirmed higher fluence-based rate constants for ARG removal towards increasing amplicon size and also indicate that the entire plasmid needs to be intact for horizontal gene transfer by transformation (Yoon et al., 2018). Moreover, McKinney et al. (2012) observed minor destruction of intracellular ARGs than extracellular ARGs and strong positive correlation to adjacent CC pyrimidine counts leading to high proportion of undamaged ARGs. The authors also pointed out that gram positive bacteria (VRE) are less sensitive to UV exposure than gram negative bacteria (McKinney et al., 2012). These results demonstrate that fundamental understanding of underlying principles is needed to assess the fate of ARGs in different processes and resulting consequences for their spread in the environment. Molecular methods with qPCR might provide valuable information if their respective limitations are taken into account during data interpretation while assessing a specific process.

4.5.3.2 Ozonation

The German drinking water directive allows a maximum ozone dosage of 10 mg/L for disinfection (TrinkwV, 2018). In addition to disinfection, ozone is widely applied for oxidation of trace organic compounds, where also simultaneous hydroxyl-radical reactions might take place (Lazarova et al., 2013). Recommended ozone dosages for removal of trace organic compounds are in the range of 0.4 to 1.0 mg O_3/mg DOC depending on removal objectives and target compounds. Recent studies indicated that germicidal effects of ozone depend on microbial community type (Alexander et al., 2016; Czekalski et al., 2016) as well as water quality parameters such as alkalinity, dissolved organic carbon (DOC), and the presence of particles (TSS/turbidity), which can negatively affect the stability of ozone and protect microorganisms attached to the surface of particles (Czekalski et al., 2016). The disinfection efficiency of ozone depends on the ozone exposure, i.e. the integration of ozone concentration over contact time, which is highly affected by the specific water quality. Especially in wastewater

matrices, DOC, suspended solids (SS), residual nitrite concentrations, and particulate matter from activated sludge treatment can result in a rapid depletion of ozone and limited efficiency for disinfection (Czekalski et al., 2016; Pak et al., 2016; Lee and von Gunten, 2016; Zucker et al., 2015). Elevated ozone doses to improve disinfection efficiency, however, are often limited by the formation of the carcinogenic by-product bromate (Hübner et al., 2015). Consequently, full-scale ozone treatment of wastewater (0.55 mg O_3/mg DOC) resulted in limited inactivation of 1–2 log units CFU/100 mL of ARB (Czekalski et al., 2016). Cultivation based analyses suggested higher removal efficiencies. For cultivable tetracycline resistant E. coli, 1.5, 2.5 and 5.5 log units CFU/ 100 mL could be achieved using elevated ozone dosages of 3, 5 and 7 mg O_3/L, respectively, in a laboratory-scale study (Oh et al., 2014). In contrast to ARB, limited removal of ARGs has been reported during wastewater (Alexander et al., 2016; Czekalski et al., 2016) and drinking water ozonation (Xu et al., 2016). This was mainly in agreement with results from taxonomic gene markers for specific bacterial strains (Alexander et al., 2016). Only very high ozone dosages resulted in more efficient removal of genes (Zhuang et al., 2015). To date, the exact mode of action of ozone on microbial cells is not completely understood. Several studies reported damage of cell membranes during ozonation, even before DNA damage might occur (Czekalski et al., 2016). However, Patil et al. (2011) concluded in their study that cell lysis was not the major mechanism of microbial inactivation. In addition, cell damages caused by ozone can be repaired due to high activity of cellular repair enzymes (von Sonntag and von Gunten, 2012). Michael-Kordatou et al. (2018) suggested further optimization potential of the ozonation process (regarding ozone dose and contact time) for enhanced AMR removal. Further studies are needed to better understand ARB inactivation mechanisms and damage of ARGs in ozonation processes.

4.5.3.3 Chlorination

Chlorination is the most common applied water and wastewater disinfection process worldwide. The maximum permitted dose is 4.7 mg/L free Cl_2 for calcium hypochlorite ($Ca(OCl)_2$) and 6 mg/L free Cl_2 for chlorine gas (Cl_2) and sodium hypochlorite (NaOCl) during drinking water treatment according to the German drinking water directive (TrinkwV, 2018). Usually applied chlorine dosages to disinfect WWTP effluents are in the range of 5–10 mg/L with 30 min contact time (Zhuang et al., 2015). The disinfection efficiency of chlorine depends on the chlorine exposure, which is often calculated simply as dosage or reactor effluent concentration multiplied by contact time. As inactivation rates for chlorination of numerous bacteria strains are well documented in literature, the same mechanisms for inactivation of ARB and not-resistant

bacteria can be expected. Unfortunately, many studies investigating the fate of antibiotic resistance during chlorination do not report specific operational information like chlorine exposure or process relevant water quality data such as DOC, suspended solids, NH4-N concentrations, floc structure, and pH value. Studies investigating ARB removal efficiencies during chlorination confirm experiences from disinfection of non-resistant bacteria achieving 3 log units removal at relevant dosages (Huang et al., 2011). However, also less efficient removal has been reported for ARB (Munir et al., 2011; Oh et al., 2014). In addition, at chlorine dosages 2.5 mg/L of sodium hypochlorite, the removal efficiency of the detected ARB was incomplete and a regrowth of the ARB was observed (Huang et al., 2011). Similar tolerance of ARB against low chlorine dosages was reported by Yuan et al. (2015). Sulfadiazine and erythromycin resistant bacteria were not eliminated until chlorine exposure was increased up to 60 and 150 mg Cl_2 min/L. Similar to UV disinfection and ozonation, little removal of ARGs was reported during chlorination (Zhang et al., 2015; Yuan et al., 2015; Fahrenfeld et al., 2013; Yang et al., 2016) with some exceptions from experiments with high or unspecified dosages (Xu et al., 2016; Zhuang et al., 2015). Yuan et al. (2015) hypothesized that the chlorination process cannot damage the plasmid structure so that transformability of ARGs is still feasible. The study of full-scale WWTP with chlorination (applying a dose of 8–9 mg/L chlorine at 30 min contact time) by Liu et al. (2018) revealed an increase of relative abundance of intracellular and extracellular ARGs.

4.6 Conclusion

This review provides a comprehensive overview regarding the current knowledge about analysis, occurrence and treatment of AMR in the anthropogenically impacted water cycle with the intention to identify knowledge gaps and future research needs. Numerous researchers have demonstrated elevated levels of antibiotic resistant bacteria and especially resistance genes in municipal wastewater effluents in contrast to receiving urban streams as well as pristine or low impacted water bodies. These findings confirm that discharge from wastewater treatment plants can result in significant contributions of AMR to the aquatic environment. This discharge of pathogenic bacteria carrying antibiotic resistance into surface waters might pose a potential threat to human health, especially if receiving rivers are subject to downstream use, for instance for recreational purposes or drinking water abstraction. However, the acute and long-term risk resulting from the release of resistance genes into the aquatic environment but also the presence of naturally-occurring resistance background, requires further studies. Further research is needed to better understand

potential pathways and threat of water-borne resistance genes to human health, even if they are not linked to living pathogenic bacteria. This information is needed to inform a risk-based determination of removal targets for wastewater treatment. The regulatory discussion on AMR should also include restrictions to the occurrence of antibiotics, since already low µg/L concentrations might constitute a selective pressure for adaption of antibiotic susceptible to antibiotic resistant bacteria in WWTPs and in specific cases maybe even in the aquatic environment. In addition, the adaption to AMR in WWTPs and aquatic environment needs additional research to fully characterize potential risks. Another important task is the application of standardized analytical techniques to determine occurrence of antibiotic resistance and to verify removal efficiencies by different treatment processes. Cultivation methods enable the detection of living organisms with antibiotic resistance but are limited to the determination of viable and cultivable bacteria. In contrast, qPCR provide a rapid detection method for antibiotic resistance genes reporting thus an "antibiotic resistance potential", but are lacking information on the acute risk, as results are not linked to pathogens. In order to provide reliable information on treatment efficiency for different processes from qPCR analysis, underlying mechanisms for inactivation need to be well understood, considering also the gene intactness to mitigate overestimations of ARG abundances. New qPCR technologies, such as digital PCR, may furthermore help to establish new standards based on absolute gene occurrences. Evaluation of removal efficiency using qPCR requires a solid understanding of processes since different investigated gene lengths of the same ARG may render the comparison of studies difficult without further validation. In addition, monitoring of fate and transport of AMR should focus on different antibiotic classes. Resistance for broad spectrum antibiotics, with occurrence in aquatic systems can be used as indicators to evaluate removal efficiencies in different processes, whereas AMR to antibiotics of last resort are more relevant to assess associated risks. Thus, the following indicator ARGs are suggested to monitor for AMR: resistance genes of broad-spectrum antibiotics *sul1, sul2* and *tet* genes (*tetA, tetB, tetO* and *tetW*) as well as resistance genes of antibiotics of last resort *vanA* and *blaVIM*. For ARB, the following suitable indicators are proposed*: Fecal coliforms, P. aeruginosa, Enterococci, and Enterobacteria*. While ample information is available regarding the fate of AMR in different conventional and advanced treatment processes, systematic studies to determine the impact of key operational and site-specific parameters (e.g., sampling conditions, dry or wet weather, wastewater parameters, antibiotic concentrations, degree of wastewater contributions in rivers) and mechanisms responsible for the observed attenuation (e.g., photo-reactivation effect) are often lacking. Conventional wastewater treatment plants are capable of reducing AMR by several orders of magnitude. Reported removal

efficiencies, however, are highly variable and likely depend on treatment type, operational settings applied, and other still unknown factors.

Micro- and ultrafiltration employed in membrane bioreactors can provide an efficient barrier to antibiotic resistance in wastewater. Also advanced non-submerged membrane treatment of wastewater effluent will probably result in similar performance. Key parameter for the retention of bacteria and genes is the pore size of the membrane, but also the formation of cake layers might contribute to overall performance. Available results from granular media filtration studies are of preliminary nature since relevant operational parameters were often not specified. However, some results indicate significant potential for filter optimization to achieve removal of several orders of magnitude. Studies on biologically active systems operating with longer retention times (e.g., groundwater recharge, bank filtration) are lacking.

The removal of ARGs by oxidation and disinfection is closely related to the mode of action of the respective oxidants. Disinfection processes provide an efficient barrier for pathogenic bacteria independent whether they carry AMR. However, it is noteworthy that ozonation of wastewater often is designed for removal of trace organic chemicals at which dose it cannot be considered a reliable disinfection process as it typically achieves approximately 1–2 log inactivation for bacteria. Furthermore, combinations of advanced treatment processes should be examined regarding their removal of antibiotic resistance.

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5 REMOVAL OF ANTIBIOTIC MICROBIAL RESISTANCE BY MICRO- AND ULTRAFILTRATION OF SECONDARY WASTEWATER EFFLUENTS AT PILOT SCALE

The following chapter addresses ***research hypothesis #2.1***: *The increase of a fouling layer with progressive filtration time will lead to a higher AMR removal efficiency.* Furthermore, ***research hypothesis #2.2:*** *Higher AMR feed abundance results in significantly higher AMR abundance in UF filtrate* is tested. Lastly, ***research hypothesis #2.3*** *Comparing AMR removal efficiencies of membranes with large pore size differences (UF = 20 nm vs. MF = 450 nm) will result in significantly different AMR removal* is addressed.

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5.1 Abstract

Low-pressure membrane filtration was investigated at pilot scale with regard to its removal of ARGs in conventional secondary treated wastewater plant effluents. While operating microfiltration (MF) and ultrafiltration (UF) membranes, key operational parameters for AMR studies and key factors influencing AMR removal efficiencies of low-pressure membrane filtration processes were examined. The main factor for AMR removal was the pore size of the membrane. The formation of the fouling layer on capillary membranes had only a small additive effect on intra- and extrachromosomal ARG removal and a significant additive effect on mobile ARG removal. Using feeds with different ARGs abundances revealed that higher ARG abundance in the feed resulted in higher ARG abundance in the filtrate. Live-Dead cell counting in UF filtrate showed intact bacteria breaking through the UF membrane. Strong correlations between *16SrRNA* genes (as surrogate for bacteria quantification) and the *sul1* gene in UF filtrate indicated ARB likely breaking through UF membranes.

Keywords: microfiltration; ultrafiltration; standard filtration mode; antibiotic resistance genes; *16SrRNA* gene, total cell counts

5.2 Introduction

Antibiotics, antibiotic resistant bacteria (ARB), and antibiotic resistance genes (ARGs) present in wastewater effluents can contribute to elevated levels of those constituents in the receiving aquatic environment (Alexander et al. 2015; Kristiansson et al., 2011; Rizzo et al., 2013). This can result in an increase in the abundance of AMR in surface waters after receiving conventional WWTP effluents (Hiller et al., 2019). This topic has been intensively studied in the past for urban and low impacted surface water analyzing either ARB by cultivation method or ARGs by qPCR technique (Hiller et al., 2019). The spread of AMR in the environment is facilitated by horizontal gene transfer, which describes the gene transfer by conjugation, transformation and transduction (Giedraitiené et al., 2011). The horizontal gene transfer is occurring naturally, not only between similar bacterial strains and between gram-negative and gram-positive bacteria, but also between pathogenic and non-pathogenic bacteria (Courvalin, 1994). That is the reason why both ARB and ARGs promote the increase of antibiotic resistance in the aquatic environment. Therefore, release of ARB and ARGs into the aquatic environment should be reduced.

Advanced wastewater treatment processes are capable to remove AMR to levels similar to ‘low impacted surface water’ concentrations (Hiller et al., 2019). One possible technical solution is the use of membrane filtration such as microfiltration (MF) and ultrafiltration (UF). These technologies have been established predominantly as membrane bioreactor (MBR) process applications (Du et al., 2020). Here, low-pressure membrane filtration is applied to replace the secondary clarifier as the solids separating step of the biological treatment stage. The implementation of a full-scale membrane filtration process in a conventional biological nutrient removal facility concerning ARB and ARGs removal requires a mechanistic understanding of the membrane filtration process. Most ARB are larger (0.2 to 2 µm) than MF or UF pores and therefore should be efficiently retained by MF or UF. In contrast, mobile ARGs which can be encoded in mobile genetic elements such as plasmids, integrons, transposons, or bacteriophages are usually too small to be sufficiently removed by size exclusion alone (Slipko et al., 2019; Breazeal et al., 2013). While previous studies confirmed the penetration of mobile ARGs through the membrane pores the question is raised if all bacteria and all intracellular ARGs are retained by UF or is it possible that intact bacteria including ARB can pass the membrane pores. Furthermore, the fact of mobile ARGs breaking through the membrane pores hypothesizes whether higher ARGs abundance in the feed can result in higher ARGs abundance in the filtrate. Certainly, the term low-pressure membranes significantly differ in their pore size distribution ranging from MF (e.g., 450 nm) to UF (e.g., 20 nm) resulting in different AMR removal efficiencies (Breazeal et al., 2013). Beside different

pore sizes of MF and UF, filtration processes applied differ from cross flow mode with continuous water- or air-cross-flow to dead-end filtration mode with separate backwash mode to minimize the build-up of a fouling layer. However, the fouling layer could cover pores potentially resulting in an increased ARGs removal.

While plenty information is available on the reduction of ARB and ARGs in different membrane filtration studies, key operational parameters (e.g., sampling protocols, dry or wet weather conditions, wastewater constituents, or operational parameters like flux, TMP, membrane integrity confirmation etc.) are not comprehensively reported. For example, the ARG studies of Munir et al. (2011a) and Böckelmann et al. (2009) examined the membrane filtration process of full-scale WWTPs for ARG removal efficiencies whereas no flux, TMP, operation mode and weather conditions were reported. Therefore, key operational parameters and target genes should be determined for AMR examinations of membrane filtration processes. Only uniform testing methods enable a comparison of AMR removal efficiencies of membrane filtration studies.

Previous mechanistic studies on AMR removal during membrane filtration have investigated ARGs predominantly in bench scale systems, and studies investigating AMR retention of MF and UF by employing capillary membranes in parallel operation mode at pilot scale are missing. Bench scale studies investigated the effect of different pore sizes on ARGs removal was evaluated by Breazeal et al. (2013). While UF with a cut-off of 100 kDa demonstrated a 1.7 log unit rejection of *bla* genes/100 mL, a 3 log greater abatement of *bla* genes was achieved by using an UF with a cut-off of 10 kDa (Breazeal et al., 2013). Further, Chaudhry et al. (2015) reported of beneficial effects of an increasing fouling layer on virus removal. Within their study they observed an additional pathogenic virus removal between 0.5 and 1.6 log units in a full-scale membrane bioreactor (pore size 0.04 µm). However, studies on the effect of the fouling layer on AMR removal using capillary membranes are still missing. Further membrane filtration studies with respect to the abatement of mobile ARGs and their penetration through UF membranes were conducted by Slipko et al. (2019) and Krzeminski et al. (2020), whereas membranes with a molecular weight cut-off (MWCO) smaller than 5 kDa (UF, NF and RO) were applied resulting in removal efficiencies of more than 99 % of free DNA. Further UF studies resulted in bacteria removal between 36 to 98.9 % using cultivation method (Morales-Morales et al., 2003; Ren et al., 2018). In this UF study flow cytometry is applied for a more accurate bacteria removal analysis (Cheswick et al., 2019).

In this study, MF and UF were investigated as efficient technologies to reduce the dissemination of ARB and ARGs. The objective of this study was to mechanistically examine key factors that influence the AMR removal efficiency during the membrane filtration processes for wastewater treatment in standard filtration mode. We specifically

investigated the influence of the microbial load in the feed, the pore size of the capillary membranes, and the effect of the fouling layer on removal efficiencies. It was hypothesized that the smaller pore size of the UF membranes lead to higher AMR removal. Furthermore, it was tested whether feed waters with higher ARGs abundances would result in higher ARGs abundances in the corresponding filtrates. Besides, while employing capillary membranes, it was expected that a fouling layer will result in a higher AMR removal efficiency. Finally, it was investigated to what extent intact bacteria as well as AMRs from feed water break through UF membranes at pilot scale.

5.3 Materials and methods

5.3.1 WWTP Steinhäule and membrane filtration pilot unit

Pilot-scale membrane studies were performed at the wastewater treatment plant Steinhäule in Neu-Ulm, Germany with a treatment capacity of 445.000 population equivalents. The WWTP Steinhäule is designed for 2,600 L/s (flow at wet weather conditions), which is double the dry weather flow. At this facility, wastewater is treated by four treatment stages – mechanical, biological, chemical, and physical stages. After secondary treatment, the physical stage is comprised of a contact reactor where 10 mg/L of powdered activated carbon (PAC) is continuously fed in order to remove trace organic chemicals. A subsequent clarifier is employed to separate the PAC followed by a tertiary filtration step. Settled activated carbon from the clarifier is returned to the contact reactor for better utilization of the PAC. Secondary effluent (SE) as well as tertiary effluent (SE+PAC+SF) were used as feed water qualities for subsequent membrane filtration studies. The overall wastewater treatment process at WWTP Steinhäule is illustrated in Figure 5-1A. Feed water constituents are presented in Table 10-3. All AMR examinations were executed using a membrane filtration pilot plant. The membrane filtration pilot plant consisted of two parallel trains. Every train comprised of two pre-filters (400 µm cut-off), feed tank (reservoir), membrane module, and by-pass filtrate/ backwash tank. Pump and flocculant tank enabling continuous flocculant dosing. Chemical enhanced backwash (CEB) was performed with one acid tank and pump as well as two base tanks and two pumps (Figure 5-1B).

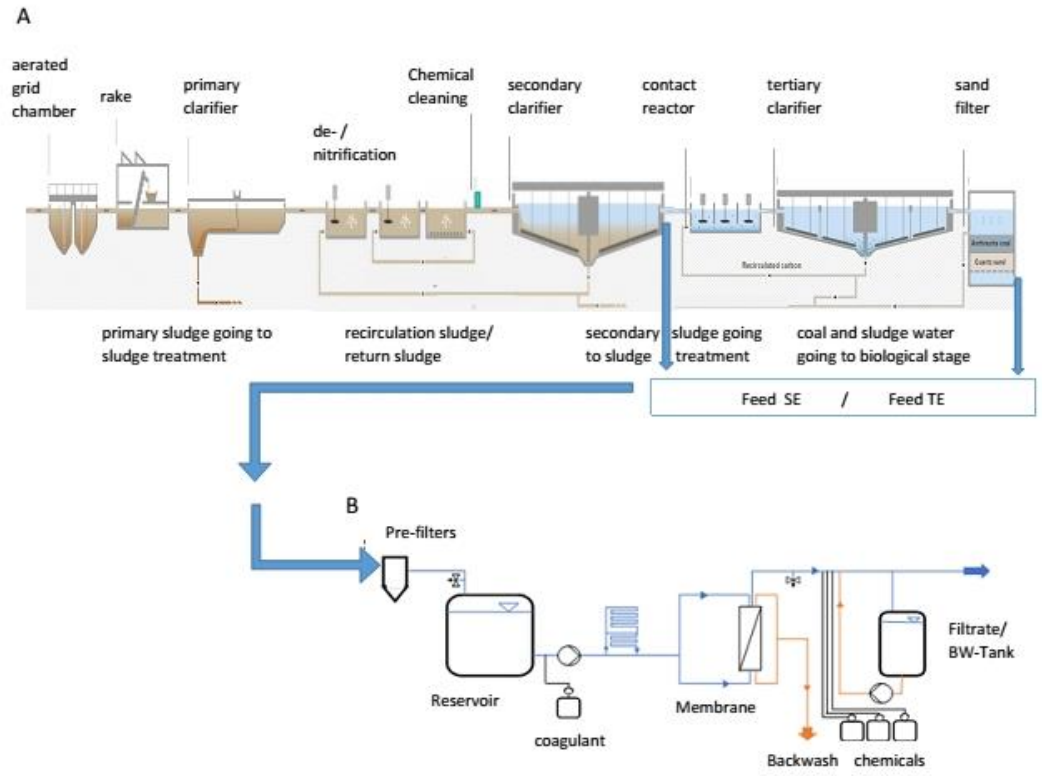

Figure 5-1: Schematic diagram of the overall wastewater treatment at WWTP Steinhäule (5-1A). Schematic flow diagram of one train of the membrane filtration pilot plant is shown in Figure 5-1B.

Membrane modules with pore sizes of 20 nm (UF, 80 m² surface are) and 450 nm (MF, 22 m² surface area) were selected for AMR studies. Both membrane modules were made of hydrophilized polyethersulfone and had a contact angle of 52°. While UF membrane module had 7 capillaries per fiber, the MF membrane module consisted of one capillary per fiber. Both membrane modules were operated in an inside-out, dead-end filtration mode in parallel. Microfiltration operated at a flux of 70 LMH. The ultrafiltration operated at fluxes of 40 and 70 LMH during the AMR studies.

The filtration cycle of the membrane filtration process is described in the following section: The standard operation mode of the membrane filtration process was 60 minutes. In this mode, the pilot plant operated at constant sustainable flux at 40 or 70 LMH under reversible fouling conditions, whereas feed wastewater was pumped from the reservoir through the membrane module to the filtrate side (Figure 5-1). A final coagulant (polyaluminum chloride solution, DIN 883, PLUSPAC FD ACH, Feralco Deutschland GmbH, Germany) dose of 2 mg/L was continuously fed into the feed line directly prior to the feed side of the membrane module. The continuous coagulant dosing was applied as fouling control. Coagulation reduces the occurrence of reversible fouling and increases the filtration efficiency (Yoo, 2018). After 60 minutes, the standard operation mode ended and both feed pump and coagulation pump were switched off. Standard backwash

mode was activated, whereas hydraulic backwashing was executed by applying filtrate water from the backwash-tank to the module at an outside-in mode. The backwash mode lasted for 45 seconds at a flux of 230 LMH. After 23 backwash modes, a chemical enhanced backwash (CEB) mode was performed. The CEB mode consisted of injecting and rinsing the UF for 90 seconds with 150 ppm sodium hydroxide at an intake flux of 120 LMH. After that, the UF module was soaked for 15 min with the injected sodium hydroxide. A final (hydraulic) backwash rinsed the chemical out at a flux of 230 LMH for 70 s. A short backwash at 70 LMH for 900 s with only filtrate was conducted directly after the sodium hydroxide CEB. Finally, the CEB procedure was repeated with sulfuric acid. After CEB procedure with sulfuric acid and a backwash to rinse the chemical out of the membrane module, the standard operation mode was initiated again.

5.3.2 Experiments and sampling conditions

In section 5.4.1, key operational parameters for membrane filtration studies were examined. AMR examinations were executed only at dry weather conditions and during standard filtration mode of the membrane filtration process. Other filtration modes, such as backwash and chemical enhanced backwash modes, were not considered. The standard filtration mode in this study is defined as the time of the membrane filtration operation, whereas the membrane filtration operates at a certain steady flux (e.g., 70 LMH) and at constant filtrate quality. In experiment I continuous filtrate quality analyses were executed in filtrate using the total cell count (TCC) as quality parameter. After 60 minutes, the filtration cycle was terminated and the backwash mode was activated in order to remove the fouling layer. In addition to the TCC analyses the transmembrane pressure (TMP) was employed as surrogate parameter for the built-up of a fouling layer within 3 and 60 minutes of standard filtration mode (experiment II). For long-term TCC measurement, one flow cytometry measurement device (Sigrist company) was connected to the feed line and one to the filtrate line of the membrane filtration pilot plant to automatically sample and measure TCC values over a period of 3 days before and after the AMR studies (experiment III). To compare the treatment variability of UF trains 1 and 2 in experiment IV, the two flow cytometry measurement devices were connected at the filtrate sides of both UF trains to automatically sampling and measuring TCC values for 2 days.

The fouling layer was examined as layer with additional AMR removal in experiment V in section 5.4.2. In order to account for possible effects of the fouling layer on AMR removal efficiency, sampling was conducted of feed and of UF filtrate after 5 and 55 minutes during standard filtration mode.

In section 5.4.3, following key factors for AMR removal were studied: Experiment VI intended to investigate for the effect of the AMR abundance in the feed and its consequences to AMR abundance in the filtrate. To analyze the relation of AMR abundance in feed and filtrate, samples were taken from the feed and filtrate side of the pilot plant. In addition, secondary effluent and tertiary effluent of WWTP Steinhäule were used as feed waters with different qualities and AMR abundances.

Pore size as an influencing factor on AMR removal efficiency of the membrane filtration process was examined in section 5.4.4 (experiment VII). The comparison of MF and UF removal efficiencies were performed by sampling filtrate qualities of both trains during parallel operation mode in consistent conditions (same flux, same material PES, same hydrophilicity of the membrane, inside-out operation, same feed, same coagulation dose, same standard filtration mode, backwash and CEB conditions). Samples for ARGs and *16S rRNA* genes as well as flow cytometry analyses were taken from feeds and corresponding UF filtrates.

In section 5.4.5, breakthrough of intact bacteria was examined as further factor influencing AMR removal efficiency. In experiment VIII, samples were taken from the feed and the corresponding filtrate from the pilot-scale UF membrane filtration (pore size of 20 nm) whereas a virgin membrane module was applied. In parallel to the pilot-scale membrane filtration, the dead and living bacteria analysis was also conducted with sterile syringe filter (Whatman® Anatop®) with a pore size of 20 nm. Both UF samples were taken and compared to exclude possible contaminations at the filtrate side. The dead and living bacteria analysis was performed using another flow cytometry from Beckman Coulter whereas gating considered all events that were larger than the added 0.2 µm beads.

To maintain sterile sampling conditions, sampling taps were flamed and stagnant water was removed prior to sampling. Grab samples for qPCR were taken and were frozen immediately after sampling at minus 20°C. Grab samples for flow cytometry (Sigrist GmbH) were manually or automatically taken and immediately analyzed at the membrane filtration pilot plant. Grab samples for flow cytometry measurement (Beckman Coulter) were manually taken and were analyzed in the laboratory within three hours.

5.3.3 AMR and microbial biomass analyses

Pre-screening studies confirmed sufficient abundances of *ermB* and *sul1* genes in the two feed water qualities in order to demonstrate ARGs removal of at least 2 log units. *VanA* gene exhibited lower abundances in the feed waters, but due to its role as antibiotic of last resort it was included in this study. Hence, the following antibiotic resistance genes were selected for AMR analyses: *ermB, sul1* and *vanA* genes. In addition, the *16S rRNA*

gene was selected as a surrogate parameter for total cells present in samples. *16S rRNA* gene quantification is practiced for bacteria quantification (Clarridge III, 2004; Revetta et al., 2010; Hembach et al., 2019).

In the laboratory, samples were thawed and an aliquot of 20 mL of the sample were freeze-dried to concentrate cells and DNA. The pellet was dissolved in 500 µl Water and extracted using the Power Soil DNA extraction kit (Qiagen), following the manufacturers protocol. The DNA was then subjected to quantitative PCR (CFX 96, Bio-Rad) with primer sets for *sul1* (Pei et al., 2006), *ermB* (Alexander et al., 2015), *16S* (Lôpez-Gutiérrez et al., 2004), and a primer probe combination for *vanA* (primer VnF and VnR from Lata et al. (2009) with probe vanAPr from Furukawa et al. (2015). For *sul1*, *ermB*, and *16S*, we employed the GoTaq qPCR Master Mix (Promega), following the reaction guidelines for a total volume of 21 µl with 1 µl of template DNA. DNA was diluted if necessary with nuclease-free water. Amplification products of the qPCR were inspected by investigating the melt-curve of each reaction. For *vanA* we used the SsoAdvanced Universal Probes Mix (Bio-Rad) following the reaction guidelines for a total volume of 16 µl. The qPCR results were calibrated using a ten-fold dilution series of a linearized plasmid (obtained by cloning using the pGEM-T easy system (Promega)) that contained a single copy variant of the listed genes across at least five orders of magnitude resulting in following efficiencies: *16S* (E = 91.9, R^2 = 0.99, slope = -3.54, intercept = 38.2), *ermB* (E = 94.6, R^2 = 0.99, slope = -3.46, intercept = 38.8), *sul1* (E = 83.4, R^2 = 0.99, slope = -3.82, intercept = 42.2), *vanA* (E = 89.3, R^2 = 0.99, slope = -3.61, intercept = 40.3). The detection limit of *vanA* and *ermB* genes were 1,000 gene copies per 100 mL, for *sul1* gene 1,750 gene copies per 100 mL as well as for *16S rRNA* gene the detection limit was 10,000 gene copies per 100 mL. The given detection limits were all above the calculated limit of detection, and were adjusted by the respective PCR efficiency and by setting a minimum of four gene copies per PCR reaction. ARG values that were below these detection limits were accounted for by using half the value of the detection limit in the bar plots. For the correlation analysis values below the detection limit were excluded.

Cell count and cell status were investigated using flow cytometry (Sigrist GmbH, Switzerland) revealing total cell count (TCC), low nucleic acid count (LNAC), and high nucleic acid count (HNAC). TCC is the sum of LNAC and HNAC. LNAC represents cells with low nucleic acid amounts, whereas HNAC provides information about cells with high nucleic acid amounts. The relation of LNAC and HNAC sample describes the microbiological fingerprint of a water sample. Santos et al. (2019) investigated in a flow cytometry study of different sampling sites of a river. Bacteria community analysis exhibited high HNAC density sampling downstream of the WWTP discharge due to high amounts of organic and nutrient from the wastewater. A higher LNAC density was

analyzed sampling river headwater with an oligotrophic environment. Even disruptions in the microbiological system can be observed analyzing LNAC and HNAC (Kötzsch and Sinreich, 2014). TCC, HNAC and LNAC values were analyzed using main fluorescent channels between 525 and 545 nm (FL1) and low pass fluorescent channels of more than 715 nm (FL2). Samples were stained with the fluorescent dye SYBR® Green. The gating was fixed to quantify LNAC, HNAC and background signals by using recommended values by the manufacturer. The detection limit of the flow cytometry was 10,000 cells per 100 mL. The fluorescent dye SYBR® Green binds to double stranded DNA (dsDNA). Hence, low nucleic acid amounts (LNA) is a sum parameter whereas double stranded DNA of small bacterial cells and virus with DNA genome (dsDNA) can be counted by flow cytometry (Kötzsch et al. 2012; Brown et al., 2015). Therefore, *16S rRNA* gene was compared to HNAC values as surrogates for bacteria quantification in wastewater.

To distinguish between live and dead cells in UF filtrate the following dyes were applied: SYTO 9 nucleic acid stain showed intact cell membranes and fluoresces bright green. The applied fluorescent channel was 525 nm (FITC-H). Propidiumiodide indicates damaged membrane cells and fluoresces red (LIVE/DEAD™ BacLight™ Bacterial Viability and Counting Kit, for flow cytometry, Thermo Fisher). The applied fluorescent channel was 690 nm (PC5.5-H). Cell analyses were differentiated by using the Sub-micron Particle Size Reference Kit (Thermo Fisher) with 0.2 µm beads. To quantify living and dead bacteria the gating was adjusted to cells that are larger than 0.2 µm. These measurements were taken by a CytoFlex instrument (Beckman Coulter, USA).

5.3.4 Statistical data analyses

Statistical data evaluation was conducted using pair samples two-tailed t-test and independent samples two-sided t-test with a significant threshold $\alpha = 0.05$. The t-test requirements were normality and homogeneity of variances. To examine the significance of mean values of different data series of 5 minutes samples and 55 minutes samples to quantify AMR removal of the fouling layer, the pair samples t-test was applied. Based on corresponding values (5 and 55 minutes of a filtration cycle), the two data series should have good correlation values.

The statistical data analyses of the examinations of different AMR abundance of the feeds resulting in different AMR abundance in filtrates were performed using independent samples t-test. Independent samples t-tests for significance analyses were also applied for AMR studies analyzing different pore sizes of MF and UF resulting in different AMR abundance in the filtrates. Pearson correlation was used in order to show the relation between *16S rRNA* genes (surrogate for bacteria quantification) and ARGs of feed and filtrate samples in experiment IX.

5.4 Results and discussion

5.4.1 Assessing standard filtration mode, fouling layer build-up, membrane integrity confirmation and treatment variability at pilot scale

To analyze particle removal during standard filtration mode operated at a constant flux of 70 LMH using tertiary effluent from a full-scale wastewater treatment plant as feed, TCC values were determined in samples collected within the first 5 minutes and after 55 minutes of the membrane filtration cycle (Figure 5-2). The analysis of the UF filtrate revealed higher TCC values within the first and second minute compared to the third, fourth, and fifth minute. Lower filtrate quality at the early start of a membrane filtration cycle was in agreement with observations reported by Chaudhry et al. (2015) observing significant higher turbidity and particle counts during the first minutes directly after completion of either backwash or chemical enhanced backwash modes. The reason for this reduced filtrate quality could be the result of particle breakthrough or might have been caused by an impaired water quality used as backwash water. The phenomenon of reduced filtrate quality occurs within the first two minutes of standard filtration mode. The reasons for this reduced filtrate quality could be the backwash mode with low quality backwash water, a reduced fouling layer on the feed side enabling higher turbidity and particle concentration, or a contamination of the UF membrane at filtrate side. However, the low filtrate quality ended within the third minute of standard filtration mode so that low filtrate quality is not a long-lasting event. Constant filtrate quality was achieved at 5 and 55 minutes of standard filtration mode. These statements can be confirmed due to a statistical evaluation. While TCC abundance was significantly different comparing UF filtrate within 1 and 5 minutes of standard filtration mode (pair samples t-test, TCC: $R = 0.999$; $dF = 2$; $p = 0.012$), the UF filtrate after 5 and 55 minutes of standard filtration mode in Figure 2 exhibited no significant differences of TCC abundances (pair samples t-test, TCC: $R = 0.794$; $dF = 2$; $p = 0.199$).

In order to quantify contaminations of the UF membrane at the filtrate side that could result in lower filtrate quality, *16S rRNA* gene analyses were compared at the beginning of the membrane filtration studies using a virgin membrane module (August 2018), after 2 months of continuous UF operation (November 2018), and after 12 months of continuous UF operation (September 2019). *16S rRNA* gene abundances of $2.40 \cdot 10^5$ per 100 mL were measured at the beginning of the membrane filtration studies within 5 and 55 minutes of standard filtration mode (August 2018). After two months and 12 months of continuous UF operation, the arithmetic mean values of *16S rRNA* gene of 5-minute samples (October 2018: $1.67 \cdot 10^5$ per 100 mL; 2019: $1.52 \cdot 10^5$ per 100 mL) and of 55-

minute samples (October 2018: $2.31 \cdot 10^5$ per 100 mL; 2019: $1.80 \cdot 10^5$ per 100 mL) showed no significant difference compared to the values measured at the beginning of the UF study. It can be concluded that the UF filtrate within 5 and 55 minutes of standard filtration mode showed no increasing *16S rRNA* gene abundance. Therefore, a secondary contamination of ultrafiltration membrane can be excluded.

Including these events during sampling would result in a more appropriate assessment of the membrane filtration performance. According to this MF and UF study to analyze AMR removal, sampling was not executed before the first 3 minutes of a filtration cycle.

Furthermore, the transmembrane pressure was used as a surrogate parameter to assess fouling layer build-up. The results of experiment II revealed that the TMP decreased during the first 5 minutes of the standard filtration mode. After 5 minutes, the TMP continuously increased (Figure 5-2). This observation further justifies the choice of a consistent sampling procedure between 5 and 55 minutes during this membrane filtration study to assess AMR removal efficiencies during the continuous build-up of a fouling layer.

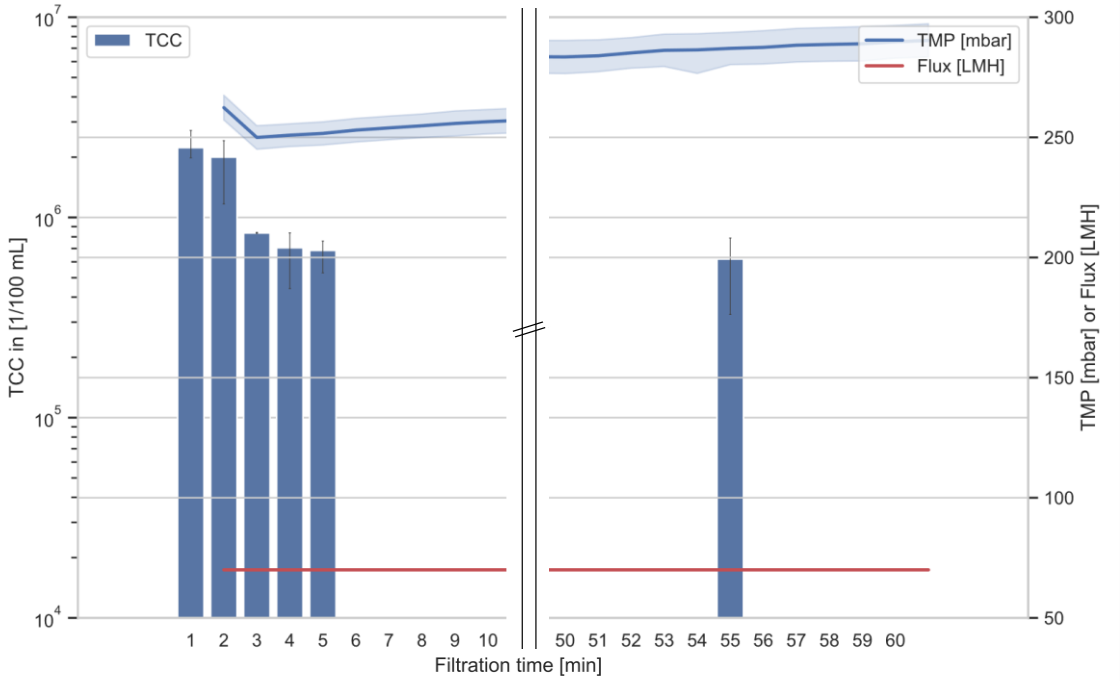

Figure 5-2: Arithmetic mean values of TCC of UF filtrate within the first 5 minutes and after 55 minutes of standard filtration cycle using tertiary effluent as feed (experiment I, n = 3).

In experiment III, long-term flow cytometry measurements were automatically analyzed in feed and UF filtrate for membrane integrity tests. While the results of the flow cytometry analysis of tertiary effluent as feed water was relatively constant (TCC $3.1 \cdot 10^8$–$3.9 \cdot 10^8$ per 100 mL; HNAC $5.0 \cdot 10^7$–$9.1 \cdot 10^7$ per 100 mL), the results of the UF

filtrate resulted in a higher deviation compared to the feed (TCC $1.8 \cdot 10^5$–$8.4 \cdot 10^5$ per 100 mL; HNAC $4.9 \cdot 10^4$–$3.1 \cdot 10^5$ per 100 mL). All in all, the flow cytometry analysis performed over 3 days during continuous UF operation suggested that the TCC removal efficiency by the UF membrane was relatively constant resulting in a reduction of about 3 log units (Figure 5-3). At the end of the entire study, the TCC analyses of feed and corresponding filtrate confirmed a 3-log removal of TCC and therefore confirming that the UF membrane was not compromised while investigating the efficacy of AMR removal. Similar TCC removal results of the UF using surface water as feed are reported by Adomat et al. (2020), who operated a UF with a pore size of 20 nm and observed about 2 log removal of TCC.

In experiment IV, the performance and variability of two UF trains operated in parallel under consistent operating conditions and employing similar membrane modules (80 m²) fed by the same feed water quality, were tested at a flux of 40 LMH. Flow cytometry measurements were analyzed in the filtrates of both UF train 1 and 2 for 2 days. The arithmetic mean values of the parallel measured TCC values in the filtrate of UF train 1 and 2 were $7.68 \cdot 10^5$ per 100 mL and $6.78 \cdot 10^5$ per 100 mL, respectively. With a variability of about 0.11 log units in TCC values of both filtrate qualities, the study revealed no observed difference. Therefore, the two membrane filtration trains exhibited a very similar TCC removal efficiency.

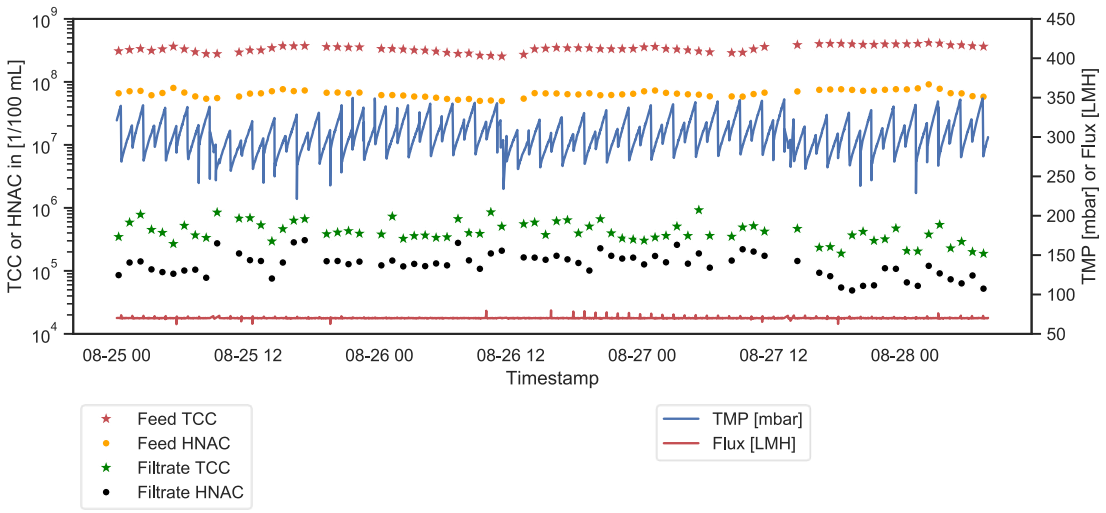

Figure 5-3: Long-term flow cytometry measurements in the tertiary effluent as feed and UF filtrate for 3 days during experiment III (the following operational parameters are illustrated: TCC and HNAC in feed and filtrate, flux and TMP).

5.4.2 Role of the fouling layer for additional AMR removal

In experiment V, the role of a growing fouling layer with progressive filtration time was investigated by performing sampling after 5 and 55 minutes during standard filtration mode. While the UF filtrate after 55 minutes of standard filtration mode exhibited slightly lower TCC values as well as *sul1*, *ermB* and *vanA* genes abundances than the UF filtrate after 5 minutes of standard filtration mode, HNAC and *16S rRNA* genes exhibited no significant difference in UF filtrate quality (Figure 5-4). Based on a confidence interval of 95 %, only *vanA* gene exhibited a significant difference between 5 min and 55 min of filtration, while the other parameters did not reveal any significant differences (see results of the paired t-test, Table 10-4).

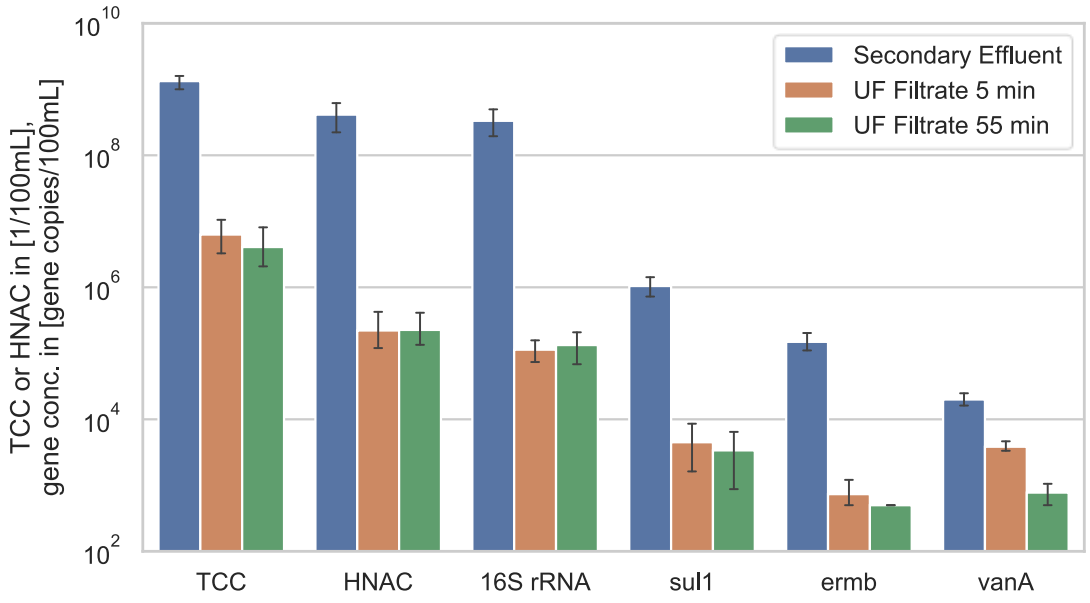

Figure 5-4: Arithmetic mean values of TCC, HNAC, 16S rRNA, ermB, sul1 and vanA genes analyzed in secondary effluent and corresponding filtrates after 5 minutes, 55 minutes and for the entire standard filtration cycle of UF operation. Error bars indicate the 95 % confidence interval. Number of samples and values below LOD are listed according to the samples secondary effluent, UF filtrate 5 min and UF filtrate 55 min. n_{TCC} = (12|3|3), LOD_{TCC} = (no values below LOD); n_{HNAC} = (9|3|3), LOD_{HNAC} = (no values below LOD); $n_{16SrRNA}$ = (12|7|7), $LOD_{16SrRNA}$ = (no values below LOD); n_{sul1} = (12|7|7), LOD_{sul1} = (0|3|4); n_{ermb} = (12|7|7), LOD_{ermB} = (0|6|7); n_{vanA} = (12|4|4), LOD_{vanA} = (0|0|4).

Considering the results of the treatment variability study (see section 5.4.1), it can be concluded that the observed removal of *16S rRNA* genes and HNAC value collected after 5 and 55 minutes are primarily a function of physical separation by pore size of the membrane module rather than driven by an additional fouling layer that is building up with progressive filtration time. Similarly, *ermB, sul1*, and TCC, showed only a marginal decrease between 5 and 55 min. In contrast, the *vanA* genes analyses revealed between 5 and 55 minutes of filtration a reduction by 87 % when the fouling layer was build-up (t-test, dF = 3; p = 0.004). This increasing fouling layer expressed in TMP increase is the

result of cake layer formation, pore constriction or partially clogged pores (Hallé 2010). However, *16S rRNA* genes and HNAC value analyzed within 5 and 55 minutes revealed no difference (t-test, *16S rRNA*: dF = 6; p = 0.548. HNAC: dF = 2; p = 0.629). It seemed that the fouled membrane module still had a high enough number of larger pores for cell breakthrough and the fouling layer did not result in any additional bacteria removal.

The fouling layer effect of an anaerobic membrane bioreactor process (MF, pore size 0.3 µm) concerning ARB removal was reported by Cheng and Hong (2017). The researchers analyzed bacteria and ARGs removal at different fouling layer conditions. Different ARGs to this AMR study were analyzed. Therefore, comparison of ARG removal efficiency is not possible. While the virgin membrane resulted in 5 log units of ARB removal, the subcritically fouled membrane exhibited lower log ARB removal due to an increase in filtration pressure. In contrast, the 5 log ARB removal was achieved by critically fouled membrane, again. The lower bacteria removal during subcritical fouled membrane (reversible fouling conditions), reported in Cheng and Hong (2017), cannot be confirmed in this fouling layer study. It seems that the smaller pore size of 20 nm of the UF in this study enabled an almost constant bacterial removal within 5 and 55 minutes of standard filtration mode. In contrast, the MF membrane had a lower bacteria removal due to higher filtration pressure. This probably bacteria deforming effect due to filtration pressure was already reported by Suchecka et al. (2003). Furthermore, the reported 5 log ARB removal was significant higher to this UF study (3.5 log units of *16S rRNA* gene). In the study of Cheng and Hong (2017), a different feed with significant higher colloid concentrations were applied. ARB and ARGs could additionally adsorb to wastewater colloids resulting in higher ARB and ARGs removal efficiency of the MF membrane. If the bacteria removal efficiency is compared between the MBR process and the UF process in this study, the AMR removal efficiency of the biological stage should be considered to the removal efficiency of the UF process. The pilot-scale study of Marti et al. (2011) also investigated bacteria and virus removal under different cake layer conditions during operation of a membrane bioreactor process in cross-flow mode (membrane area 8 m²; nominal pore size 0.4 µm). The MF operated 9 minutes in continuously cross flow mode with aeration (flux at 25 LMH) and after 1 minute in relaxation phase (filtration off). In this study, the bacteria removal was examined directly after relaxation phase within the first minute with a low fouling layer and within 9 minutes of continuously membrane filtration, whereas the membrane experienced the highest fouling condition. The study results demonstrated that bacteria removal had no correlation with TMP, which was the surrogate for fouling layer increase. *E. coli* could be efficiently reduced by 5.1 log units. This bacteria removal efficiency is in line with the bacteria removal of the MBR study of Cheng and Hong (2017).

In contrast, the growing fouling layer resulted in a significant *vanA* gene removal. The range of this removal is in the range that has been reported for the removal of viruses by a fouling layer Chaudhry et al. (2015). Like viruses, *vanA* genes may be comparatively frequent in the mobile DNA fraction. Che et al. (2019) investigated in a metagenomic sequencing study the occurrence of intra- and extrachromosomal ARGs in wastewater and confirmed that the antibiotic resistance genes of the aminoglycoside class of antibiotics (e.g., *vanA* genes) had higher extrachromosomal abundances (sum of plasmid as well as integrative and conjugative elements) than intrachromosomal abundances (chromosome) compared to resistance genes *ermB* and *sul1* gene of the macrolide (e.g. erythromycin) and sulfonamide class (e.g. sulfamethoxazole). Hence, the observed *vanA* gene removal by the fouling layer may be the result of electrostatic charge effects of the fouling layer that lead to a reduced passage of mobile DNA. Wang et al. (2021) investigated the removal of plasmid with artificial marker genes as a surrogate for extracellular and extrachromosomal ARG using a lab-scale membrane bioreactor (flat-sheet membrane with 0.2 µm of pore size). The study results demonstrated that the plasmids were predominantly removed by adsorption onto sludge particles. An additional plasmid removal was the result of the fouling layer increase. Wang et al. (2021) hypothesized that the enhancement of plasmid removal with the increasing fouling layer was the result of narrow pores or of the enhanced interaction among foulants and plasmids. The foulants, especially extracellular polymeric substances (EPS) and soluble microbial products (SMP) have negatively charged functional groups and the DNA is negatively charged due to the phosphate groups. Extracellular ARGs can have a high tendency to interact with negatively charged EPS and SMP in the presence of divalent cations like Ca^{2+} und Mg^{2+}.

The MF and UF studies were executed with coagulant dosing at the feed side of the membrane module for fouling control. The continuous coagulant dosing of 2 mg/L with Al^{3+} cations can have an additional electrostatic charge effect according to extracellular ARGs removal. The study of Chen et al. (2020) is in line with electrostatic charge effects for ARGs removal. The author investigated intra- and extracellular ARGs removal in municipal wastewater effluent by electrocoagulation. It was reported that UV disinfection of wastewater effluent resulted in an extracellular ARGs increase and following electrocoagulation could significantly reduce extracellular ARGs.

This UF study is the first study in which the fouling layer of capillary membranes in dead-end operation was examined at pilot-scale with regard to ARG removal efficiency. Conversely, the build-up of a fouling layer under hydraulically and chemically reversible fouling conditions, did not result in any significant decrease for *sul1* and *ermB* genes. However, the fouling layer may facilitate a higher removal of free, mobile ARGs.

5.4.3 Role of feed water quality for UF filtrate water quality

It was hypothesized that AMR abundance in the feed water has a direct influence on AMR abundance in the UF filtrate (experiment VI). Two different wastewater qualities, namely secondary and tertiary effluents from the WWTP Steinhäule, were selected as feed waters. The qPCR and flow cytometry analyses exhibited that advanced treatment using powdered activated carbon followed by sand filtration (tertiary effluent) resulted in significant lower TCC, HNAC as well as *16S rRNA*, *ermB*, *sul1* and *vanA* genes abundances compared to the secondary effluent (Figure 5-5) (based on a t-test, Table 10-5). The study results of experiment VI revealed that *sul1*, *ermB* and *vanA* genes could be detected in both UF filtrates. The UF filtrate of the secondary effluent had significantly higher *sul1* genes abundances than the UF filtrate of the tertiary effluent. Since *ermB* genes were detected close to the detection limit, the ultrafiltered secondary and tertiary effluents showed a similar *ermB* gene abundance. No significant different *vanA* genes were measured in ultrafiltered secondary and tertiary effluents. While the TCC value was lower in the ultrafiltered tertiary effluent than in the ultrafiltered secondary effluent, HNAC value and *16S rRNA* gene showed no difference between the two filtrates. This effect of almost constant HNAC and *16S rRNA* gene abundances in UF filtrates was already confirmed in section 5.4.2.

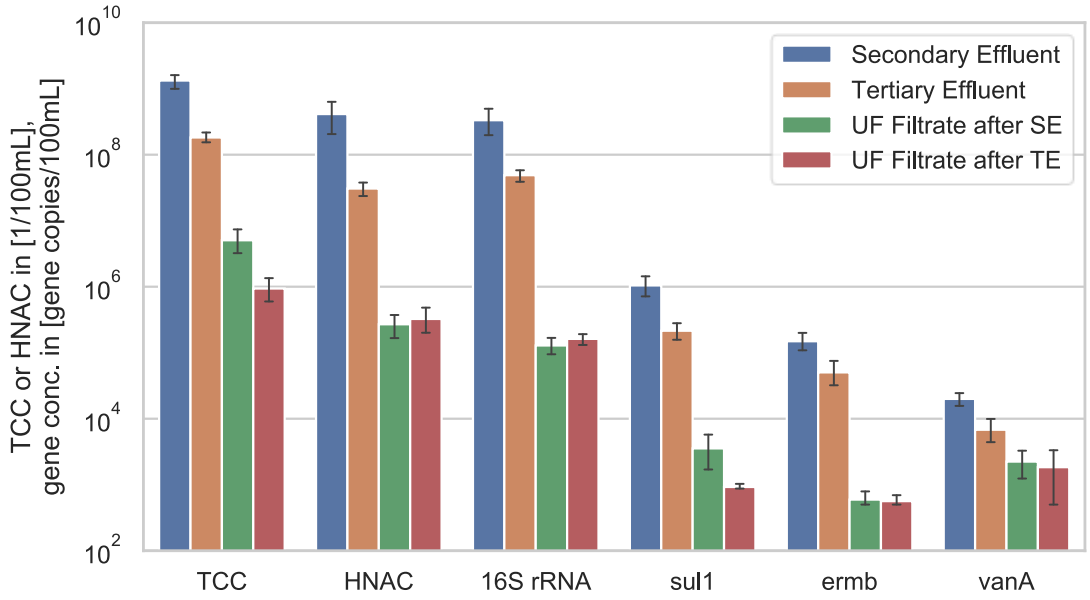

Figure 5-5: Arithmetic mean values of TCC, HNAC, 16S rRNA, sul1 ermB, and vanA genes analyzed in secondary effluent (SE), tertiary effluent (TE), and corresponding filtrates. Error bars indicate the 95 % confidence interval. Number of samples and values below LOD are listed according to the samples secondary effluent, tertiary effluent, UF filtrate after SE and UF filtrate after TE. n_{TCC} = (9|12|10|14), LOD_{TCC} = (no values below LOD); n_{HNAC} = (9|12|10|14), LOD_{HNAC} = (no values below LOD); $n_{16SrRNA}$ = (12|12|16|20), $LOD_{16SrRNA}$ = (no values below LOD); n_{sul1} = (12|12|16|20), LOD_{sul1} = (0|0|9|19); n_{ermb} = (12|12|16|20), LOD_{ermB} = (0|0|15|19); n_{vanA} = (12|10|10|11), LOD_{vanA} = (0|0|3|8).

The UF study of Du et al. (2015) is in accordance with the observations of this study. Du et al. (2015) studied ARG removal by a MBR (using a membrane with 0.1 µm to 0.4 µm mean pore size) analyzing ARGs in the influent and effluent of the MBR process as well as seasonal fluctuations of ARGs in wastewater. Seasonal fluctuations of the *sul1* gene resulted in higher *sul1* gene abundances in the feed and as a consequence in higher *sul1* gene abundance in the filtrate. To summarize, the results of *sul1* genes removal confirmed the hypothesis that higher AMR abundance in feed water results in higher AMR abundance in UF filtrate.

5.4.4 Comparison of MF and UF ARG removal efficiencies

To elucidate the effect of different pore sizes on the ARG removal efficiency, MF and UF with different pore sizes were employed in parallel operation in experiment VII. In this case the MF and UF modules were operated with secondary effluent as feed. The HNAC and *16S rRNA* gene showed a similar response to the MF and UF treatment with significantly higher removal rates with UF (3.2 log and 3.5 log removal, respectively) compared to MF (2.6 log and 2.8 log removal, respectively). The TCC, however, was not that strongly affected, pointing to a selective removal of the active cell fraction, represented by the HNAC (Lebaron et al., 2001), by the UF (Figure 5-6, see also Figure 10-1 and Figure 10-4). The UF had a significantly higher (T-test; dF = 6.8; p = 0.036) *sul1* gene removal by 2.9 log units compared to MF (2.1 log, respectively). The *ermB* gene was already approaching the lower limit of detection for both filtration units and were efficiently removed (Figure 5-6). In contrast, low *vanA* gene removal efficiencies were examined by both MF (1.1 log unit *vanA* gene) and UF (1.2 log units *vanA* gene) (Figure 10-1).

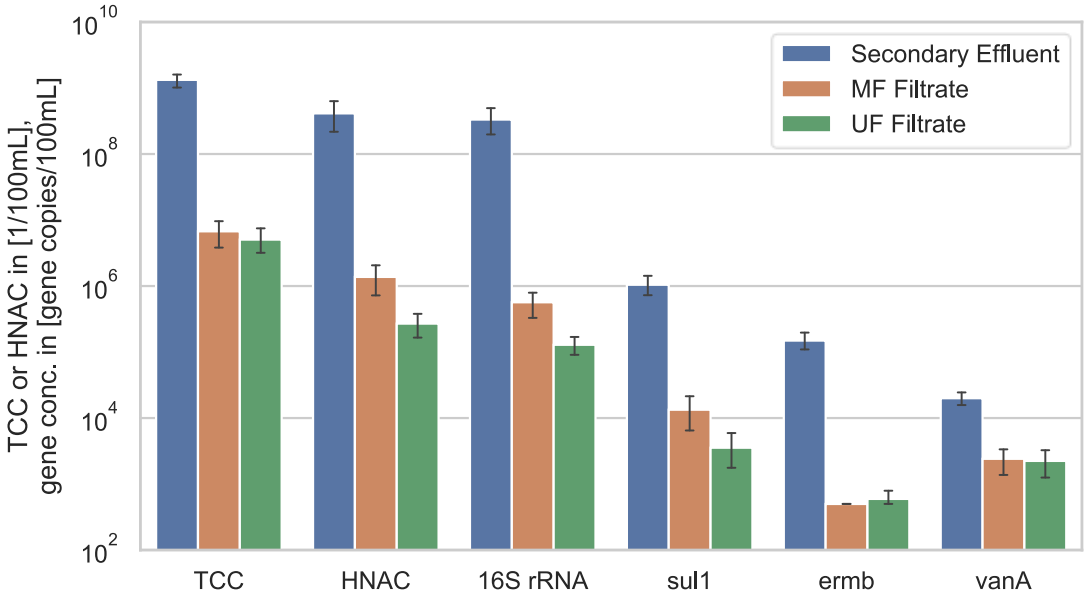

Figure 5-6: Arithmetic mean values of TCC, HNAC, 16S rRNA, ermB, sul1 and vanA genes from feed, MF and UF filtrate are presented. Error bars indicate the 95 % confidence interval. Number of samples and values below LOD are listed according to the samples secondary effluent, MF filtrate and UF filtrate. n_{TCC} = (9|7|10), LOD_{TCC} = (no values below LOD); n_{HNAC} = (9|7|10), LOD_{HNAC} = (no values below LOD); $n_{16SrRNA}$ = (16|7|16), $LOD_{16SrRNA}$ = (no values below LOD); n_{sul1} = (16|7|16), LOD_{sul1} = (0|0|8); n_{ermb} = (16|8|16), LOD_{ermB} = (0|8|15); n_{vanA} = (12|8|9), LOD_{vanA} = (0|2|3).

Previous membrane filtration studies comparing MF and UF reported similar ARG removal efficiencies. Similar to our results, a full-scale study of Munir et al. (2011a), a bench-scale study of Kappell et al. (2018) and a pilot-scale study of Hembach et al. (2019) also reported detectable *ermB* and *sul1* genes in UF filtrate. Munir et al. (2011a) examined the ARG removal of a full-scale MBR process (pore size of 40 nm) and observed *sul1* gene removal of about 3 log units. The lab-scale UF study (pore size of 17 nm) of Ren et al. (2018) also resulted in a 3 log *sul1* gene removal efficiency.

However, ARG removal by membrane filtration differs greatly between different types of ARGs. While UF samples had about 76 % lower *sul1* gene mean value than MF samples, almost the same *ermB* gene abundances were detected in MF and UF filtrate likely due to the fact that the *ermB* gene abundances were close to the detection limit. The low *vanA* gene removal efficiencies of MF as well as UF could be the result of higher mobile ARGs abundances in the feed water. Mobile or free DNA can easily penetrate through MF as well as UF pores. The breakthrough of extracellular ARGs through UF membrane pores was also reported elsewhere (Slipko et al., 2019; Krzeminski et al., 2020). ARGs removal efficiencies by MF and UF processes were already reported by Breazeal et al. (2013) where plasmid-associated ARGs in an artificial feed could be better removed with decreasing membrane pore size using laboratory-scale MF, UF and NF

skids. While MF (pore size of 0.45 and 0.1 µm) resulted in less than 1 log unit removal of *blaTEM* and *vanA* genes, UF (pore size of 100 kDa) could decrease *blaTEM* and *vanA* genes by 1.1-2.4 log units, NF (pore size of 10 kDa) reduced *blaTEM* and *vanA* genes by 4.2-5.8 log units. This UF study examined *vanA* gene removal (1.2 log units) using membrane with 20 nm pore size (about 1.200 kDa). The results are in line with the study of Breazeal et al. (2013).

This was the first study in which pilot-scale MF and UF plants were operated in parallel to investigate removal of ARGs under realistic operational conditions. UF capillary membranes with smaller pores could also increase the removal of ARGs, in our case *sul1* gene, potentially through the higher removal of active cells.

5.4.5 Distinguishing live and dead bacteria and intracellular ARG in UF filtrates

MF and UF membrane modules are specified by the manufacturer with nominal pore sizes of 450 and 20 nm, respectively, representing a pore size that should predominantly exclude passage of particles like bacteria. However, as described in the previous experiments above (e.g. Figure 5-6; see also Figure 10-2) we always measured a constant number of cells with flow cytometry (6 log units/ 100 mL in MF filtrate and 5 log units/ 100 mL in UF filtrate) and *16S rRNA* genes. Previous research mainly focused on the breakthrough of ARGs, however, bacteria breakthrough was not investigated in parallel. As microbial cells are the main ARG carriers, we distinguished dead and live bacteria in UF filtrate (experiment VIII). This was tested with the secondary and tertiary effluent to evaluate the effect of different feed water qualities. Arithmetic mean of HNAC in UF filtrate was 5.8 log units per 100 mL using tertiary effluent as feed and 6 log units per 100 mL using secondary effluent as feed (Figure 5-7). These detected HNAC values agreed well with the HNAC values analyzed by flow cytometry from Sigrist GmbH (see sections 5.4.2, 5.4.3, 5.4.4). Remarkably was the fact that 49-59 % of detected HNAC values in UF filtrate samples were live bacteria (Figure 5-7), confirming the results from above (Figure 5-7) that mostly active cells are removed by the filtration modules. The experiment analyzing dead and live bacteria in UF filtrate was executed using a virgin membrane module in the pilot plant and sterile syringe filters with the same pore size. The dead and live bacteria analyses in the filtrate of the sterile syringe filters resulted in arithmetic mean values of HNAC of 6 log units per 100 mL. The percentage of live bacteria was between 58 and 62 %. The study results of the virgin membrane modules and sterile syringe filters demonstrated similar HNAC values. Hence, bacterial contamination from the pilot plant using virgin membrane module can be excluded. Figures of live and dead bacteria analyses are illustrated in Figure 10-3.

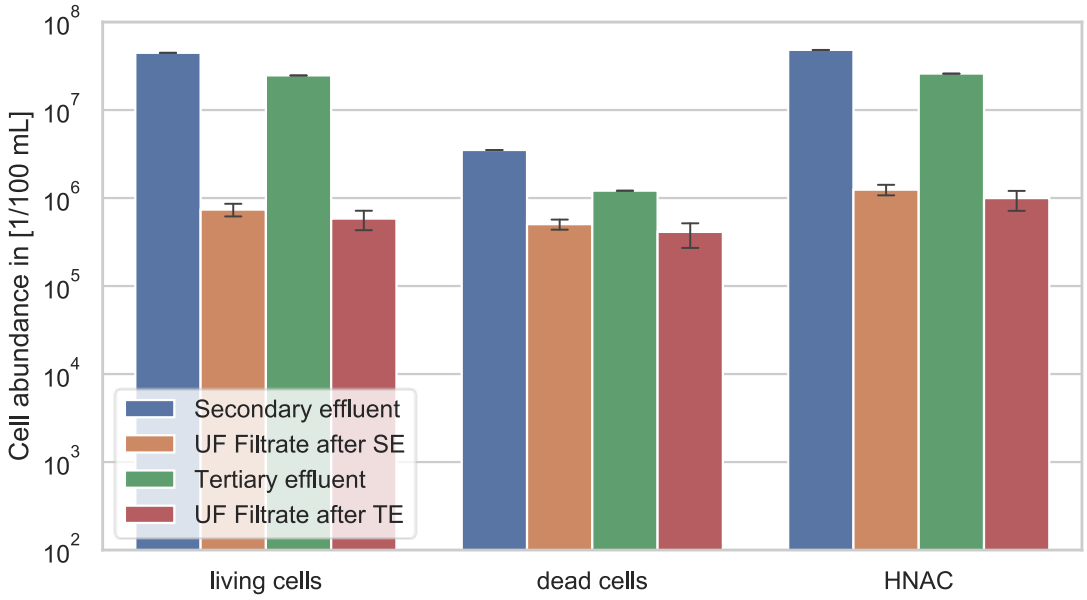

Figure 5-7: Arithmetic mean values of live and dead bacteria in secondary and tertiary effluent and corresponding UF filtrates. HNAC is the sum of live and dead cells. Error bars indicate standard deviation. $n_{\text{Secondary effluent}} = 2$, $n_{\text{UF Filtrate after SE}} = 5$, $n_{\text{Tertiary effluent}} = 2$, $n_{\text{UF Filtrate after TE}} = 5$.

The MF studies of Hahn (2004) and Liu et al. (2019) both confirm bacteria breakthrough using membranes with pore sizes between 0.2 µm and 0.1 µm. Hahn (2004) investigated in sterile 0.2 µm filters to quantify bacteria removal (size of isolated strains ranged from <1 to >10 µm in cell length) and concluded that two out of 19 bacterial strains were able to pass pores of 0.2 µm filter. The study of Liu et al. (2019) is in line with the MF study of Hahn (2004). Liu et al. (2019) examined the breakthrough of Hylemonella bacteria using 0.1 µm sterile filter. This filter also used in the study of Liu et al. (2019) had a nominal pore size of 100 nm. This range of pores were measured as largest pores (90 nm) in the UF study of ElHadidy et al. (2013b) using an UF membrane with a nominal pore size of 40 nm. According to this wide range of pore size distribution, it can be concluded that bacteria can pass larger pores of UF membranes. The UF study of Ren et al. (2018) confirmed that bacteria can breach UF membranes. Ren et al. (2018) reported an incomplete bacteria removal of 98.9 %, using an UF module with a pore size of 100 kDa.

Different mechanisms resulting in membrane breakthrough of bacteria, ARB and extracellular ARGs are summarized in the following section: There are different possibilities how pore sizes of membrane can be determined, but in all cases, it needs to be considered that a nominal pore size always represents a pore size distribution. For example, the pore size or the molecular weight cutoff can be described as a molecule of

a certain size that can be removed by 90 %. ElHadidy et al. (2013b) studied the pore size distribution of ultrafiltration membranes with nominal pore size of 40 nm by atomic force microscopy and reported pores up to 90 nm. Therefore, a small number of larger molecules or particles could theoretically migrate through the membrane. Additional factors affecting ARB and ARGs breakthrough are membrane materials (Liu et al., 2019) or the increase of flux and TMP. Liu et al. (2019) reported rejection of bacteria by membranes of similar pore size (0.1 µm), but four different materials. While Hylemonella bacteria could pass the pores of polyvinylidene fluoride and polyethersulfone filters, no transmission was detected using polycarbonate and mixed cellulose esters filters. The increase of flux and TMP resulted in plasmids (Arkhangelsky et al., 2011), viruses (Arkhangelsky et al., 2011), and bacteria (Suchecka et al., 2003) breakthrough. The breakthrough effects caused by higher flux and TMP were the result of membrane pore enlargement (Arkhangelsky and Gritis, 2008) and of bacterial cell deformation (Suchecka et al., 2003). The bacterial cell deformation seems to be strongly depended on the cell-wall structure of the bacteria. Lebleu et al. (2009) while investigating MF membranes concluded that bacteria removal depends on the kind of bacteria. While gram-positive bacteria have a thicker peptidoglycan layer and thus are less formable and better retainable by MF, gram-negative bacteria with their thin peptidoglycan layer enable their better deformation and transmission through MF pores. In addition, Slipko et al. (2019) investigated extracellular DNA breakthrough during membrane filtration. They concluded that both size exclusion and surface charge of the membrane were important for extracellular DNA retention. Hence, negatively charged membranes exhibited lower free DNA retention than neutral charged membranes. In addition, extracellular DNA like plasmids are ARG carriers and can pass membranes by elongation in converging and accelerating flow fields, which usually occurs above the immediate openings of the membrane pores (Arkhangelsky et al., 2011; Latulippe and Zydney, 2011, Schwaller et al., 2022a). The study of Arkhangelsky et al. (2011) focused on DNA transport of particles of 350 nm of diameter penetrating through UF membranes with pores as narrow as 10 nm. Arkhangelsky et al. (2011) reported of hydrodynamic strains that lead to 350 nm diameter particle breakthrough, due to elongation of those particles into long hair-shaped strands. The study of Latulippe and Zydney (2011) is in accordance with the study results of Arkhangelsky et al. (2011). In this study larger plasmids DNA from 3 to 17 kbp in size were able to filtrate through UF pores that were over an order of magnitude smaller than the plasmid DNA. High filtrate flux can cause elongation of plasmid DNA in the so-called converging flow field so that plasmid DNA breakthrough occurred (Schwaller et al., 2022a).

To summarize, both dead and live bacteria concentrations were detected in high concentrations up to 6 log units/ 100 mL in UF filtrate. Bacteria, ARB and ARGs breaking

through the UF membrane is likely the result of the pore size distribution of the membrane module, membrane materials, membrane pore enlargement and bacterial cell deformation due to high TMP. Bacterial cell deformation and transmission tendency depends on cell-wall structure of the bacteria. Transfer of extracellular ARGs bonded on free DNA like plasmids depends on size exclusion and surface charging of the membrane as well as the elongation effects in the converging flow fields above the opening of the membrane pores. Therefore, detected ARG genes abundances in UF filtrate could be the result of both, breakthrough of cells and of extracellular DNA.

To illustrate the relation of bacterial genomes measured as *16S rRNA* gene (assuming a constant copy number of 16S) and ARGs, correlations of the values were evaluated in the Figure 5-8A and Figure 5-8B using feed and filtrate samples. While good correlations between *ermB, sul1* genes and *16S rRNA* gene existed in secondary and tertiary effluents, *vanA* gene showed no correlation with the *16S rRNA* gene in the secondary effluent (Figure 5-8A). The low correlation of *vanA* gene with *16S rRNA* genes suggests that *vanA* gene might be predominantly associated with either free extracellular or extrachromosomal DNA, like plasmids. This could explain the previously observed different ARG removal efficiencies by the fouling layer study (section 5.4.2) and the low *vanA* gene removal efficiencies of MF and UF membranes (section 5.4.4). The study results of detected *sul1* genes in UF filtrate of section 5.4.2, 5.4.3 and 5.4.4 were confirmed by the evaluation of findings shown in Figure 5-8. The relation of intra- and extracellular ARGs in wastewater of a full-scale WWTP was analyzed by Liu et al. (2018), who showed that the lowest correlation of the *16S rRNA* gene abundance with 22 analyzed ARGs was the *vanA* gene, while the highest *16S rRNA* gene correlations were achieved with *sul1*, *sul2, tetM* and *ermB* genes. This is also in line with a study by Che et al. (2019) who investigated mobile and chromosomal antibiotic resistomes in WWTP influent, activated sludge, and WWTP effluent by metagenomic sequencing. The authors found that between 41 to 66 % of the ARGs detected in all wastewater compartments were associated with extrachromosomal mobile plasmids, integrative and conjugative elements (ICEs), whereas only 21 to 36 % of detected ARGs belonged to intrachromosomal group (Che et al., 2019).

To summarize, the study results of experiment IX showed that (living and dead) bacteria were capable of breaking through the pores of the UF membrane. Correlations of *sul1* gene and *16S rRNA* gene revealed that intracellular *sul1* gene likely penetrated through the pores of the UF membrane with the bacterial cell.

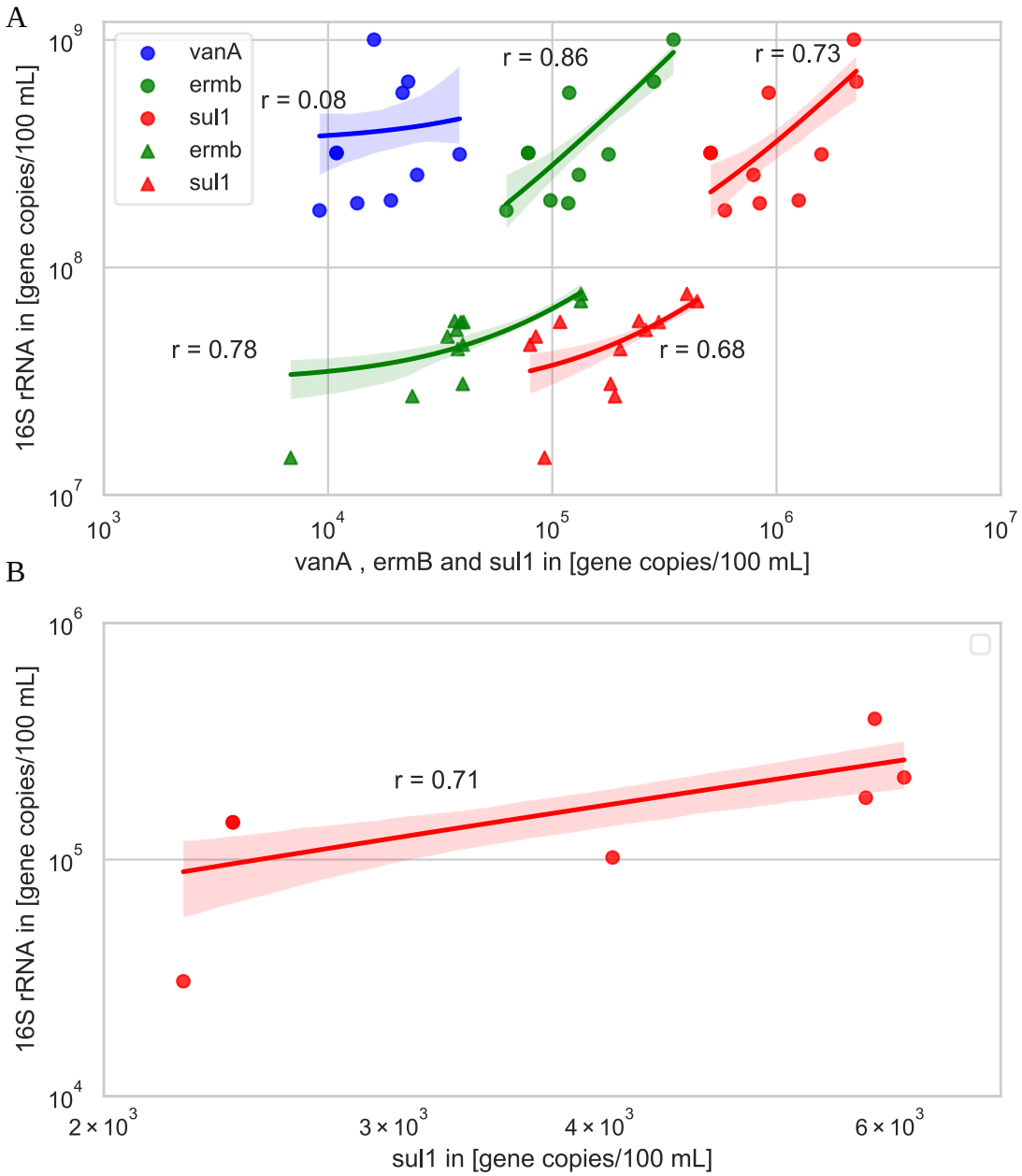

Figure 5-8: Pearson correlation of 16S rRNA gene with sul1, ermB and vanA genes analyzed in secondary effluent (circular markers) and tertiary effluent (triangular markers) (A). Pearson correlation of 16S rRNA and sul1 genes measured in UF filtrate samples using secondary effluent as feed (B). For both figures, the shaded area indicates the 95 % confidence interval.

5.5 Conclusions

Pilot scale membrane filtration studies using real wastewater of WWTPs should consider the following key operation parameter for AMR analyses: Dry weather flow of the WWTP should be applied for AMR analyses. Sampling is carried out at standard filtration mode (constant steady flux and constant filtrate quality). Fouling layer examinations for AMR analyses is executed at constant TMP increase. Membrane integrity is demonstrated before and after AMR examinations. Treatment variability of two trains of the pilot plant is checked before AMR studies.

The AMR examinations, using MF and UF, revealed a significant reduction of *ermB* genes (by 2.7 and 2.8 log units) and *sul1* genes (by 2.1 log units and 2.9 log units) using secondary effluent as feed. In contrast, with regard to *vanA* gene MF and UF achieved only a moderate reduction by 1.1 log units and 1.2 log units. These significant different degrees of removal of *ermB*, *sul1* and *vanA* genes by MF and UF were the result of different factors that were the focus of this study. Overall, the main factor for ARGs removal was the pore size of the applied membranes. While no significant additional AMR removal on intra- and extrachromosomal ARGs (e.g., *sul1* and *ermB* genes) was detected by the fouling layer, predominantly mobile ARGs (e.g., *vanA* gene) could be significantly decreased. The ARG abundance in the feed water is another factor influencing AMR removal. The higher the ARGs abundance in the feed water the higher was the ARGs abundance in the filtrate water. Beside of ARGs abundance in the feed water the AMR removal efficiency of the membrane filtration also depends on the relation of intra- and extracellular ARGs abundance. It was found that predominantly intra- and extrachromosomal ARGs (e.g., *sul1* and *ermB* gene) can result in higher ARGs removal efficiencies of the membrane filtration process while predominantly extracellular or extrachromosomal ARGs (e.g., *vanA* gene) can result in lower ARGs removal efficiencies. Lastly, dead and live bacteria as well as ARB can break through the membrane, which raises the question to what extent ARB-associated regrowth can occur on the filtrate side. This effect would reduce ARGs removal efficiency of MF and UF. Therefore, further investigations concerning ARB-associated regrowth at filtrate side are required.

5.6 Acknowledgement

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6 IMPACT OF BACKWASH AND CEB MODES ON RETENTION OF BIOMASS AND ANTIBIOTIC RESISTANCE GENES DURING ULTRAFILTRATION OF WWTP EFFLUENTS

The following chapter addresses ***research hypothesis #3.1***: CEB will result in higher total biomass and ARGs abundance in UF filtrate within the first minute of standard filtration mode than backwash. Furthermore, the ***research hypothesis #3.2***: Lower cake layer after backwash and CEB mode will result in higher AMR abundances in UF filtrate is presented. In addition, ***research hypothesis #3.3*** is addressed: Backwash mode and CEB mode will result in higher AMR abundance in UF filtrate due to contaminated filtrate from the fitrate tank. Lastly, **research hypothesis #3.4** is tested: CEB event using sodium hypochlorite instead of sodium hydroxide will result in significantly lower biomass and ARG abundances in UF filtrate.

This chapter has been published with some editorial changes as follows:

Hiller, Christian X.; Drewes, Jörg E. (in review): Impact of backwash and CEB modes on retention of biomass and antibiotic resistance genes during ultrafiltration of WWTP effluents. Membranes.

Author contributions: Christian Hiller developed the research objective, executed the experiments and wrote the manuscript as first author. Jörg E. Drewes supported the funding acquisition, supervised the study, and reviewed the manuscript.

6.1 Abstract

The impacts of various operational modes of ultrafiltration on the reduction of antimicrobial resistance (AMR) from conventional WWTP effluent were investigated using a pilot-scale ultrafiltration (UF) facility. In particular the focus of this investigation was to elucidate the role of backwash and chemical enhanced backwash (CEB) on AMR retention while employing capillary UF membrane modules. While AMR removal during standard filtration mode has been investigated in depth, this study is the first to report the retention dynamics of AMR directly after backwash and chemical enhanced backwash (CEB) within the first minutes of standard filtration mode characterized by highly transient conditions. High nucleic acid count (HNAC), total cell count (TCC), and *16S rRNA* gene abundance were analyzed as a proxy for total biomass in addition to relevant antibiotic resistance genes such as *sul1*, *ermB* and *vanA* genes. Findings revealed that the abundance of HNAC, TCC and *16S rRNA* gene measured within the first minute directly after backwash and CEB were up to one order of magnitude higher compared to the abundance after 5 and 55 minutes of standard filtration mode. While *ermB* and *vanA* genes exhibited no increase after backwash and CEB, *sul1* gene increased significantly within the first minute. However, the *sul1* gene increase in the filtrate after backwash and CEB mode was caused by microbial regrowth in the backwash tank contaminating the backwash water and as a consequence severly affecting the filtrate quality of the first minutes of standard filtration mode. This has practical implications for filter-to-waste protocols and to properly maintaining backwash water quality to prevent regrowth and horizontal gene transfer where a high microbial filtrate water quality is desired for downstream uses.

Keywords: Antibiotic resistance genes; wastewater treatment; ultrafiltration; backwash mode; chemical enhanced backwash mode

6.2 Introduction

The anthropogenically-impacted water cycle is characterized by water abstraction for drinking and process water from natural resources while these sources are also impacted by upstream agricultural, industrial and domestic activities. Not-metabolized antibiotics as well as antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) find their way from different sources (e.g., slaughterhouses, residential areas, hospitals) via waste discharges into the impacted water cycle. It has been reported that antibiotic concentrations occurring in wastewater can constitute a continuous selection pressure on bacteria to develop antibiotic resistance (Salcedo et al., 2015; Gullberg et al., 2011). Antibiotic resistance can be also transferred from ARB to autochthonous bacteria and other facultative pathogenic bacteria via horizontal gene transfer (Davison, 1999). Thus, the anthropogenically-impacted water cycle is a highly relevant conveyance pathway of antibiotic resistance with potential adverse effects on various endpoints (Chow et al., 2015).

Multiple studies investigating AMR removal by conventional biological wastewater treatment facilities have confirmed a rather inefficient AMR retention (Rizzo et al., 2013; Alexander et al., 2015, Hembach et al., 2017). However, advanced treatment processes, in particular membrane filtration processes, can achieve very high ARGs removal efficiencies and therefore could assist in mitigating the spread of antimicrobial resistance into the aquatic environment (Hembach et al., 2019; Hiller et al., 2019). Previous membrane filtration studies investigated ARGs removal efficiencies were commonly focusing on membranes employing different pore sizes and average filtrate quality while operating at standard filtration mode (Breazeal et al., 2013; Böckelmann et al., 2009; Morales-Morales et al., 2003; Munir et al., 2011). In a previous study of the authors, we did confirm high AMR removal efficiencies of UF membranes as a function of pore size, but also elucidated the role of the fouling layer, AMR abundance in the feed water, and considered intra- and extrachromosomal distribution of ARGs in the feed water on AMR removal (Hiller et al., 2022). However, the effect of backwash and chemical enhanced backwash modes concerning AMR retention under relevant operational conditions at scale has not been investigated so far.

Hydraulic backwash and CEB modes are essential steps to maintain a sustainable operation during dead-end ultrafiltration to recover not only transmembrane pressure (TMP) and a desired specific flux, but also to remove the fouling layer. Regular applied backwash provides hydraulic cleaning that is utilizing alterations in hydrodynamics to generate shear forces and turbulences for loosening foulants at the membrane surface as well as reopen clogged pores of the membrane module. The extent of fouling that is not recovered during regular backwash, can be further reduced by using additional chemicals

during chemical enhanced backwash (CEB). Chemical cleaning can result in hydrolysis, a modified solution pH, as well as oxidation and disinfection effects that depends on the individual CEB chemical. The effect of chemical and hydraulic conditions during CEB and backwash mode can result in biofilm detachment (Gomes et al., 2021; de Vries et al., 2021). Beside of biofilm removal, backwash and CEB can reduce colloids in the range of 3 nm to 20 nm size at the feed side of the membrane module representing the major foulants and responsible for build-up of rapid flow resistance (Filloux et al., 2012). Furthermore, Akhondi et al. (2017) studied the impact of backwash mode and reported that backwashing was more effective by recovering foulants from the larger pores than the smaller pores. The preferred recovery of larger pores could result in lower AMR and biomass removal efficiency within the first minutes of standard filtration mode. Low filtrate quality directly after backwash and CEB mode within the first minutes of standard filtration mode was already measured analyzing TCC and HNAC (Hiller et al., 2022). The reduced retention efficiency immediately following backwash is the reason why water treatment plants applying filter-to-waste protocols for the first filtrate water after backwash (Soucie and Sheen, 2007; Cescon and Jiang, 2020). Therefore, this study is also addressing whether filter-to-waste should also be applied for ultrafiltration processes in order to prevent the spread of AMR. However, filter-to-waste protocols come at the expense of higher OPEX and CAPEX, thus, alternative measures should be discussed to optimize investment costs and operation costs while maintaining a high filtrate quality.

This study is the first to report the impact of hydraulic and chemical cleaning on the filtrate quality of standard mode of filtration within the first minutes compared to 5 and 55 minutes. It is hypothesized that *CEB results in higher total biomass and ARGs abundance in UF filtrate within the first minute of standard filtration mode.* Furthermore, it is expected that *higher total biomass and ARGs abundance in the filtrate are the result of particles breaching the feed side of the membrane module due to a reduced fouling layer.* In addition, it is expected that hydraulic and chemical cleaning procedures can result in possible bacterial and AMR regrowth at the filtrate side leading to *an increase in total biomass and ARG abundance in UF filtrate with progressive filtration time*. Based on these observations, mitigation measures are suggested for engineering practice.

6.3 Experimental Design

6.3.1 WWTP Steinhäule and membrane filtration pilot plant

In the wastewater treatment plant (WWTP) Steinhäule in Neu-Ulm, pilot-scale membrane filtration studies were conducted using different effluents of the WWTP. The

WWTP Steinhäule (Germany) was designed for an overall treatment capacity of 445.000 population equivalents. With a design capacity of 2,600 L/s at wet weather flow conditions, the maximum flow of the WWTP Steinhäule was designed double the dry weather flow. The WWTP Steinhäule treats wastewater by four treatment stages – mechanical, biological, chemical, and physical processes. The physical stage downstream the biological stage consists of a contact reactor. 10 mg/L of powdered activated carbon (PAC) is continuously fed to the wastewater of the contact reactor in order to remove trace organic chemicals. A clarifier separates the PAC after the contact reactor. A tertiary filtration step is the last treatment process. When powdered activated carbon settles in the clarifier, the PAC is returned back to the contact reactor for better carbon utilization. The flow diagram of the WWTP Steinhäule is illustrated in Figure 6-1A. Typical wastewater parameter of the feeds are summarized in Table SI1 (Supplemental Information). Secondary effluent (SE) as well as tertiary effluent (TE) were applied as feed water for the membrane filtration pilot plant. The pilot plant was built up with two parallel trains. Every train consisted of two pre-filters with 400 µm cut-off, feed tank used as reservoir, membrane module, and by-pass filtrate/ backwash tank (Figure 6-1B).

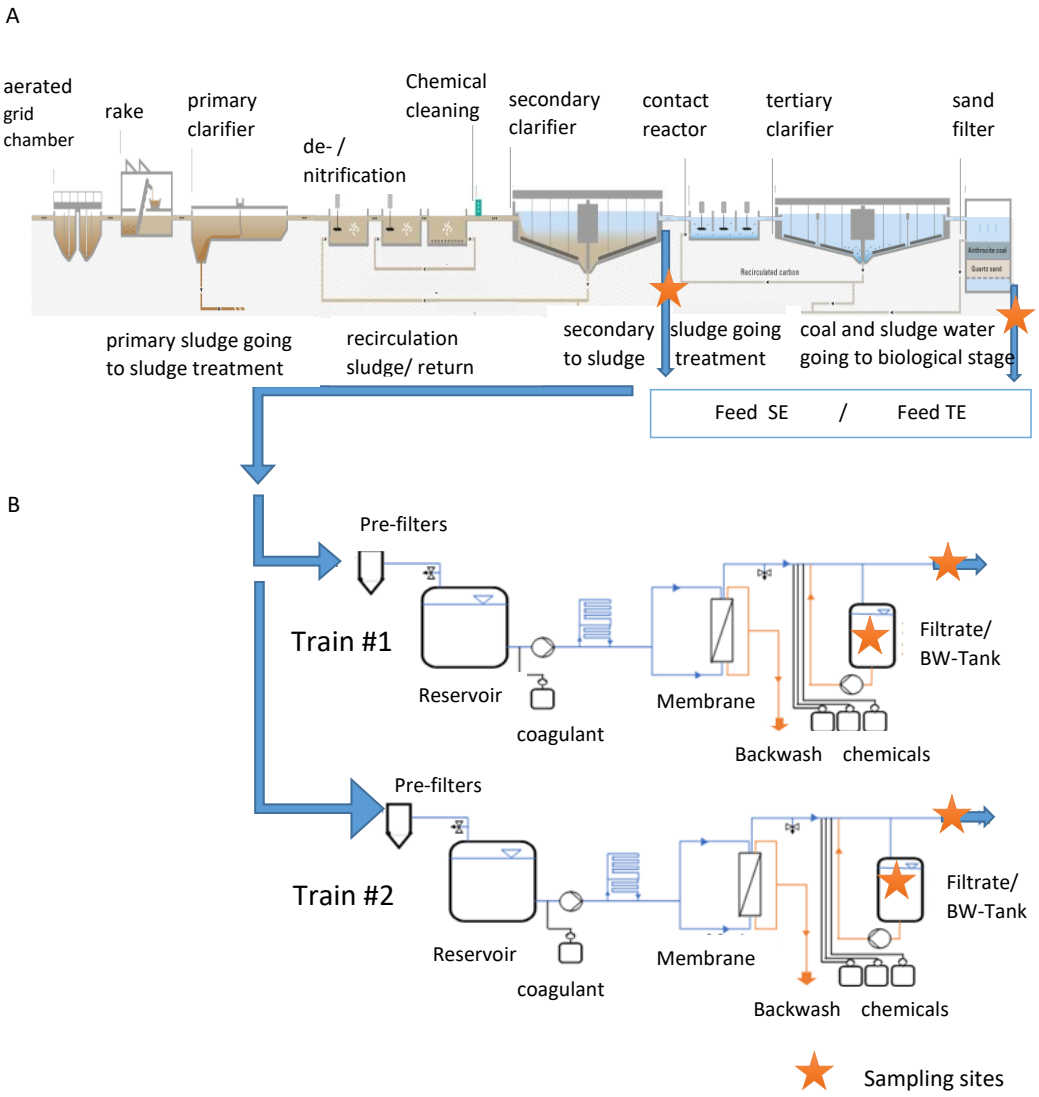


Figure 6-1: Conceptional flow diagram of the overall wastewater treatment at WWTP Steinhäule (A) and the membrane filtration pilot plant (B). Secondary effluent (SE) and tertiary effluent (TE), consisting of advanced treatment of secondary effluent using activated carbon and sand filtration, were used as feeds for membrane filtration pilot plant. Samples were taken from SE and TE, the corresponding filtrates of train #1 and #2 and from the filtrate tanks of train #1 and train #2.

Commercial UF membrane modules with pore sizes of 20 nm (80 m² surface area) were applied for this study. Membrane modules were made of hydrophilized polyethersulfone, characterized by a contact angle of 52°. The UF membrane modules had 7 capillaries per fiber and were operated in an inside-out, dead-end filtration mode in parallel.

The continuous UF filtration operation in dead-end mode resulted in a fouling layer increase at the feed side of the membrane and therefore also in an increase of the transmembrane pressure (TMP) and higher power requirement of the pumps in order to

maintain a constant flux level. To mitigate the extent of fouling, backwash and CEB mode are regularly applied during membrane operation. The filtration cycle of the UF process can be described as follows:

The standard filtration mode of the membrane filtration was 60 minutes. During standard filtration mode, the pilot plant operated at a constant sustainable flux (under reversible fouling conditions) at 40 or 70 LMH (Figure 6-1B) whereas feed water is pumped from the feed side to the filtrate side of the membrane module. In order to have a fouling control measure, a coagulant dose (polyaluminum chloride solution, PLUSPAC FD ACH, Feralco Deutschland GmbH, Germany) of 2 mg/L was continuously given to the feed line of the pilot plant. Coagulation can increase the filtration efficiency by reducing the occurrence of reversible fouling [20]. The standard filtration mode ended after 60 minutes whereas feed pump and coagulation pump were switched off. After that, backwash mode was the next step, whereas hydraulic backwashing was conducted by pumping filtrate water from the bypass backwash-tank to the module. After 45 seconds of backwash mode at a flux of 230 LMH, the standard filtration mode started again. This protocol of backwash mode and standard filtration mode is repeated 23 times. After that, the CEB mode is activated instead of a backwash mode. During the CEB mode, 150 ppm sodium hydroxide is applied to the UF module for 90 seconds at a flux of 120 LMH. After a soaking time of 15 min of the UF module, a final (hydraulic) backwash rinsed the sodium hydroxide out of the UF module at a flux of 230 LMH for 70 s using filtrate water from the backwash tank. In order to refill the filtrate tank, a short filtration at 70 LMH for 900 s was executed after the sodium hydroxide CEB. Finally, the 150 ppm of sulfuric acid was applied repeating the same protocol like the sodium hydroxide CEB. After finishing the CEB protocol, the standard filtration mode was started again.

6.3.2 Experiments and sampling conditions

AMR analyses was conducted according to Hiller et al. (2022) and were performed only under dry weather flow conditions. Membrane integrity was checked before and after AMR examinations using flow cytometry measurements and analyzing UF filtrate before and after AMR studies. Treatment consistency of the two pilot-scale trains was checked before AMR studies. In order to assess the influence of hydraulic backwash and CEB on AMR removal efficiency within the first minutes of standard filtration mode the following hypotheses are proposed:

> #1: CEB results in higher total biomass and ARGs abundance in UF filtrate within the first minute of standard filtration mode.

In order to test this hypothesis, samples were taken immediately after the backwash and the chemical enhanced backwash mode within the first minute, after 5 minutes, and

after 55 minutes during standard filtration mode using feed secondary effluent over a period of several weeks. Furthermore, sampling every minute within the first 5 minutes of standard filtration mode was extended and total biomass as well as ARGs abundance were analyzed.

> #2: Higher total biomass and ARGs abundance in the filtrate are the result of particles breaching the feed side of the membrane module due to a reduced fouling layer.

In order to test this hypothesis, virgin membranes with the same pore size of 20 nm without a fouling layer were employed and analyzed for total biomass and ARGs analyzes in UF filtrate. To elucidate whether the fouling layer can contribute to an additional total biomass and ARGs removal, the total biomass and ARGs analyses of the first hypothesis are compared with analyses of the second hypothesis.

> #3: Total biomass and ARG abundance are increasing in UF filtrate tank with progressive filtration time.

To test the hypothesis, UF filtrate samples were taken from the backwash tank within 14 days of continuous UF operation. The samples were analyzed for biomass and ARGs concentrations.

If filtrate water quality is decreasing in the filtrate tank, this filtrate quality should be characterized by higher total biomass and ARGs abundance in filtrate samples. Therefore, filtrate samples were taken directly after backwash and CEB events within the first minute of standard filtration mode after 1 and 3 weeks of continuous UF operation. A clean-in-place (CIP) of the membrane modules and the filtrate tank was executed with sodium hypochlorite for 24 hours before and after the trials 1, 2 and 3 as well as during the regrowth study focusing on the filtrate tank. This also included the use of different CEB chemicals and their impact on total biomass and ARGs abundance in UF filtrate. Sodium hydroxide and sodium hypochlorite were used as CEB chemicals in these examinations. Sodium hydroxide's disinfection impact is driven by hydrolysis of amides (protein denaturation) caused by OH^- ions. Other CEB chemical like sodium hypochlorite could be more effective during chemical enhanced backwash. Therefore, it was hypothesized that a CEB event using sodium hypochlorite instead of sodium hydroxide can result in significantly lower total biomass and ARG abundances in the UF filtrate. To analyze the impact of the two CEB chemicals, both trains were operated in parallel with the same feed tertiary effluent but different CEB chemicals (sodium hydroxide in train #1 and sodium hypochlorite in train #2). CEB was performed once a day. Sampling for total biomass and ARGs was conducted for filtrates of both trains within the first minute of standard filtration mode.

6.3.3 AMR analyses and microbial biomass analyses

Sufficient abundances of *ermB* and *sul1* genes in the feed waters were confirmed in pre-screening studies so that membrane filtration studies can demonstrate removal efficiencies of these ARGs of at least 2 log units. *VanA* gene was included in this AMR study due to the fact that it represents an important antibiotic of last resort. *16S rRNA* gene was used as surrogate parameter for total cell counts in samples ([21], [22], [8]).

In the laboratory, an aliquot of 20 mL of the thawed sample was freeze-dried to accumulate cells and DNA. The further protocol for qPCR analyses was applied following the protocol of Hiller et al. [14]. For *vanA* and *ermB* genes, the detection limits were 1,000 gene copies per 100 mL, for *sul1* gene 1,750 gene copies per 100 mL as well as for *16S rRNA* gene 10,000 gene copies per 100 mL. The aforementioned detection limits were all above the calculated limit of detection. The detection limits were used by the respective PCR efficiency and by establishing a minimum of four gene copies per PCR reaction. In the bar plots, all ARG values below the detection limits were considered by applying half the value of the detection limit.

Total cell count (TCC), low nucleic acid count (LNAC), and high nucleic acid count (HNAC) were analyzed using flow cytometry (Sigrist GmbH, Switzerland). The sum of LNAC and HNAC is TCC. HNAC value represents cells with high nucleic acid amounts, whereas LNAC value characterizes cells with low nucleic acid amounts. The relation of HNAC and LNAC provides information about the microbiological fingerprint of a water sample. Santos et al. [26] executed a flow cytometry study with river water samples resulting high HNAC abundances sampling downstream of the WWTP discharge according to high concentrations of organic and nutrient from the wastewater. River headwater samples revealed a higher LNAC abundance characterizing oligotrophic conditions. LNAC and HNAC analyses can even detect disruptions in the microbiological system [27]. TCC, HNAC and LNAC values were analyzed following the protocol of Hiller et al. [14]. 10,000 cells per 100 mL was the detection limit of the flow cytometry. Double stranded DNA (dsDNA) was bound by fluorescent dye SYBR® Green. Therefore, LNAC provides information about double stranded DNA of small bacterial cells and viruses with DNA genome (dsDNA) which can be analyzed by flow cytometry [28]. That is the reason why *16S rRNA* gene and HNAC value were both selected in this study to quantify bacteria in wastewater samples.

6.3.4 Statistical data analyses

Statistical data evaluation was executed using independent and paired samples two-sided t-test with a significant threshold of $\alpha = 0.05$. Normality and homogeneity of variances were the t-test requirements.

In chapter 6.4.1, the statistical data analyses of the examinations of different biomass and ARGs abundance of the first minute filtrate samples after backwash and CEB were performed using independent sample t-test. Based on a confidence interval of 95 %, the values of total biomass and ARGs abundance, taken within the first minute, fifth minute and fifty-fifth minute are illustrated in Figure 6-3. Every sampling event consisted of three samples. The total biomass and ARGs analyses of the first five minutes of standard filtration mode as illustrated in Figure 6-4 are based on a confidence interval of 67 %. To examine the significance of mean values of different data series of samples from the first, second, and third minute, the pair samples t-test was applied. Every sampling event consisted of three samples.

The study results presented in Figure 6-5 (chapter 6.4.2) are evaluated with respect to a confidence interval of 67 %. The total biomass and ARGs abundance of filtrate samples of the virgin membrane study and the third minute samples of the UF study with a fouling layer (Figure 6-4) were executed using independent sample t-test. Every sampling event included three samples.

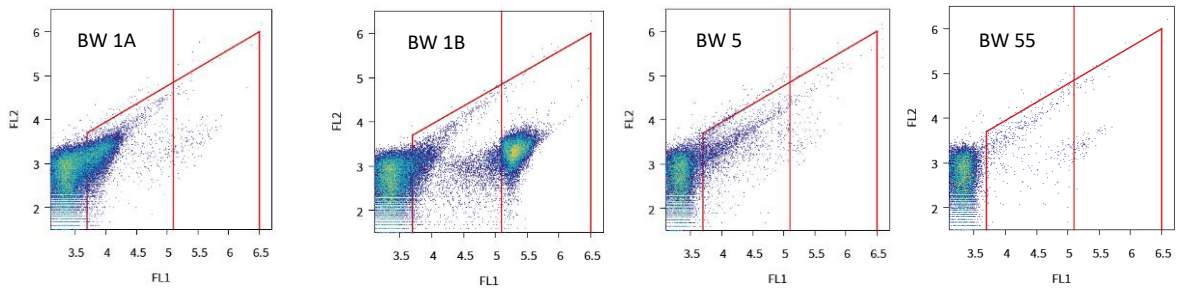
In chapter 6.4.3, the total biomass and ARGs analyses of samples after the first minute, taken after 1 week and after 3 weeks of continuous UF operation, are presented in Figure 6-6. The study results are shown based on a confidence interval of 95 %. Independent sample t-tests were applied for significance analyses. The statistical data analyses were executed considering at least two samples per sampling event and trial. The sampling campaign consisted of three trails.

The different CEB chemicals and their impact on total biomass and ARGs abundance were analyzed in chapter 6.4.4. The study results were illustrated in Figure 6-7 based on a confidence interval of 67 %. The statistical data analyses were performed using independent sample t-test. Three CEB events were conducted whereas three first minute samples were taken from train #1 and train #2.

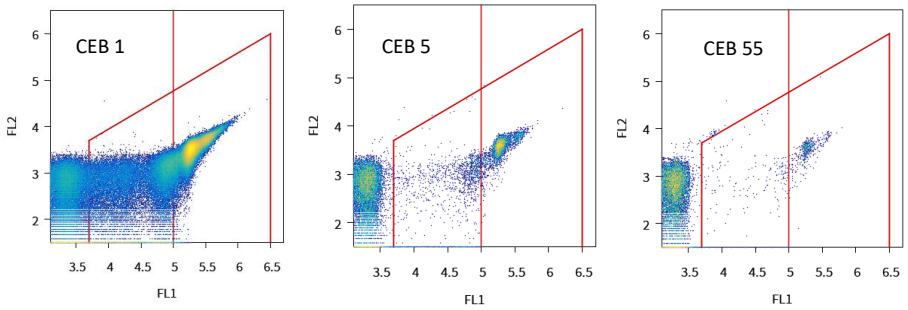
6.4 Results and discussion

6.4.1 Role of backwash and CEB on ARGs and total biomass increase in UF filtrate

To investigate the effect of backwash and CEB on UF filtrate quality during standard filtration, ARGs as well as total biomass (expressed by determining *16S rRNA* gene and HNAC and TCC via flow cytometry) were measured in filtrate samples. While HNAC represents bacterial cells in the wastewater sample, the TCC value is the sum of bacterial cells and viruses. TCC and HNAC values of UF filtrate samples collected after different filtration times are presented in Figure 6-2.



UF filtrate:	1 minute after BW	1 minute after BW	5 minutes after BW	55 minutes after BW
TCC [1/mL]:	126,037	261,144	32,688	6,966
HNAC [%]:	1.31	67.61	3.67	15.14



UF filtrate:	1 minute after CEB	5 minutes after CEB	55 minutes after CEB
TCC [1/mL]:	2,391,522	58,544	8,286
HNAC [%]:	77.81	72.78	70.19

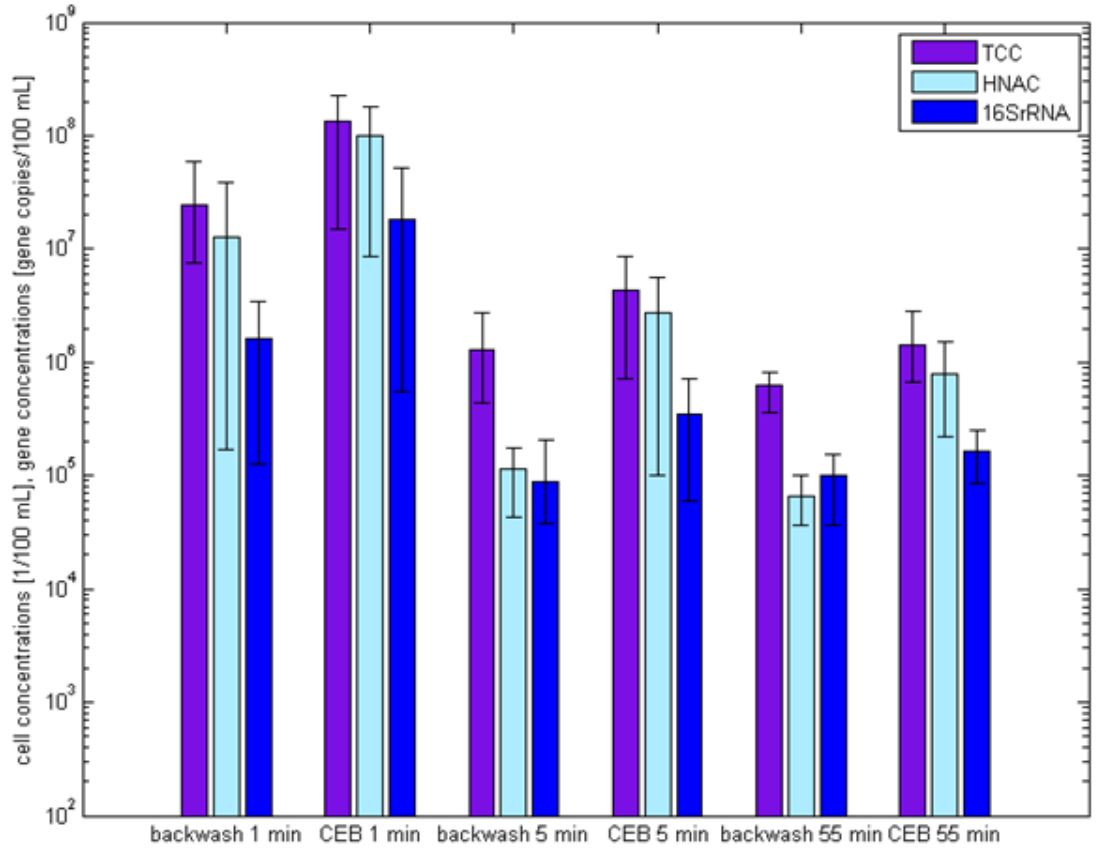
Figure 6-2: TCC and HNAC measurements of UF filtrate samples within the first minute (BW 1A, BW 1B and CEB 1), 5 minutes (BW 5, CEB 5), and 55 minutes (BW 55, CEB 55) of standard filtration mode after backwash and CEB events. BW 1A and BW 1B are two different first minute samples after backwash with significant different HNAC values..

Significantly higher TCC concentrations were determined after backwash within the first minute compared to the fifth and fifty-fifth minute during standard filtration. In general, HNAC abundances were low after backwash with less than 16 % of TCC abundances during standard filtration of 60 minutes (BW1B, BW5 and BW55). However, quite striking were the unexpected high HNAC abundance of more than 67% of TCC abundance in a sample also taken within the first minute of standard filtration after 8 days of continuous UF operation, although the operational parameters were not changed during this examination. The observed HNAC increase is further examined in chapter 6.4.3. Sampling immediately after CEB during 60 min of standard filtration resulted in very high TCC and HNAC abundances within the first and fifth minute (CEB1, CEB5). HNAC abundances after CEB were consistently above 70 % of TCC abundances from the first to the last minutes of the 60-minute standard filtration (Figure 6-2).

It was suggested that the increase of ARGs was attributed to the increase of TCC and HNAC after backwash and CEB. Therefore, further studies were performed analyzing

TCC, HNAC, *16S rRNA* gene as well as ARGs abundances in UF filtrate directly after CEB and backwash events. These results are shown in Figure 6-3.

A



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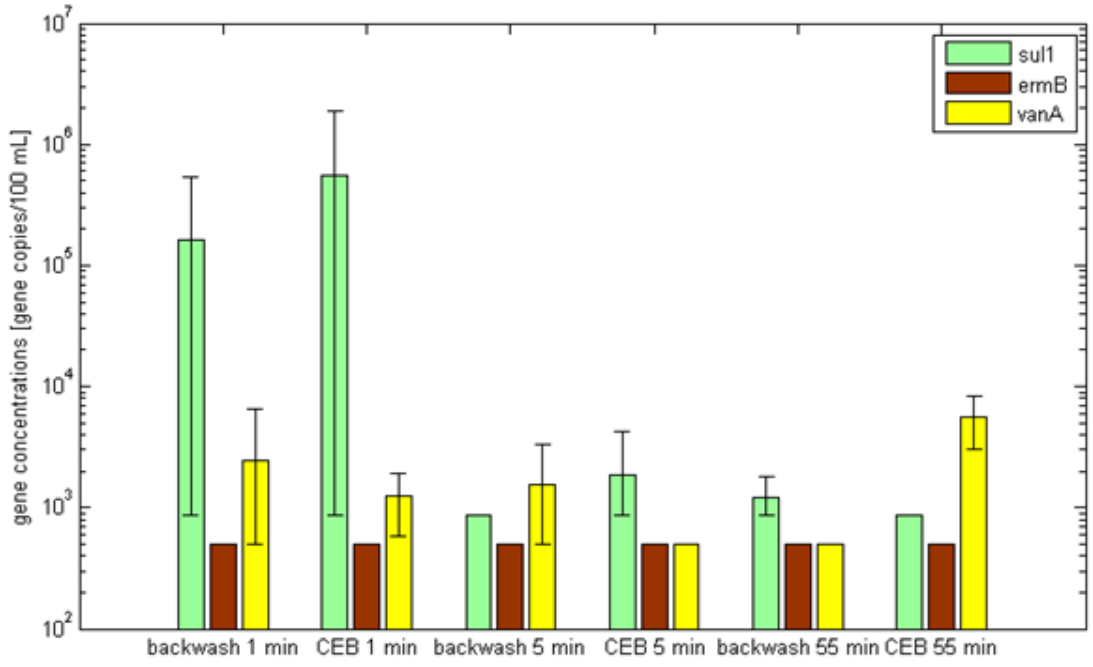


Figure 6-3: TCC, HNAC, and *16S rRNA* gene abundances (A) and *sul1*, *ermB*, and *vanA* gene abundances (B) in UF filtrate operated at a flux of 70 LMH **after backwash and after CEB** within 1, 5 and 55 minutes of continuous UF operation using secondary effluent as feed. Error bars indicate the 95% confidence interval.

A comparison of samples collected directly after backwash events revealed decreasing TCC, HNAC and *16S rRNA* gene concentrations of more than 1 log unit with progressive filtration time. While *ermB* and *vanA* genes abundances varied by two and three orders of magnitude per 100 mL in all samples taken during membrane filtration after backwash and CEB, *sul1* gene abundance was detected up to 3 log units higher in samples collected after the first minute immediately after backwash and CEB compared to 5 and 55 minute samples. A statistical analysis of arithmetic mean values of analyzed parameters of total biomass and ARGs revealed no significant difference of mean values in samples collected after the first minute after hydraulic backwash and CEB (Table 10-8). Quite striking was the fact that especially all analyzed values of the samples taken within the first minute of membrane filtration operation exhibited a higher standard deviation compared to the samples taken after 5 and 55 minutes of standard filtration mode. The reason for this observation is further examined in chapter 3.3.

The total ARGs removal efficiencies of the UF during 5 and 55 minutes of standard filtration were reported by Hiller et al. (2022). While *ermB* and *vanA* genes were removed by 2.5 and 1.1 log units, respectively, *sul1* gene was removed by 2.7 log units. Hence, the *sul1* gene increase, detected in the filtrate sample after the first minute, would suggest that the UF did not achieve any *sul1* gene removal within the first minute of standard filtration mode.

The study results pointing to significantly higher detected biomass parameter within the first minute compared to 5 and 55 minutes of standard filtration are in line with findings reported by Chaudhry et al. (2015). Chaudhry et al. (2015) examined the removal of adenovirus, norovirus GII, and F+ coliphage during full-scale membrane bioreactor operation. In this study, four virus removal mechanisms had been proposed: virus incorporation into suspended solids, which are retained by the membrane, virus retention by the clean membrane after backwash, virus removal by the cake layer, and virus inactivation effects caused by predation or enzymatic breakdown. Therefore, the observed higher biomass parameter in this study could be the result of the reduced cake layer that was removed due to mechanical forces during backwash mode. Hydraulic backwash changes the cake layer formation so that clogged pores are opened and deposits are abraded at the surface of the membrane. Chaudhry et al. (2015) reported more than 1 log unit higher adenovirus concentrations and about 0.2 log unit higher norovirus GII abundances in permeate samples directly after backwash. In contrast, F+ coliphage had

no different concentration measured in samples directly after backwash and after 1 day of standard filtration operation. The difference in the reported individual virus removal was due to the fact that F+ coliphage was quantified applying an infectivity assay while adenovirus and norovirus GII were analyzed using nucleic acid-based methods. PCR-based methods cannot distinguish between infectious and noninfectious viruses. Beside of virus removal, Chaudhry et al. (2015) observed more than 10 times higher turbidity values and more than 12 times higher particle counts immediately after a CEB event compared to the standard filtration mode. Furthermore, backwash events resulted in up to 2 times higher turbidity and 3 times higher particle counts compared to standard filtration mode. All in all, Chaudhry et al. (2015) observed significant higher adenovirus, norovirus, turbidity and particle counts after CEB and backwash compared to standard filtration. The reason for these significant higher adenovirus, norovirus, turbidity and particle counts after backwash and CEB was not examined in that study. A particle breakthrough or biofilm sloughing effects could be the reason for this effect. However, these observed effects after backwash and CEB require further investigations.

To summarize, the total biomass and ARG abundance in samples after the first minute after CEB revealed no significant higher values compared to the first minute samples after backwash. Thus, hypothesis #1 is rejected. However, both backwash and CEB can result in significant higher total biomass and sul1 gene abundance within the first minute compared to fifth and fifty-fifth minute of standard filtration mode. The reason for this total biomass and sul1 gene increase is being discussed in the following sections.

In order to quantify how long elevated levels of biomass and ARGs might last immediately after backwash, samples for biomass and ARGs analyses were collected during the first 5 minutes of standard filtration time. These findings are presented in Figure 6-4.

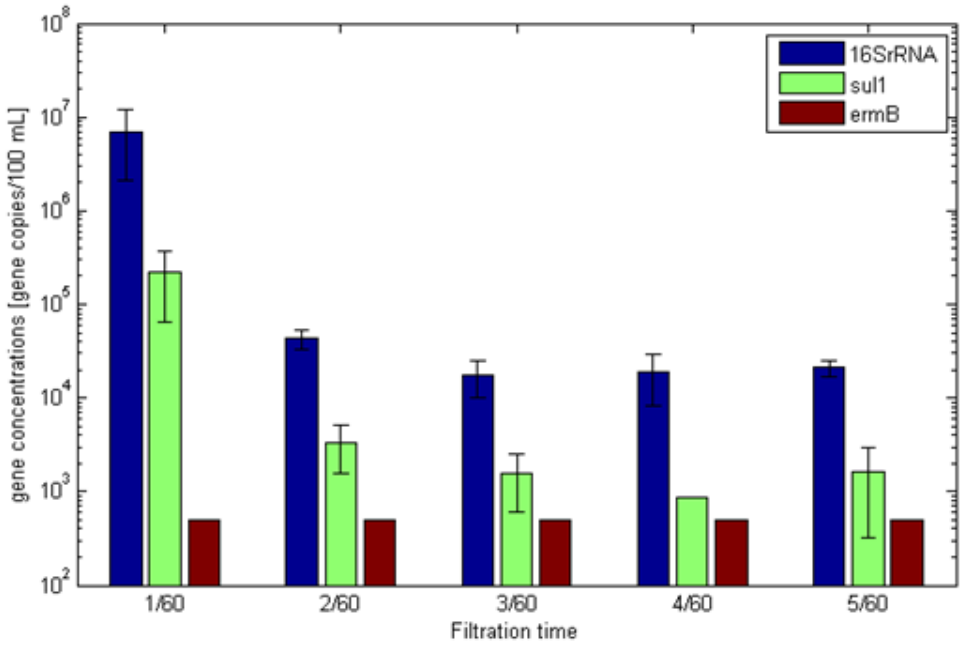


Figure 6-4: UF operation at a flux of 70 L/m² x h after backwash using secondary effluent as feed. *16S rRNA*, *ermB* and *sul1* genes abundances were analyzed in UF filtrate within the first minutes of standard filtration time. Error bars indicate standard deviation.

Backwash resulted in higher *16S rRNA* and *sul1* genes abundances in samples collected after the first and second minute of standard filtration mode compared to the samples taken within the third minute (Table 10-8). This examination revealed that the backwash effect and impact on UF filtrate quality during standard filtration is not a long-lasting effect.

Indeed, the question can be raised what is the reason for the *16S rRNA* and *sul1* genes increase and the high standard deviation of measured values in samples after the first minute (Figures 6-3 and 6-4). While *sul1* gene increased up to 2 log units, *ermB* gene was analyzed below the limit of detection, similar to the results of Figure 6-3. The relatively short effect of the first and second minute of standard filtration with higher *16S rRNA* and *sul1* genes (Figure 6-4) could be the result of both particle breakthrough effects from the feed or possible effects at the filtrate side of the membrane module. Breakthrough effects of intact bacteria penetrating through membranes with given pore sizes of 20 nm was examined in our previous study (Hiller et al., 2022). This study resulted in live bacteria abundance up to 6 log units per 100 mL and sul1 genes abundances up to 3 log units per 100 mL measured in UF filtrate that penetrated through the membrane module. Hiller et al. (2022) concluded that there are different possibilities how pore sizes of membrane modules can be determined, but generally a given pore size always represents a pore size distribution. Therefore, a small fraction of larger molecules or particles could theoretically penetrate the membrane. Further factors influencing ARB and ARGs

breakthrough are membrane materials (Liu et al., 2019) and a flux or TMP increase. The flux and TMP increase resulted in bacteria (Suchecka et al., 2003), viruses (Arkhangelsky and Gitis, 2008) and plasmids (Arkhangelsky et al., 2011) breakthrough. The breakthrough effects are the result of bacterial cell deformation (Suchecka et al., 2003) and membrane pore enlargement (Arkhangelsky and Gitis, 2008).

The breakthrough effect caused by bacterial cell deformation and membrane pore enlargement could explain the detected HNAC as well as *16S rRNA* gene and *sul1* gene abundances in UF filtrate measured within the third and fifth minute of standard filtration mode (Figure 6-4). To exclude that significant higher *16SrRNA* and *sul1* genes abundances within the first and second minute are the result of a reduced fouling layer, further investigations were performed in section 6.4.2.

6.4.2 Role of the fouling layer reduction due to backwash and CEB on ARGs and total biomass increase in UF filtrate

A virgin membrane module with a pore size of 20 nm was employed to analyze the total biomass and ARG removal of a membrane module without cake layer effects like pore clogging and deposits on the membrane surface. The virgin membrane module was operated for 1 minute to rinse the preservative out of the membrane module. Samples were collected within the second, third and fourth minute using tertiary effluent as feed. During the fifth and sixth minute, the feed line and the virgin membrane module was filled with secondary effluent. Sampling while applying secondary effluent as feed was executed within the seventh, eighth and ninth minute. The study results of total biomass and ARGs analyses in feeds and corresponding filtrates of the virgin membrane module operation are presented in Figure 6-5.

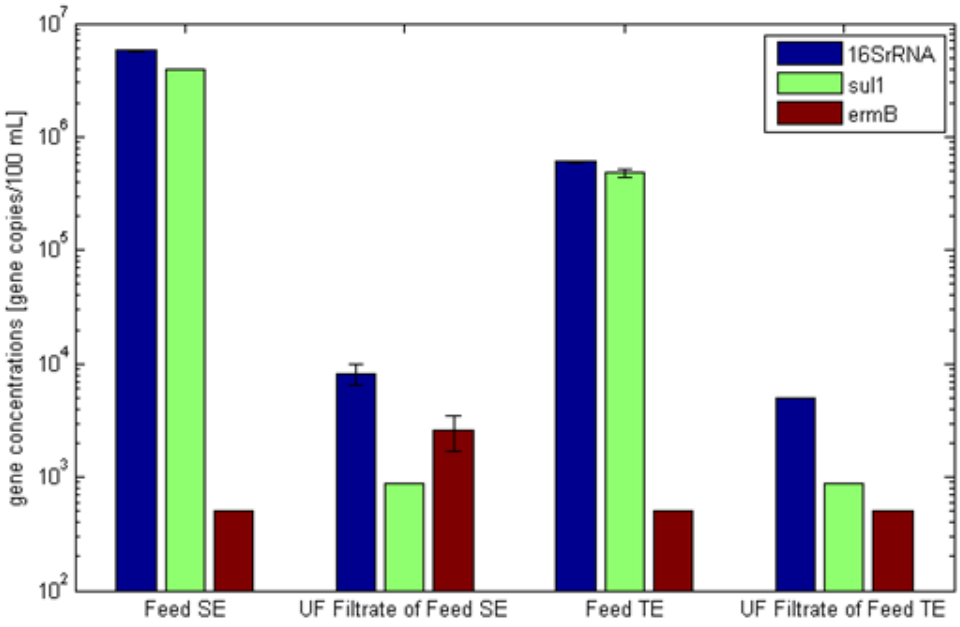


Figure 6-5: UF operation using virgin membrane modules. Feeds SE and TE and the corresponding UF filtrates were analyzed for *16SrRNA*, *ermB* and *sul1* genes abundances. Error bars indicate standard deviation.

The results using the virgin membrane module revealed that UF filtrate analyses had similar *16S rRNA* and *sul1* genes abundances like the fouled membrane module within the third minute of standard filtration mode using tertiary effluent as feed (Figures 6-4 and 6-5). The comparison of mean values of *16S rRNA* gene (t-test, dF = 2; p = 0.171) and *sul1* gene (all values were below the limit of detection) measured in the filtrate samples after the first minute compared to third minute filtrate sample of fouled membrane module revealed no significant difference (Table 10-9).

The backwash mode reduced the fouling layer that had been discussed in section 6.3.1. This fouling layer reduction also resulted in a decrease of the TMP (Figure 10-5). However, this decrease of the TMP or reduced fouling layer did not accompany with significantly higher abundances of *16SrRNA* and *sul1* gene during the first minute of standard filtration.

This observed removal efficiency of the reduced fouling layer directly after backwash and CEB is in line with our previous study (Hiller et al., 2022). This study was the first to examine AMR removal of the fouling layer within 5 and 55 minutes of standard filtration mode. The study results revealed that *ermB*, *sul1*, and TCC showed only a marginal decrease between 5 and 55 minutes of standard filtration. *16S rRNA* genes and *sul1* gene analyzed within 5 and 55 minutes exhibited no difference.

To summarize, significant higher *16S rRNA* gene and *sul1* gene abundances of about 7 and 5 log units of section 6.4.1 after backwash and CEB measured in filtrate samples

after the first minute is neither the result of a reduced fouling layer, nor is it originating from the feed side. Therefore, hypothesis #2 can be rejected. If this increase of total biomass can be excluded coming from the feed side, the question is raised how this significant higher *16S rRNA* and *sul1* genes abundances at the filtrate side could occur. This notion could only be possible due to potentially microbial regrowth effects at the UF filtrate side after backwash and CEB that might originated from the UF filtrate tank. This hypothesis is tested in section 6.4.3.

6.4.3 Role of the water quality of the filtrate tank for backwash and CEB mode and consequences on ARGs and total biomass increase in UF filtrate

To test hypothesis #3 that bacterial and AMR associated regrowth can occur in backwash tanks, UF filtrate samples from the backwash tank were regularly taken for 2 weeks of continuous membrane filtration operation using secondary effluent as feed. Prior to this investigation, a 24-hour CIP with 150 ppm of sodium hypochlorite was performed to disinfect the backwash tank. The blank filtrate sample after CIP had a TCC abundance of 54,600 per 100 mL (Figure 10-6).

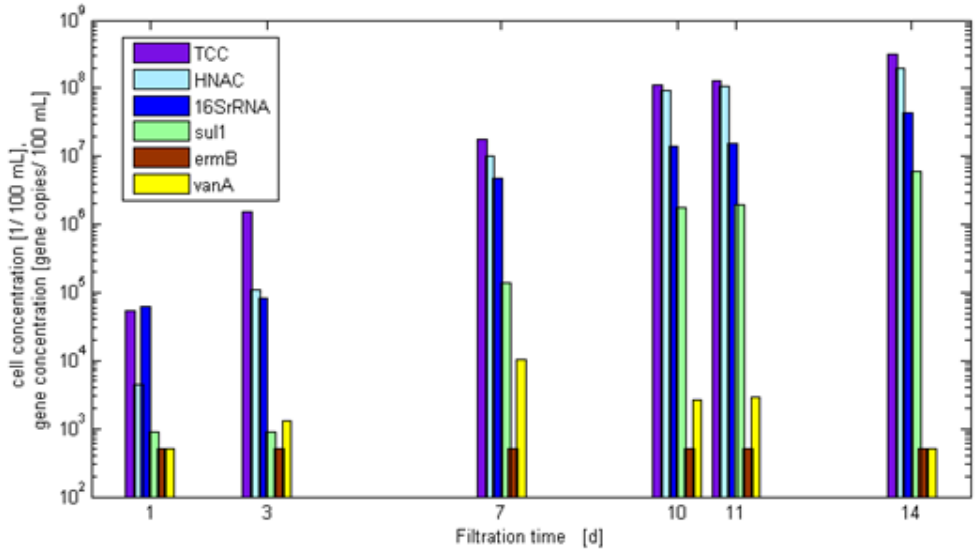


Figure 6-6: Biomass (TCC, HNAC and *16SrRNA* gene) and ARGs (*sul1*, *ermB* and *vanA* genes) analyses in UF filtrate samples taken from the backwash tank during 14 days of continuous UF operation.

The filtrate samples from the backwash tank taken during 14 days of continuous UF operation exhibited significant increasing biomass parameter (Figure 6-6). TCC, HNAC and *16S rRNA* gene increased in UF filtrate samples of the backwash tank within 14 days of continuous UF operation by about 3.8, 4.7 and 2.8 log units, respectively. While *ermB* and *vanA* genes exhibited no increase, *sul1* gene increased significantly by about 3.8 log units. It can be concluded that the time depending increase of total biomass parameter and

the *sul1* gene in the filtrate water of the filtrate tank was the result of ARG associated regrowth.

The observed bacterial regrowth effect is in line with the study of Yi et al. (2019). Yi et al. (2019) studied a gravity driven membrane system (pore size 0.157 µm) for water purification and measured bacterial regrowth via heterotrophic bacteria plate count (HPC) in the filtrate line in the absence and presence of stagnant water. HPC values at the beginning of the batch filtration were between 10 to 1,000 times higher in the presence of stagnant water compared to filtration without stagnant water. Yi et al. (2019) stated that stagnant water was a significant factor for increasing HPC values and a promoter for bacterial regrowth.

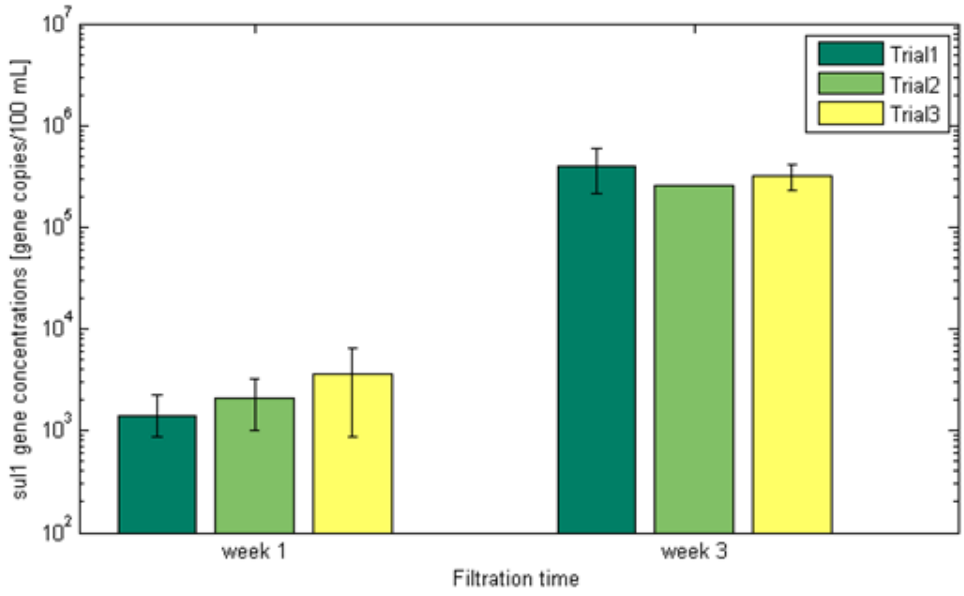
The question is raised why high *sul1* gene abundance could be measured in UF filtrate tank while *ermB* and *vanA* gene abundance were only detected close to the limit of detection. The TCC, *16S rRNA* gene and *sul1* gene abundances in UF filtrate during standard filtration mode was examined in the study of Hiller et al. (2022) as well. Findings of that study suggested that *sul1* gene resistant bacteria can breach UF membranes during standard filtration mode. This might have resulted in increasing *sul1* gene abundances in the filtrate also feeding the UF filtrate tank by 2 log units after 3 weeks of continuous operation. Furthermore, the backwash tank was not completely emptied while performing the backwash. Some filtrate water remained as stagnant water after every backwash in the tank. During this time *sul1* gene resistant bacteria could increase in UF filtrate tank by vertical or horizontal gene transfer. However, the selection of individual antibiotic resistant bacteria and antibiotic resistance genes was already detected after advanced wastewater treatment processes (Alexander et al., 2016; Jäger et al., 2018). Even microbial community shifts could be detected downstream of wastewater discharge measured in surface water (Mansfeldt et al., 2020), this ultrafiltration study is the first to report an ARG associated regrowth in UF filtrate. Other factors could also be responsible for a selective pressure to bacterial adaption to antibiotic resistance in UF filtrate tank. For example, antibiotic concentrations below minimum inhibitory concentration (MIC) could play a role for bacterial adaption to antibiotic resistance (Gullberg et al., 2011). Bacterial mutation to antibiotic resistance could occur in wastewater as well (Malekian et al., 2022).

To summarize, hypothesis #3 stating that ARG associated regrowth occurs in UF filtrate tank with continuous filtration time can be confirmed. Beside of stagnant water and *sul1* gene resistant bacteria breakthrough, further extrinsic factors like remaining

antibiotic concentrations or bacterial mutations could be the result for the observed ARG associated regrowth effect in the filtrate tank.

Since ARG associated regrowth occurred in the UF filtrate tank, the pilot plant applied filtrate water for backwash with decreasing filtrate quality with progressive filtration time. After backwash, the membrane module is filled with backwash water from the filtrate tank. This backwash water could adversely impact first minute samples of standard filtration by low filtrate quality. Therefore, investigations were performed measuring *16S rRNA* and *sul1* genes abundances in samples taken within the first minute of standard filtration mode within 1 and 3 weeks of continuous UF operation. The sampling campaign was repeated three times. The results are presented in Figure 6-7.

A



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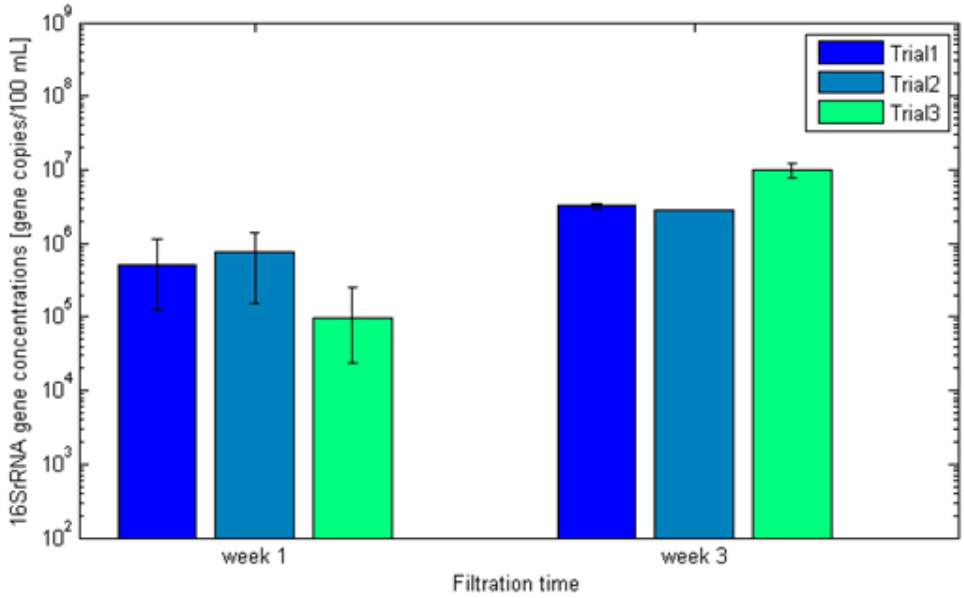

Figure 6-7:*Sul1* gene (A) and *16S rRNA* gene (B) abundances measured in first minute samples after 1 and 3 weeks of continuous UF operation. The sampling campaign was repeated three times. Error bars indicate 95% confidence interval.

Samples taken directly after backwash events within the first minute of standard filtration mode of week 1 samples as well as week 3 samples increased by more than 0.5 log unit for *16S rRNA* gene and by more than 2 log units for the *sul1* gene (Figure 6-7). An independent sample t-test of analyzed *sul1* gene and *16S rRNA* gene of week 1 samples and week 3 samples exhibited that the mean value of *sul1* gene (t-test, dF = 4.0; p = 0.012) and *16S rRNA* gene (t-test, dF = 4.14; p = 0.035) measured in week 3 samples were significantly higher than in week 1 samples (Table 10-10). The comparison of first minute and fifth minute samples of standard filtration after 3 weeks UF operation also revealed significantly higher *sul1* gene (t-test, dF = 4.0; p = 0.012) and *16S rRNA* gene (t-test, dF = 4.0; p = 0.029) abundances. The *16S rRNA* gene increase between first and third week of continuous UF operation might explain the different graphical plots ("BW1A" and "BW1B") analyzed by flow cytometry of Figure 6-2. While graphical plot "BW1A" was executed during UF operation after 4 days, graphical plot "BW1B" with significant higher HNAC value was observed during UF operation after 8 days whereas the sample after the first minute was contaminated with low filtrate quality during backwash. Furthermore, the 16S *rRNA* and *sul1* genes abundances with high standard deviation illustrated in Figure 6-3 are the result of common evaluation of samples after the first minute taken between week 1 and week 3 of continuous UF operation. Finally, the results of the investigations depicted in Figures 6-3 and 6-4 also demonstrated up to more than 2 log units higher *sul1* gene concentrations in first minute samples compared to the third week samples of Figure 6-7. Thus, it can be concluded that the UF filtrate water originating from the filtrate tank had a strong influence on filtrate quality of the first minute of standard filtration.

6.4.4 Role of the CEB chemicals during CEB on ARGs and total biomass increase in UF filtrate

It was hypothesized that a CEB event using sodium hypochlorite instead of sodium hydroxide can result in significantly lower biomass abundances in UF filtrate. Sodium hypochlorite is applied in water as a disinfection agent (Somani et al., 2011). ARG removal in chlorination processes was also reported in Liu et al. (2022). To test this hypothesis, studies were undertaken with the pilot-scale UF unit operated with two parallel trains receiving the same feed water quality. The CEB event was performed once

a day. A CIP was executed for the membrane modules and filtrate tanks before the investigation. The results of these studies are presented in Figure 6-8.

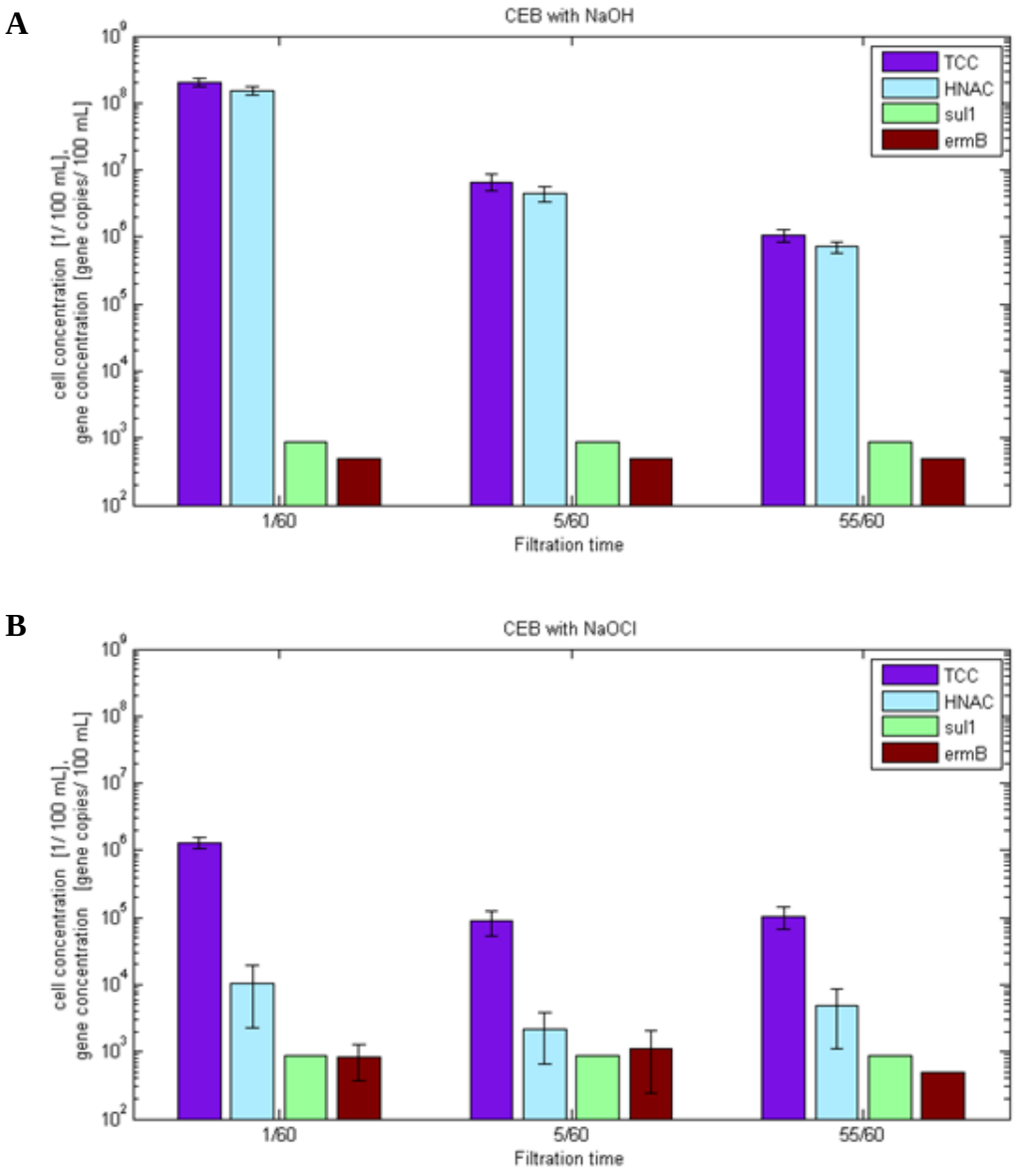

Figure 6-8: TCC, HNAC, *sul1* and *ermB* genes abundances of UF standard filtration mode at a flux of 40 LMH **after CEB** with sodium hydroxide 150 ppm (A) and with sodium hypochlorite 150 ppm (B). Tertiary effluent from WWTP Steinhäule was used as feed. Error bars indicate standard deviation.

Train #1 employing sodium hydroxide during CEB revealed high TCC and HNAC abundances exhibiting more than 7 log units per 100 mL in the UF filtrate within the first minute of membrane filtration. In contrast, strong TCC and HNAC reduction to 6 log units per 100 mL was detected after CEB using sodium hypochlorite within the first minute of standard filtration of train #2. The CEB effect using sodium hydroxide was still detectable with still 6 log units per 100 mL of TCC and HNAC values after 5 minutes of

standard filtration (Figure 10-7). CEB using sodium hypochlorite resulted in significantly lower TCC and HNAC abundances within the first, 5 and 55 minutes of membrane filtration compared to CEB using sodium hydroxide (independent samples t-test, Table 10-11). In contrast, AMR analyses of UF filtrate of trains #1 and #2 resulted in no significant increase. The study was executed after a clean-in-place of both filtrate tanks. The fouling layer measured as TMP significantly decreased after CEB using sodium hypochlorite compared to sodium hydroxide (Figure 10-8). It seems that sodium hypochlorite has a better cleaning efficiency for removal of a fouling layer and bacteria compared to sodium hydroxide. However, lower TMP and lower fouling layer did not result in any significant higher ARGs abundances in the UF filtrate.

In this UF study, the application of sodium hypochlorite instead of sodium hydroxide resulted in more than 4 log units of HNAC value reduction within the first minute of standard filtration mode. The study of Jadoun et al. [41] confirms the findings of this study. Jadoun et al. (2018) analyzed biofilm associated-bacteria at the membrane surface and the cleaning effect with sodium hypochlorite. 0.5 hour of clean-in-place with 100 ppm sodium hypochlorite of the UF membrane revealed a 2.5 log reduction in the number of heterotrophic bacteria and almost completely eliminated fecal coliforms and *E. coli* (5 – 5.5 log units).

To summarize, the comparison of TCC and HNAC abundances after CEB using sodium hydroxide (train #1) and sodium hypochlorite (train #2) revealed high TCC and HNAC abundances that only appeared during CEB using sodium hydroxide. Therefore, CEB using sodium hydroxide seems to have a low disinfection efficiency in mitigating TCC and HNAC abundance. Hence, hypothesis #3 can be confirmed that CEB using sodium hypochlorite instead of sodium hydroxide can result in significantly lower biomass abundances in UF filtrate. However, an ARG reduction accompanied by total biomass reduction was not observed.

6.5 Conclusion

Investigating backwash and CEB during UF operation resulted in significantly increased *16S rRNA* gene abundance, measured as surrogate for bacteria quantification, and *sul1* gene abundances in UF filtrate within the first and second minute compared to 5 and 55 minutes of standard filtration. In contrast to the *sul1 gene*, the antibiotic resistant genes *ermB* and *vanA* did not exhibit this behavior. The UF operation of a virgin membrane module demonstrated similar *16S rRNA* gene and *sul1* gene abundances in the UF filtrate compared to a fouled UF membrane module sampled within 3 and 55 minutes of standard filtration. Thus, breakthrough of *16S rRNA* gene of more than 6 log units per

100 mL and *sul1* gene of more than 5 log units per 100 mL from the feed to the filtrate is likely not the cause of high gene abundance in the filtrate. Instead, it was demonstrated that *sul1* gene associated regrowth occurred in the by-pass filtrate tank resulting in a declining filtrate quality. However, abundance of *ermB* and *vanA* genes in the filtrate tank remained constant at a low level. Using water from this filtrate tank resulted in significant higher *sul1* gene and *16S rRNA* gene abundances within the first and second minute of standard filtration with progressive operation time.

Full-scale capillary UF plants are usually equipped with filtrate tanks that are operated in by-pass mode. In order to maintain a constant filtrate quality and constant removal efficiency during a complete filtration cycle (here 60 minutes), the question is raised what kind of countermeasure is needed. On the one hand, UF filtrate of the first and second minute of membrane filtration operation could be discharged by filter-to-waste. The consequences would be a lower recovery and overall economic performance of the UF plant. Further measures could be the modification of the CEB mode and application of sodium hypochlorite instead of sodium hydroxide. Sodium hypochlorite could have an additional disinfection effect in the UF filtrate tank so that bacterial regrowth is hindered in the UF filtrate tank. However, sodium hypochlorite is known for its accelerating ageing effect on polymer membranes (Puspitasari et al., 2010). Other measures like additional cleaning effects (i.e., chlorination, regular clean-in-place, etc.) of the UF filtrate tank are possible measures as well. Furthermore, the UF filtrate tank operation could be modified from by-pass to continuous flow mode. In this case, stagnant water would not occur and the increase of bacterial population and ARGs associated regrowth could be kept at a lower level. These additional measures should be studied during future investigations.

6.6 Acknowledgements

The authors gratefully acknowledge partial support through a grant provided by the German Ministry of Education and Research (BMBF) as part of the research project ‘HyReKa’ (grant number 02WRS137*)*. We also would like to thank Dr. Christian Wurzbacher and Heidrun Mayrhofer at the Technical University of Munich for technical support.

7 FACTORS AFFECTING ANTIBIOTIC RESISTANCE GENE ASSOCIATED REGROWTH IN WWTP EFFLUENTS AFTER MEMBRANE FILTRATION

The following chapter revealed examinations to test ***research hypothesis #4.1***: AMR associated regrowth in UF filtrate can occur using secondary effluent as feed. Furthermore, ***research hypothesis #4.2***: Advanced wastewater treatment of the feed wastewater prior ultrafiltration will result in no ARG associated regrowth in the filtrate water in the bypass filtrate tank is tested. In addition, ***research hypothesis #4.3*** is tested: Typical erythromycin and sulfamethoxazole concentrations of secondary effluent will not result in bacterial adaption on to antibiotic resistant bacteria at UF filtrate side. Furthermore, the **research hypothesis #4.4:** The filtrate tank in continuous flow mode can reduce ARG associated regrowth effect compared to bypass mode is tested. Lastly, **research hypothesis #4.5 is addressed:** Continuously dosing of 0.5 mg/L sodium hypochlorite in UF filtrate can successfully prevent ARG associated regrowth in UF filtrate tank during continuous flow mode using secondary effluent as feed.

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Author contributions: Christian Hiller developed the research objective, executed the experiments and wrote the manuscript as first author. Christian Wurzbacher supported partially during the writing and reviewed the manuscript. Jörg E. Drewes supported the funding acquisition, supervised the study, and reviewed the manuscript.

7.1 Abstract

The spread of antibiotic resistance genes (ARG) during regrowth in ultrafiltration (UF) filtrate using secondary treated effluent and advanced treated effluents (activated carbon treatment with subsequent porous media filtration) as feed water was investigated at pilot scale. ARG associated regrowth in the UF filtrate was investigated as a function of hydraulic retention time in the filtrate tank, feed water with different ARG abundance and antibiotic concentrations. While efficient AMR removal during standard filtration mode has been reported in previous studies, this study is the first to examine factors for ARG associated regrowth in UF filtrate. ARG associated regrowth can strongly influence the total AMR removal efficiency during UF. *16S rRNA* gene was analyzed for total biomass and s*ul1*, *ermB* and *vanA* genes as representative antibiotic resistance genes. The integron *intl1* gene was analyzed as typical carrier for ARGs. The study findings demonstrated that environmental concentrations of the antibiotics sulfamethoxazole and erythromycin present in secondary effluents, exhibited no ARG associated regrowth in UF filtrate. In contrast, an increase by more than 1 log unit for both *16S rRNA* genes and *sul1* genes were observed using secondary effluent as feed water in the filtrate tank with a hydraulic retention time of 3 hours while operating at continuous flow mode. The highest ARG associated regrowth was detected in temporarily stagnant water in the UF filtrate tank while operating in bypass mode. While ultrafiltered secondary effluent resulted in significant *16S rRNA* gene and *sul1* gene increase by about 3 and 4 log units, the ultrafiltered advanced treated effluent exhibited no *sul1* gene associated regrowth in the filtrate tank while operating in bypass mode. Therefore, ARG abundance in the feed had the strongest influence on ARG associated regrowth in the UF studies. Consequently, a potential strategy to prevent ARG associated regrowth is the pretreatment of the feed with activated carbon followed by porous media filtration. Alternatively, ARG associated regrowth was also inhibited in ultrafiltered secondary effluent by continuous post-treatment of feeding of 0.5 mg/L of sodium hypochlorite after ultrafiltration.

Keywords: Antibiotic resistance genes; wastewater treatment; ultrafiltration; quality of backwash water; antibiotic concentrations; hydraulic retention time

7.2 Introduction

The urban water cycle is one of the most important pathway for distribution of antibiotic resistance in the aquatic environment (Chow et al. 2015, Rizzo et al. 2013). Indeed, several studies of AMR removal efficiencies of WWTPs with mechanical and biological treatment reporting inefficient AMR retention (Alexander et al. 2015; Hembach et al. 2017). Advanced treatment processes, especially membrane filtration processes, revealed the highest ARGs removal efficiencies to prevent the spread of antimicrobial resistance via the urban water cycle (Hembach et al. 2019; Hiller et. al 2019).

Previously published laboratory- and pilot-scale UF membrane filtration studies reported high ARG removal efficiencies between 2 and 7 log units depending on individual ARGs (Breazeal et al., 2013; Böckelmann et al., 2009; Munir et al., 2011). Beside this effective removal, ultrafiltration studies of Munir et al. (2011) and Morales-Morales et al. (2003) detected ARGs above the limit of detection in UF filtrate. The authors of this study (Hiller et al. 2022) investigated the impact of different factors affecting AMR removal efficiency including the pore size of the membrane, the role of the fouling layer, and the AMR abundance and the intra- and extracellular distribution of ARGs in the feed water. Remarkably was the fact that strong correlations between *sul1* gene and bacteria abundance (measured as *16S rRNA* gene) existed in UF filtrate using secondary effluent as feed ($R^2 = 0.60$). The breakthrough of up to 6 log units per 100 mL of live bacteria through ultrafiltration pores could be confirmed by flow cytometry examinations. Considering these findings of live bacteria and some documented ARG breakthroughs through the UF membrane raises the question whether ARGs could be spread further during bacterial regrowth in UF filtrate. This study investigates extrinsic factors for ARG associated regrowth effects in UF filtrate that have not been examined previously.

During full-scale ultrafiltration, the filtrate water stored in a standalone filtrate tank is applied for backwash (BW) and chemical-enhanced backwash (CEB) modes. In general, UF facilities employing capillary membranes are operated predominantly with filtrate tanks in bypass mode than in continuous flow mode. The authors revealed in a previous study (Hiller et al. in review) that operating a filtrate tank in bypass mode, where filtrate water is stagnant until the next BW and CEBs, resulted in ARG associated regrowth in the filtrate tank in bypass mode. However, the observed ARG associated regrowth in the UF filtrate tank could take place not only due to stagnant water conditions but also due to higher *sul1* gene resistant bacteria breaking through the membrane. Therefore, in this study different feed water qualities with different ARG abundances and antibiotic concentrations were compared. These factors were not studied so far as

promoter for ARG associated regrowth at the filtrate side impacting backwash water quality.

In addition, residual antibiotic concentrations in filtrate water could constitute a selective pressure for antibiotic resistant bacteria. Thus, the term minimal selective concentrations (MSCs) define the selective potential of antibiotic concentrations in the environment to trigger bacterial adaption towards antibiotic resistant bacteria (Gullberg et al., 2011; Bruchmann et al., 2013; Sandegren, 2019). Sandegren (2019) reported that streptomycin (1,000 µg/L), tetracycline (15 µg/L) and ciprofloxacin (23 µg/L) had significant different MSCs. These reported MSCs values are higher than the predicted no effect concentrations (PNECs) for the aquatic environment of the European Water Framework Directive (WFD), where adverse effects (e.g., bacterial selection to antibiotic resistance) higher as this PNEC value will most likely occur. The PNECs for streptomycin, tetracycline, ciprofloxacin, erythromycin and sulfamethoxazole were 16, 1, 0.064, 1 and 16 µg/L, respectively. However, researcher reported antibiotic concentrations in WWTP effluents between 0.07 and 1 µg/L for erythromycin as well as between 0.09 and 16 µg/L for sulfamethoxazole (Miao et al., 2004; Bhandari et al., 2008; Alexander et al., 2015). The antibiotic concentrations at PNEC levels in ultrafiltered wastewater and its possible effect to trigger ARG associated regrowth has not been studied so far. Therefore, sulfamethoxazole and the corresponding ARG (*sul1* gene) as well as erythromycin and the ARG (*ermB* gene) were selected to investigate ARG associated regrowth in UF filtrate during this study.

Where wastewater is treated by membranes for example to facilitate water reuse, UF filtrate is buffered in a large reservoir or is transported via a conveyance system to the end user. In this case, the hydraulic retention time could take several hours to days during which ARG associated regrowth can occur. Thus, hydraulic retention time (HRT) is another extrinsic factor that can impact ARG associated regrowth effects in UF filtrate. Previous studies by Shamsaei et al. (2013) and Abhijith et al. (2021) confirmed that bacterial regrowth is directly related to water age (and therefore the hydraulic retention time). However, ARG associated regrowth in UF filtrate as a function of the hydraulic retention time has not been studied so far.

This membrane filtration study is striving for a deeper understanding of factors affecting ARGs associated regrowth in UF filtrate. It is hypothesized that antibiotic concentrations in the range of the PNEC values result in no ARGs associated regrowth in UF filtrate. ARG associated regrowth in UF filtrate is assumed to increase with higher ARG abundance of the feed. Furthermore, the hydraulic retention time of UF filtrate during storage is regarded as an additional extrinsic factor for ARG associated regrowth.

Finally, optional advanced treatment processes and post-treatment strategies were tested for their potential to mitigate ARGs associated regrowth in UF filtrate.

7.3 Experimental Approach

7.3.1 WWTP Steinhäule and membrane filtration pilot plant

UF pilot plant studies were conducted at the Wastewater Treatment Plant (WWTP) Steinhäule in Neu-Ulm, Germany. The WWTP Steinhäule has a treatment capacity of 445,000 population equivalents and was designed for 2,600 L/s at flow at wet weather conditions, which is the double of the dry weather flow. Four treatment stages (mechanical, biological, chemical, and physical) treat wastewater at the WWTP Steinhäule. The physical stage was designed with a contact reactor whereas continuous 10 mg/L of powdered activated carbon (PAC) dosing reduces trace organic chemicals. A clarifier downstream the contact reactor lets the PAC settled to the ground whereas the PAC is returned to the contact reactor for better utilization of the PAC. A final step, the porous media filtration reduces suspended solids from the wastewater. Secondary treated effluent (SE), advanced treated effluent with activated carbon treatment followed by clarification (AWT_{PAC}), and advanced treated effluent with activated carbon treatment and porous media filtration ($AWT_{PAC\text{-}Filtration}$) were used as feed water for subsequent membrane filtration studies. The WWTP Steinhäule and the wastewater treatment process is illustrated in Figure 7-1A. The Table 10-12 presents typical wastewater constitutions of the WWTP Steinhäule. A membrane filtration pilot plant was applied at WWTP Steinhäule whereas all AMR studies were conducted. The pilot plant had two parallel trains. Each train had two pre-filters (400 µm cut-off), a feed tank applied as reservoir, membrane module, and a by-pass filtrate/ backwash tank. Continuous flocculant dosing was performed with a pump and a flocculant tank. CEB was conducted with two pumps and two tanks for the different CEB chemicals (sodium hydroxide and sulfuric acid). For the ARG regrowth studies examining the hydraulic retention time of the filtrate water, two filtrate tanks #1 and #2 (with 0.5 mg/L sodium hypochlorite dosing), operated in continuous flow mode, were installed at the filtrate side of train #1. A filtrate tank #3 in continuous flow mode was configured at the filtrate side of train #2 (Figure 7-1B).

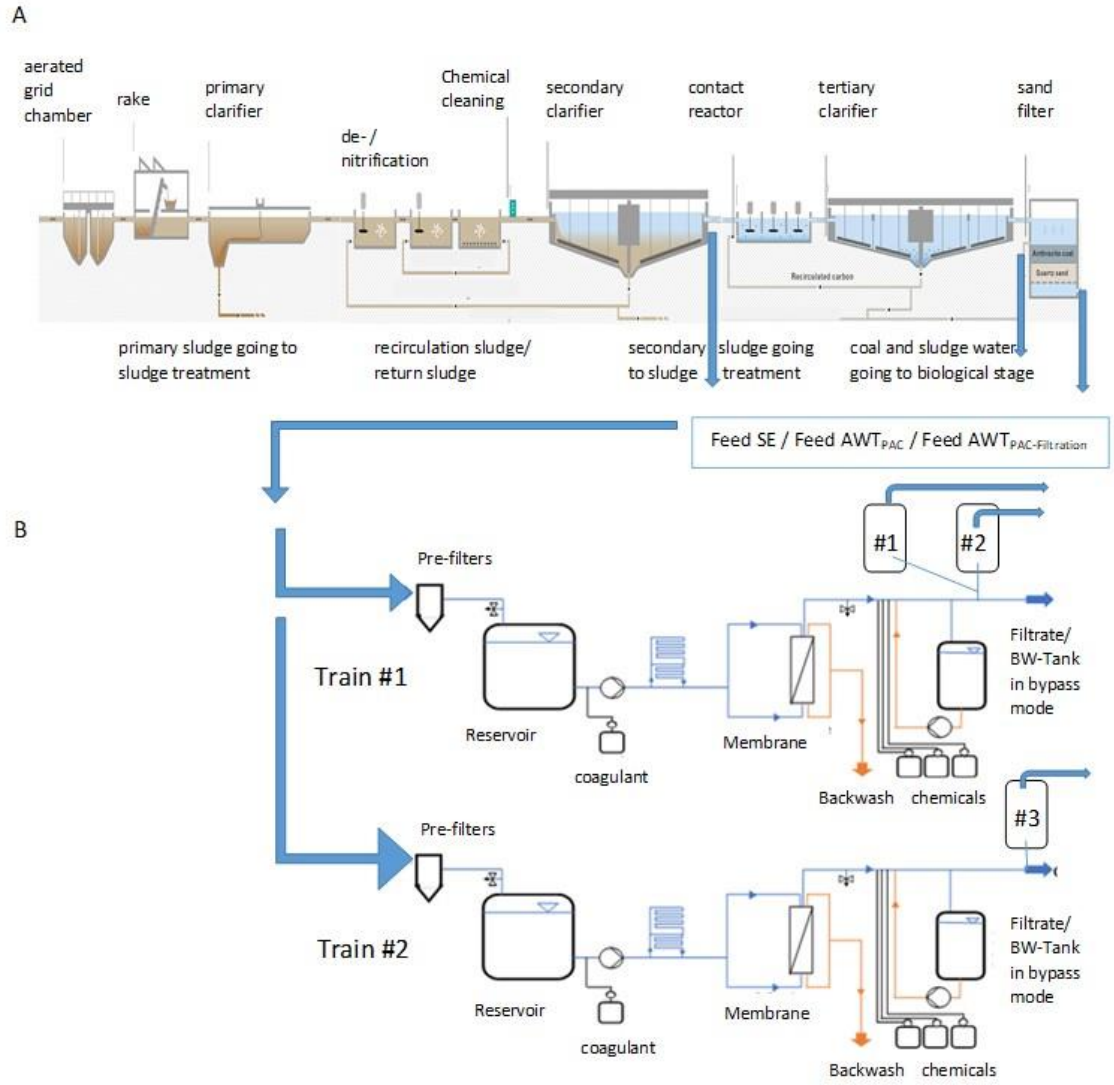

Figure 7-1: Schematic flow diagram of the wastewater treatment at WWTP Steinhäule (A). Secondary treated effluent (SE), advanced treated effluent with activated carbon treatment followed by clarification (AWT_{PAC}), and advanced treated effluent with activated carbon treatment and porous media filtration ($AWT_{PAC-Filtration}$) were used as feed of the membrane filtration pilot plant. Schematic flow diagram of the pilot plant is shown in Figure 1B.

Ultrafiltration and microfiltration membrane modules with pore sizes of 20 nm (UF, 80 m² surface area) and 450 nm (MF, 22 m² surface area) were applied for AMR studies. The membrane modules were operated in parallel at a flux of 40 and 70 LMH (inside-out and dead-end filtration mode). The material of the membrane modules were hydrophilized polyethersulfone, characterized by a contact angle of 52°.

The standard filtration mode of the membrane filtration pilot plant lasted 60 minutes. In this mode and at the applied fluxes, the pilot plant operated at sustainable fluxes under reversible fouling conditions. Feed water was pumped from the reservoir at the feed side through the membrane module to the filtrate side (Figure 7-1B). To maintain a moderate

TMP increase (Yoo 2018), 2 mg/L of coagulant (polyaluminum chloride solution, PLUSPAC FD ACH, Feralco Deutschland GmbH, Germany) was continuously dosed into the feed side of the membrane module. After 60 minutes of standard filtration mode, the feed and coagulation pumps were switched off. After that, the BW mode was activated. Hydraulic backwashing was conducted whereas filtrate water from the backwash tank is pumped through the membrane. After 45 seconds at a flux of 230 LMH, the backwash mode was finished. This described standard filtration and backwash protocol consisted of 23 repetitions. After that, a CEB mode was executed instead of a backwash mode. The CEB mode consisted of applying a 150 ppm sodium hydroxide rinsing of the membrane module for 90 seconds at a flux of 120 LMH. After that, the UF module had a 15 min soaking with sodium hydroxide. At a flux of 230 LMH a hydraulic backwash rinsed the sodium hydroxide out for 70 s applying filtrate water from the backwash tank. After that, the membrane filtration pilot plant operated at 70 LMH for 900 s so that the filtrate tank was refilled. After sodium hydroxide saoking, the sodium hydroxide protocol was repeated with sulfuric acid. When the CEB mode was finished, the standard filtration mode was started again.

7.3.2 Experiments and sampling conditions

AMR analyses were executed as described in Hiller et al. (2022). In this study, we attempted to perform AMR removal studies only during dry weather flow conditions. Membrane integrity was successfully confirmed before and after AMR examinations of the UF filtrate before and after AMR studies. Treatment variability of two trains of the pilot plant was checked before initiating the AMR studies.

In chapter 7.4.1, the factors temporarily stagnant filtrate water and feed water quality with different ARG abundances were examined in bypass mode concerning ARG associated regrowth. While a previous study by the authors (Hiller et al. in review) already reported bacterial and ARG associated regrowth in UF filtrate using secondary effluent as feed, these examinations were repeated in this study for statistical purposes. Furthermore, we hypothesized that also ARG abundance in the feed water, strongly influences the ARG associated regrowth in the bypass filtrate tank. Therefore, advanced treated effluent ($AWT_{PAC\text{-}Filtration}$) of the WWTP Steinhäule, characterized by Hiller et al. (2022) to exhibit significant lower biomass and ARG abundances than SE as feed, were applied in this study. Regular filtrate samples were taken from the bypass filtrate tank during 14 and 19 days of continuous membrane operation using secondary and advanced treated effluent as feed. During standard filtration mode, the temporarily stagnant water phase between backwash cycles lasted 58 minutes in the filtrate tanks. Every sampling campaign consisted of samples taken from filtrate tank of train #1 and train #2 whereas both trains operated in parallel at a flux of 70 LMH. Clean-in-place (CIP) procedures of

the membrane modules and the filtrate tanks were executed applying 150 ppm sodium hypochlorite for 24 hours before and after each examination.

In order to investigate the impact of residual antibiotic concentrations on regrowth of antibiotic resistant bacteria in UF filtrate, sulfamethoxazole and erythromycin were continuously spiked in the UF filtrate tank during continuous flow mode of train #2 while train #1 was not spiked with antibiotics (chapter 7.4.2). These UF studies were executed using the advanced treated effluent $AWT_{PAC\text{-}Filtration}$ as feed where powdered activated carbon already reduced sulfamethoxazole and erythromycin occurrences to concentrations close to the limit of detection (Table 10-13). A stock solution with autoclaved water with 1 mg/L sulfamethoxazole and 0.1 mg/L erythromycin was prepared (Table 10-14) and analyzed after 0, 24, 48 and 120 hours to confirm that the stock solution provided a constant sulfamethoxazole concentration. A dosage pump continuously delivered the stock solution to the filtrate tank so that concentrations of 10 µg/L sulfamethoxazole and 1 µg/L erythromycin were achieved in the filtrate tank in continuous flow mode of train #2. The amount of stock solution that was pumped into the filtrate tank was checked several times every day and was adjusted as needed. UF filtrate samples were taken from the filtrate tanks of train #1 and #2 operated in continuous flow mode for 17 days during continuous UF operation at a flux of 40 LMH. Filtrate samples were analyzed for biomass and ARGs abundances.

To analyze the impact of hydraulic retention time in the filtrate tank for ARG associated regrowth, three filtrate tanks were installed at the UF pilot plant that operated in continuous flow mode each with a hydraulic retention time of 3 hours (Chapter 7.4.3). The UF pilot plant was operated with two UF trains in parallel. Filtrate tanks #1 and #2 received UF filtrate from train #1 that was operated with SE as feed. Filtrate tank #3 received UF filtrate from train #2 that was operated with AWT_{PAC}. The difference between filtrate tank #1 and #2 was the fact that additional 0.5 mg/L sodium hypochlorite was continuously applied to tank #2. UF filtrate samples were taken every three days from the three filtrate tanks in continuous flow mode for 31 days of continuous UF operation at a sustainable flux of 70 LMH. The samples were analyzed for biomass and ARGs abundances. Virgin filtrate tanks were applied for this examination. Prior to executing the study, CIP was executed whereas the membrane modules were exposed to 150 ppm sodium hypochlorite for 24 hours.

7.3.3 AMR and microbial biomass analyses

The following antibiotic resistance genes were selected: *ermB, sul1,* and *vanA* genes. The integron *intl1* gene was analyzed as typical carrier for ARGs. Furthermore, the *16S*

rRNA gene was employed as surrogate parameter for bacterial cells quantification in samples (Clarridge 2004; Revetta et al. 2010; Hembach et al. 2019).

In the laboratory, each water sample was filtrated through a 0.22 µm Sterivex filter (Millipore, Sigma-Aldrich, Munich, Germany). The filters were stored until further analysis at -20°C. DNA was extracted from the filtered biomass at the membrane using the Power Soil DNA extraction kit (Qiagen), following the manufacturers protocol. The further protocol for qPCR analyses was executed following the protocol of Hiller et al. (2022). The detection limit of *sul1* gene and *intl1* gene were 1,750 gene copies per 100 mL, *vanA* and *ermB* genes were 1,000 gene copies per 100 mL. The detection limit of *16S rRNA* gene was 10,000 gene copies per 100 mL. ARG values that were below the limit of detection were taken into account in the bar plots by using half the value of the detection limit.

7.3.4 Statistical data analyses

Statistical data evaluation was conducted using paired sample two-sided t-test with a significant threshold of $\alpha = 0.05$ whereas the t-test requirements were normality and homogeneity of variances. To examine the significance of mean values of different data series of ARG associated regrowth studies in UF filtrate tanks in continuous flow mode, pair samples t-test were executed with analyzed parameter of the filtrate samples of the filtrate tanks.

7.4 Results and discussion

7.4.1 Role of temporarily stagnant UF filtrate and feeds with different ARG abundance as factors for ARG associated regrowth

According to manufacturer information, full-scale UF plants are predominantly operated with filtrate tanks in bypass mode rather than in continuous flow mode resulting in temporarily stagnant water phases between backwash cycles. Full-scale filtrate tanks in bypass mode are designed to hold a capacity of 2-3 times of backwash volumes.

To analyze the effect of stagnant water and feeds with different ARG abundance on filtrate quality after microfiltration and ultrafiltration, ARGs as well as total biomass (*16S rRNA* gene) were measured in the filtrate tanks in bypass mode using secondary (Figure 2A) and advanced treated effluents as feeds over 14 and 19 days of continuous operation (Figure 7-2B).

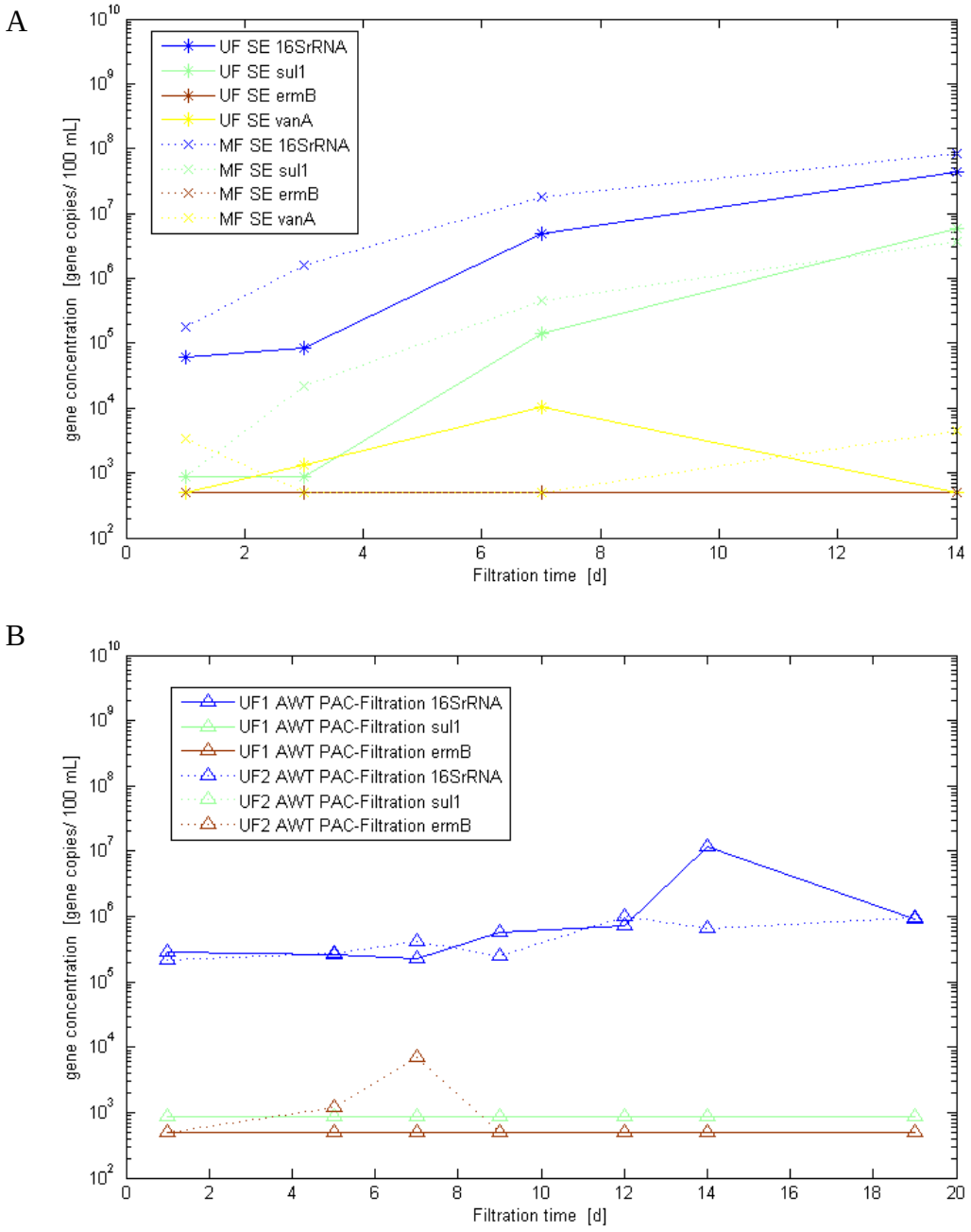

Figure 7-2: (A) Abundance of *16S rRNA*, *sul1*, *ermB* and *vanA* genes in filtrate from filtrate tanks of train #1 and train #2. Train #1 (MF) and train #2 (UF) were operated in parallel at a flux of 70 LMH and 60 minutes filtration time using secondary effluent (SE) as feed. (B) A second experiment was performed with advanced treated effluent (AWT$_{PAC\text{-}Filtration}$) as feed and UF filtrate analyses in the filtrate tanks of train #1 and train #2.

While *ermB* and *vanA* genes abundances did not significant increase in the filtrate tanks of train #1 and train #2, the abundance of *sul1* and *16S rRNA* genes significantly increased (about 4 and 3 log units) in the filtrate tanks after microfiltration (train #1) and ultrafiltration (train #2) (Figure 7-2A). This suggests that *sul1* gene resistant bacteria increased with progressive filtration time in both filtrate tanks. In addition, all measured ARGs abundances were temporarily detectable (> LOD). Treating SE by MF or UF

demonstrated that ARG associated regrowth can appear in temporarily stagnant water (Figure 7-2 A). In contrast, operation using advanced treated effluent as feed with significant lower initial ARGs abundances (Figure 7-2B) revealed no significant ARG associated regrowth in both filtrate tanks after 19 days of continuous membrane operation. Under these conditions, *16S rRNA* gene slightly increased by 0.5 to 1 log unit, while *sul1* and *ermB* genes did not increase.

As previously reported by Hiller et al. (2022), sul*1* gene resistant bacteria can likely breach UF membranes during standard filtration mode. After completion of a backwash, the bypass tank was refilled with filtrate and the water remained stagnant during the remainder of the 60-min filtration cycle. No fresh water was delivered to the tank and no water was withdrawn during this stagnant phase. This phase might have provided an opportunity for *sul1* gene resistant bacteria to increase in UF filtrate tank by vertical or horizontal gene transfer.

ARG associated regrowth in UF filtrate due to temporarily stagnant water and ARG abundance differences in feed water quality has not been investigated so far. Bacterial regrowth was reported by Yi et al. (2019), who studied a gravity driven membrane system (pore size 0.157 µm) for water purification. The bacterial regrowth was measured by heterotrophic bacteria plate count (HPC) in the filtrate of the membrane filtration lines both in the absence and presence of stagnant water. While the membrane filtration lines were operated for 28 days, the average filtration time of each membrane filtration study was around 1 hour per day. To study the stagnant water effect of the filtrate, the filtrate water of the membrane filtration line A had a “standstill time” for the next 23 hours of the day. Membrane filtration line B was operated in parallel with membrane filtration A, but the filtrate was allowed to drain off so that no stagnant filtrate water was formed. The stagnant filtrate water phase resulted in HPC values measured in the filtrate that were up to 1,000 times higher in the presence of stagnant water compared to the batch filtration trial without stagnant water. Yi et al. (2019) concluded that stagnant water was a significant factor for the observed increasing heterotrophic plate counts and a promoter for bacterial regrowth.

In this study, no ARG associated regrowth was detected in the filtrate tanks of both trains using advanced treated effluent as feed. The upstream wastewater treatment, especially the activated carbon and subsequent porous media filtration process, might have resulted in a significant additional chemical and bacterial removal resulting in lower chemical and bacterial loads compared to the secondary effluent. This pretreatment resulted in a decreased ARG associated regrowth potential in the UF filtrate. The

performance of porous media filtration process to decrease AMR abundance in the wastewater is presented according to the study of Lüddeke et al. (2015). Lüddeke et al. (2015) analyzed the sand filtration with pre-coagulation for ARB removal efficiencies. Antibiotic resistant *E. coli*, *Enterococci* and *Staphylococci* were quantified by cultivation method. The study results demonstrated that sand filtration can decrease ARB abundance by about 2.6 log units *E. coli*, 2.6 log units *Enterococci*, and 3.6 log units *Staphylococci*, respectively.

To summarize, temporarily stagnant water and ARG abundance in the feed are relevant extrinsic factors for ARG associated regrowth in the corresponding filtrate. Therefore, advanced wastewater treatment of the feed with powdered activated carbon and porous media filtration may mitigate regrowth potential and ARG reappearance in the filtrate

7.4.2 Role of antibiotic concentrations in UF filtrate as factor for possibly promoting ARG associated regrowth

In order to exclude that very low antibiotic concentrations can affect ARG associated regrowth in UF filtrate, train #1 was operated in continuous flow mode without spiking elevated antibiotic concentrations in UF filtrate while the filtrate tank of train #2 was continuously spiked with sulfamethoxazole and erythromycin, resulting in concentration of 10 µg/L sulfamethoxazole and 1 µg/L erythromycin in the filtrate. Both trains were operated with advanced treated effluent ($AWT_{PAC\text{-}Filtration}$) as feed.

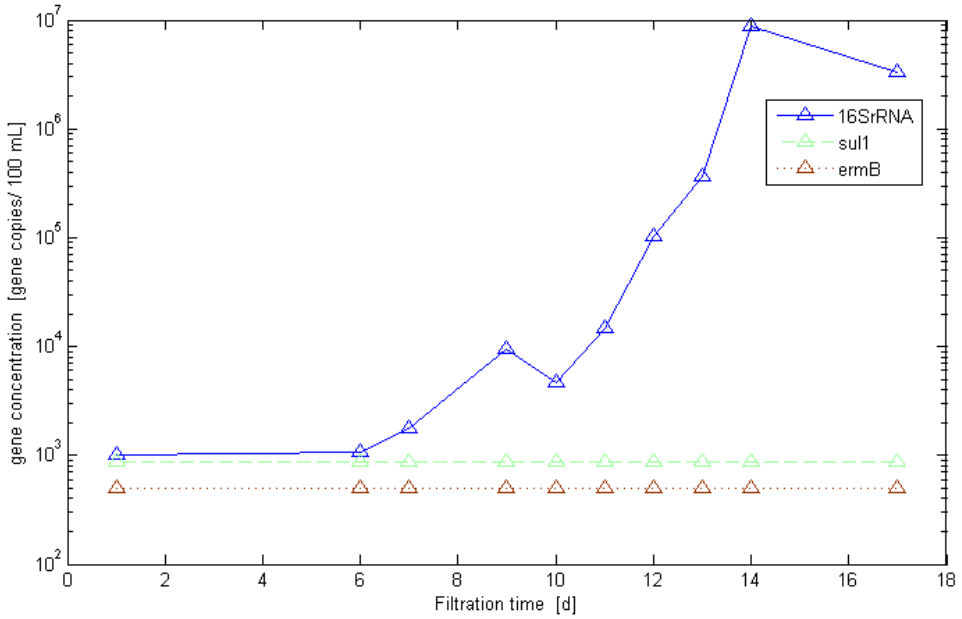

Figure 7-3: Abundances of *16S rRNA*, *ermB* and *sul1* genes in the UF filtrate from the filtrate tank of train #2 using advanced treated effluent as feed while spiking antibiotics at elevated concentrations.

Although in this experiment, where the filtrate was spiked with elevated concentrations of antibiotics, regrowth was detected based on *16S rRNA* data (similar as in the control train #1, Figure 10-9), but ARG levels stayed at the limit of detection after 17 days for train #2 (Figure 7-3). No ARG associated regrowth was also detected in UF filtrate tank of train #1 (Figure 10-9) whereas no antibiotic spiking was applied. The *ermB* gene and *sul1* gene abundances measured in the UF filtrate tanks of both trains were similar to abundances analyzed in the filtrate samples without considering storage in the filtrate tank (see study by Hiller et al. 2022). Therefore, it can be concluded that within 17 days of continuous UF operation the applied antibiotic concentration in the microgram per L range provided no selective pressure for ARG transfer in the filtrate.

Several studies still focused on minimal selective concentrations (MSCs) of antibiotics and bacterial adaption to antibiotic resistance. The following studies are in line with the results of this study: Bengtsson-Palme and Larsson (2016) studied MSCs using 111 antibiotics from the public European Committee on Antimicrobial Susceptibility Testing (EUCAST) database. Bengtsson-Palme and Larsson (2016) reported MSCs for erythromycin (16 µg/L) and for sulfamethoxazole (1,000 µg/L) that were significantly higher than the evaluated concentrations of 1 µg/L erythromycin and 10 µg/L sulfamethoxazole applied in this study. Significant higher MSCs of erythromycin (between 500 and 750 µg/L) were also reported by Stanton et al. (2020). However, other studies reported antibiotic concentrations in WWTP effluents that had significant lower antibiotic concentrations than the spiked antibiotic concentrations in this study (Alexander et al., 2015; Michael et al., 2013).

To summarize, slightly elevated erythromycin and sulfamethoxazole concentrations typically observed in secondary treated effluents (ref) seem to have no selective pressure to bacteria present at UF filtrate side. Therefore, it can be expected that antibiotic concentrations lower than 10 µg/L sulfamethoxazole and 1 µg/L erythromycin have no selective pressure to bacteria in municipal wastewater.

7.4.3 Role of the hydraulic retention time of the UF filtrate on ARG associated regrowth

The UF pilot plant was operated with UF train #1 and UF train #2 in parallel. During dry weather flow, the feed of train #1 was secondary effluent and the feed of train #2 was

advanced treated effluent with activated carbon treatment followed by clarification (AWT_{PAC}). At the WWTP Steinhäule the activated carbon stage is actually designed to treat wastewater during dry weather conditions, whereas the sand filtration process is designed for wet weather conditions. Under wet weather conditions, the secondary effluent of train #2 of WWTP Steinhäule is only treated by the sand filtration process not by the activated carbon stage. While secondary effluent was the feed of train #1 during wet weather events, the feed water quality of train #2 of the UF pilot plant changed within this UF study from advanced treated effluent to a blend of secondary and advanced treated effluent during wet weather events.

As stagnant water seems to be one of the important factors for regrowth, the hydraulic retention time was examined as further factor for ARG associated regrowth using filtrate tanks in continuous flow mode instead of filtrate tanks in bypass mode. It is expected that filtrate tank in continuous flow mode can reduce ARG associated regrowth compared to the filtrate tank operated in bypass mode. To study the hydraulic retention time, three filtrate tanks were operated in parallel at the UF pilot plant mimicking continuous flow mode. While filtrate tank #1 and #2 were continuously filled with ultrafiltered secondary effluent (SE) from train #1, filtrate tank #3 was continuously supplied with ultrafiltered advanced treated effluent (AWT_{PAC}) from train #2. Filtrate tank #2 was additionally treated with 0.5 mg/L sodium hypochlorite.

Since full-scale filtrate tanks in bypass mode are designed to hold a capacity of 2-3 times of backwash volumes, a backwash volume has to be provided, as well, when filtrate tanks in bypass mode should be replaced into filtrate tanks in continuous flow mode. In this case, the filtrate water of full-scale filtrate tanks in continuous flow mode should be regularly changed within about 0.8 to 1.2 hours of hydraulic retention time at dry weather. During low dry weather inflow in the summer time, whereas low groundwater level resulted in low infiltration water, the hydraulic retention time of up to 3 hours could be achieved in filtrate tanks in continuous flow mode. Therefore, the hydraulic retention time of the filtrate tanks was set at 3 hours.

The bacterial abundance was measured in the filtrate tanks in continuous flow mode (Figure 7-4).

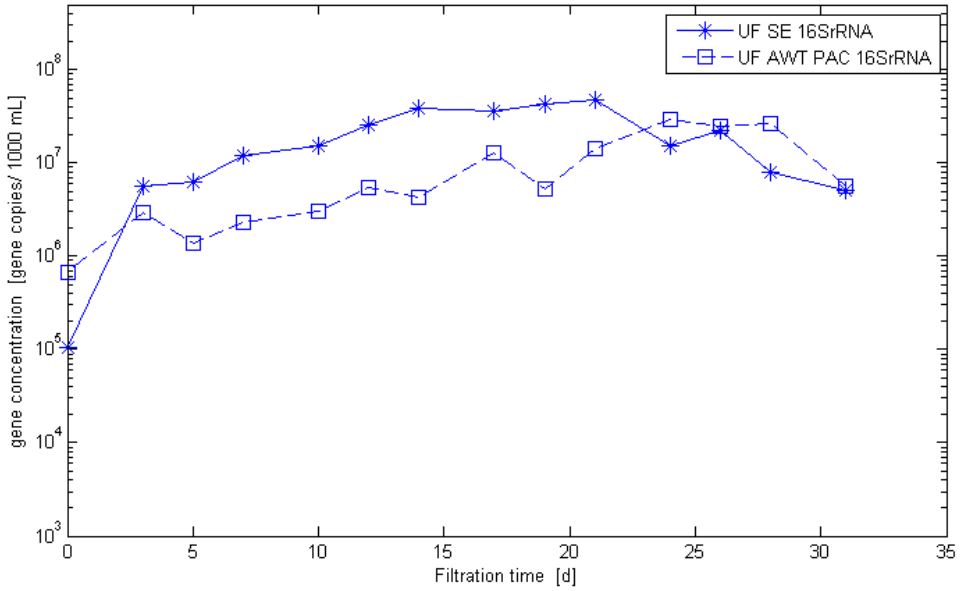

Figure 7-4: Abundances of *16S rRNA* genes in ultrafiltration filtrate after a hydraulic retention time of 3 hours using secondary effluent (UF SE) and advanced treated effluent (UF AWTPAC) as feed water.

At the beginning of the regrowth studies, the first filtrate sample measured directly after the initial filling of filtrate tanks #1 and #3 exhibited *16S rRNA* gene abundances, measured as surrogate for bacteria quantification, between 5 and 6 log units (Figure 7-4). These initial *16S rRNA* gene abundances represent typical measured *16S rRNA* gene abundance in UF filtrate during standard filtration mode as measured in our previous study (Hiller et al., 2022). *16S rRNA* gene abundances significantly increased with progressive filtration time and a hydraulic retention time of 3 hours by more than 1 log unit in the first 21 days in both filtrate tanks #1 and #3 (Figure 7-4). Despite the filtrate tank in continuous flow mode, it seems, the analyzed bacterial regrowth occurred due to an accumulation of penetrated bacteria through the UF membrane in the filtrate tank in continuous flow mode. A bacteria growth effect due to a hydraulic retention time was also observed by Boe-Hansen et al. (2002). A drinking water distribution system was examined for bacterial growth in the drinking water. Boe-Hansen et al. (2002) resulted a bacterial growth rate of 0.30 day^{-1} in the water.

Within this filtrate tank study, the tanks in continuous flow mode were flowed from bottom to the top so that stagnant water zones in the tanks should be avoided. However, the question is raised whether the filtrate tanks in continuous flow mode were operated under complete mixing conditions or whether occasional stagnant water zones within the filtrate tank in continuous flow mode existed. Stagnant water was examined in chapter 7.4.1 showing a significant bacteria regrowth effect. The research question about completely mixing conditions of a water tank Gualtieri et al. (2010) modeled in a study

reporting about 82 % of the tank volume was under complete mixing conditions. Hence, occasional stagnant water zones within the filtrate tank in continuous flow mode could occur and contribute to bacterial regrowth.

This bacterial regrowth measured in the filtrate tanks during continuous flow mode resulted in a reduced *16S rRNA* gene removal efficiency analyzed between influent UF and effluent of the tank of the ultrafiltration process with progressive filtration time (Figure 10-10). While the *16S rRNA* gene removal efficiencies without considering storage in a tank were quantified with 3.6 and 3.3 log units using secondary effluent and advanced treated effluent as feeds (mean values of removal efficiencies based on feed and filtrate samples, illustrated in Figure 10-12), a hydraulic retention time of 3 hours decreased the overall removal efficiency to about 1.8 log units (feed SE) and 2.5 log units (feed AWT_{PAC}) after 19 days (characterized by almost dry weather conditions) of continuous UF operation (Figure 10-10). The higher *16S rRNA* gene abundance in ultrafiltered secondary effluent resulted in a higher regrowth rate and a lower *16S rRNA* gene removal efficiency with progressive filtration time compared to samples of ultrafiltered advanced treated effluent.

Nutrient and dissolved organic carbon (DOC) concentrations were also analyzed during the regrowth study in the UF filtrate samples (Table 10-15). While nitrate and total phosphate were detected at the same concentration in ultrafiltered SE and ultrafiltered AWT_{PAC}, the DOC concentration in ultrafiltered AWT_{PAC} was significantly lower than in ultrafiltered SE. However, during this study almost the same increase of *16S rRNA* gene in both filtrates by more than 1 log unit from day 3 to day 21 was observed (Figure 7-4) suggesting that the remaining DOC concentration at UF filtrate side was not a limiting factor for ARG associated regrowth.

However, it can be concluded that the increase of *16S rRNA* gene by more than 1 log unit during progressive filtration time is depended upon the hydraulic retention time in the filtrate tank. The application of AWT_{PAC} as feed with lower biomass abundance maintained about 1 log unit lower *16S rRNA* gene abundance in the corresponding filtrate compared to ultrafiltered SE. However, from day 22 until day 31 the *16S rRNA* gene exhibited almost the same abundance in the filtrate tanks of both trains. This is likely a result of wet weather conditions (Figure 10-11), where similar *16S rRNA* gene abundances of both filtrates between day 22 until day 31 were observed due to the same feed water quality. While the feed water quality, measured as *16S rRNA* gene, was higher in SE (424,275,000 gene copies/ 100 mL) than in AWT_{PAC} (219,750,000 gene copies/ 100 mL) within dry weather conditions after 12 days of continuous UF operation, the feeds had almost the same abundance (SE: 154,300,000 gene copies/ 100 mL and AWT_{PAC} 158,400,000 gene copies/ 100 mL) within rainy weather conditions after 26 days of continuous UF operation.

The *sul1* gene as well as *intl1* gene analyses in the filtrates with progressive filtration time are illustrated in Figure 7-5.

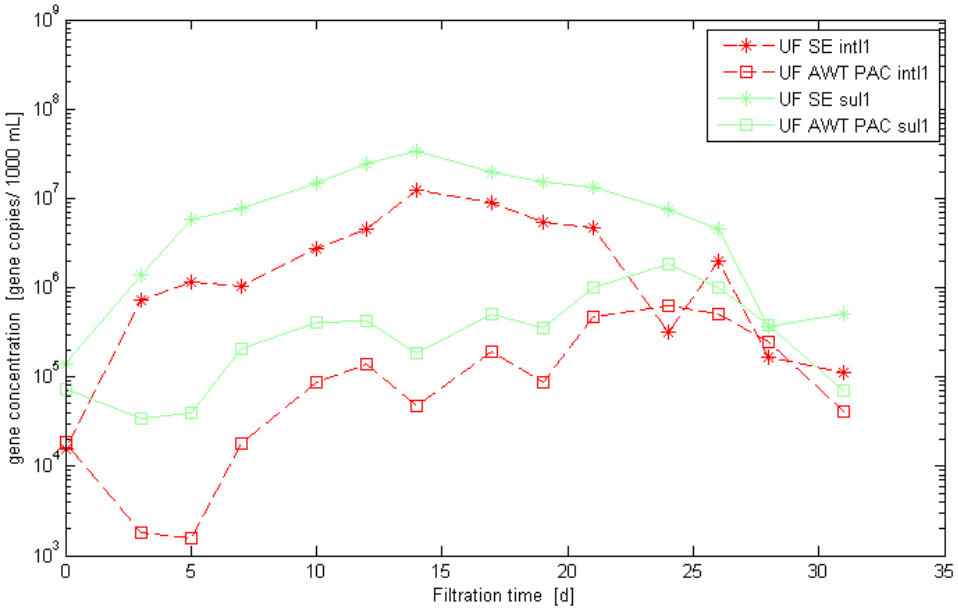

Figure 7-5: Abundances of *16S rRNA* genes in ultrafiltration filtrate after a hydraulic retention time of 3 hours using secondary effluent (UF SE) and advanced treated effluent (UF AWTPAC) as feed water.

The data series until day 21 of ultrafiltered SE samples revealed higher mean values of *16S rRNA* gene as well as *sul1* and *intl1* genes than the respective mean values of ultrafiltered AWTPAC samples. During the 21st days, *sul1* gene and *intl1* gene of ultrafiltered SE in tank #1 exhibited about 1 log unit higher abundances than samples of ultrafiltered AWTPAC in tank #3. During wet weather conditions and the associated dilution effect between day 22 until day 31 resulted in very similar decreased *sul1* gene and *intl1* gene abundances in both UF filtrates.

While the *intl1* and *sul1* gene removal efficiencies without any storage time were also quantified with 2.6 and 2.7 log units using SE as feed as well as 3.0 and 3.0 log units using AWTPAC as feed (mean values of removal efficiencies based on feed and filtrate samples, illustrated in Figure 10-12), the *intl1* and *sul1* genes removal efficiencies after a hydraulic retention time of 3 hours, decreased to about 0.7 and 0.8 log units (SE as feed) as well as 2.1 log units (AWTPAC as feed) after 19 days (a period characterized by almost dry weather conditions) of continuous UF operation (Figure 10-13). Equivalent to *16S rRNA* gene removal efficiency, *intl1* and *sul1* genes removal efficiencies were significantly lower with progressive filtration time using SE compared to AWTPAC as feed.

The data series of the Figure 7-4 and 7-5 were proved for significance mean values using pair samples T-tests (Table 10-16). The data series of ultrafiltered secondary

effluent samples had between day 0 and day 21 significant higher *16SrRNA* gene as well as *sul1* and *intl1* genes mean values than the data series of ultrafiltered activated carbon treated secondary effluent (AWT_{PAC}) samples ($P < 0.05$).

The hydraulic retention time resulted in an increase of *16S rRNA*, *intl1* and *sul1* genes with progressive filtration time in ultrafiltered SE and AWT_{PAC}, while *ermB* and *vanA* genes did not change in all measured filtrates with progressive filtration time. Beside feed pretreatment, it was hypothesized that applying a continuous 0.5 mg/L sodium hypochlorite dosage as a post-treatment strategy of UF filtrate tank #2 can successfully prevent ARG associated regrowth using SE as feed. The sodium hypochlorite dosage resulted in significant decrease of *sul1* and *intl1* genes close to or below the limit of detection and *16S rRNA* gene decrease by about 1 log unit during the 31 days of continuous UF operation (Figure 10-14). This prevention of bacterial regrowth is accompanied with a constant high *16S rRNA*, *intl1* and *sul1* gene removal efficiencies varying between 4 to 5 log units (Figure 10-15).

To conclude, the hypothesis that the hydraulic retention time of the filtrate tank can affect bacteria, *sul1* and *intl1* gene abundance in UF filtrate can be confirmed. Furthermore, pretreatment steps to reduce bacteria and ARG abundance in feed water could significantly reduce the ARG associated regrowth effect in the filtrate tank in continuous flow mode.

7.5 Conclusion

Findings of this study revealed that *sul1* gene, *intl1* gene and bacteria can increase in abundance in UF filtrate with progressive filtration time and therefore can strongly decrease total removal efficiency of the UF process. Examined extrinsic factors for ARG associated regrowth were the temporarily stagnant filtrate water in backwash tanks, ARG abundance levels of the feed, antibiotic concentrations, and the hydraulic retention time of filtrate water in backwash tanks. Antibiotics occurring at typical environmental concentrations in secondary treated effluent revealed no selective pressure for bacteria in the filtrate. While three hours of hydraulic retention time in the backwash tank in continuous flow mode resulted in more than 1 log unit higher *sul1* gene and bacteria abundances, temporarily stagnant filtrate water during bypass operation strongly promoted *sul1* gene and bacteria to increase by more than 3 log units. Since analyzed ARGs like *ermB* gene and *vanA* gene did not increase in the filtrate, this does not exclude that other not detected ARGs could also increase in the filtrate. However, findings revealed that ultrafiltration of secondary effluent resulted in *sul1* gene and *intl1* gene associated regrowth in the filtrate and backwash tanks operated in bypass mode.

In order to reduce or prevent ARG associated regrowth at UF filtrate side, technical optimizations within the UF process were examined. Full-scale UF plants should operate filtrate tanks in continuous flow mode instead of bypass mode. Additional treatment of secondary effluent with activated carbon and sand filtration could significantly reduce ARG and bacteria abundances in the feed so that lower ARG and bacteria abundance in the filtrate occurred resulting in no ARG associated regrowth at the filtrate side. As an alternative strategy, continuously dosing 0.5 mg/L sodium hypochlorite at filtrate side is viable post-treatment process to successfully prevent ARG associated regrowth. These strategies are recommended whereas wastewater is treated by low-pressure membranes for water reuse, where UF filtrate is stored in buffer tanks or large reservoirs or being transported via a conveyance system to the end user.

7.6 Acknowledgement

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8 PLANNING OF A FULL-SCALE MEMBRANE FILTRATION PLANT AT THE WWTP STEINHÄULE

This chapter presents investigations related to ***research objective #5***: The pilot-scale studies focused on different UF operational conditions at the WWTP Steinhäule. This chapter presents a conceptional design for a full-scale membrane filtration plant to reduce the spread of antibiotic resistance through discharges of the WWTP Steinhäule.

8.1 Abstract

The membrane filtration process is considered an advanced treatment technology to prevent the spread of AMR by discharge of WWTP effluents. The membrane filtration pilot-scale studies were executed at the WWTP Steinhäule, which employs mechanical, biological, chemical and physical treatment steps. The following aspects were investigated in lieu of considering a full-scale implementation at this facility. Where is the best position for the implementation of the membrane filtration process? What kind of pore size of the membrane module is needed to achieve a high AMR removal efficiency under economic conditions? How economic are operational conditions maintaining a high AMR removal efficiency? What strategies are needed to prevent AMR associated regrowth? The study results revealed that the best place for the implementation of the full-scale membrane filtration plant was right after tertiary treatment. In this case, *sul1* gene resistant bacteria were significantly removed within the activated carbon stage and subsequent sand filtration so that no ARG associated regrowth at the filtrate side occurred. The application of an inge MF module™ had a pore blockage with significant higher TMP build-up during continuous operation whereas a higher backwash flux could not solve this shortcoming. The operation of a Microdyn-Nadir UF module™ revealed a low flux of about 38 LMH using a constant coagulation dosis of 9 mg/L PAC. The application of the inge UF modules™ revealed the highest sustainable flux of 70 LMH while constantly dosing 2 mg/L PAC. The inge UF modules were further considered for implementation of a full-scale UF facility at the WWTP Steinhäule.

Keywords: Microfiltration; ultrafiltration; inside out and outside in membrane modules; operational modes with backwash; backflush; chemical enhanced backwash; coagulation dosing; invest and operation costs

8.2 Introduction

Municipal wastewater treatment in Germany is currently requiring three treatment stages: mechanical, biological and chemical treatment stage. In addition to the mechanical, biological and chemical treatment stages, the WWTP Steinhäule in Neu-Ulm is already employing an advanced treatment stage for the elimination of trace organic chemicals (TOrCs). TOrCs in the secondary effluent are adsorbed onto the surface of powdered activated carbon. The spent carbon is fed to the sewage sludge incinerator via surplus sludge removal. Beside the removal of TOrCs, it is assumed that antibiotic resistant pathogenic bacteria removal is an additional important parameter that needs to be addressed. Viable process technologies for wastewater disinfection include ozonation, UV irradiation, and membrane filtration.

Membrane filtration technology is a physical process that is already used in wastewater treatment as separation step through pressure-driven filtration. However, membrane filtration processes have so far been used in the wastewater treatment predominantly as membrane bioreactor process (MBR) mainly employing microfiltration membranes. The MBR process is operated with a high dry matter content, low flux, high membrane pressure, high pumping costs and low backwash intervals compared to capillary membranes. The use of capillary membranes with regard to the removal of bacteria and ARGs under different operating conditions in a conventional secondary effluent, after a powdered activated carbon (PAC) treatment step, and in the effluent of a PAC process with subsequent sand filtration were investigated. The pilot-scale membrane plant with capillary membranes was operated with the aforementioned effluents at the WWTP Steinhäule, whereby these effluents have low turbidity of FNU < 1-5 m^{-1}, which would favor economical operation of capillary membranes.

Within the scope of this chapter, two ultrafiltration pilot plants were tested in parallel at the WWTP Steinhäule. Three hollow fiber modules (UF and MF modules) as polymer membranes with inside-out and outside-in filtration were compared with each other for economic efficiency. The optimum operating conditions of the different pilot-scale plants were analyzed. These investigations should reveal the achievable sustainable flux, determination of the critical flux, optimization of backwash procedures, and the optimization of using conditioning agents.

8.3 Material and methods

8.3.1 Wastewater treatment at WWTP Steinhäule

Pilot-scale membrane studies were performed at the wastewater treatment plant Steinhäule in Neu-Ulm, Germany with a treatment capacity of 445.000 population equivalents. The WWTP Steinhäule is designed for a flow of 2,600 L/s (at wet weather conditions), which is double the dry weather flow. At this facility, wastewater is treated by four treatment stages – mechanical, biological, chemical, and physical stages. After

secondary treatment, the physical stage is comprised of a contact reactor where 10 mg/L of powdered activated carbon (PAC) is continuously fed in order to remove trace organic chemicals. A subsequent clarifier is employed to separate the PAC followed by a tertiary filtration step. Settled activated carbon from the clarifier is returned to the contact reactor for better utilization of the PAC. The overall wastewater treatment process at WWTP Steinhäule is illustrated in Figure 8-1.

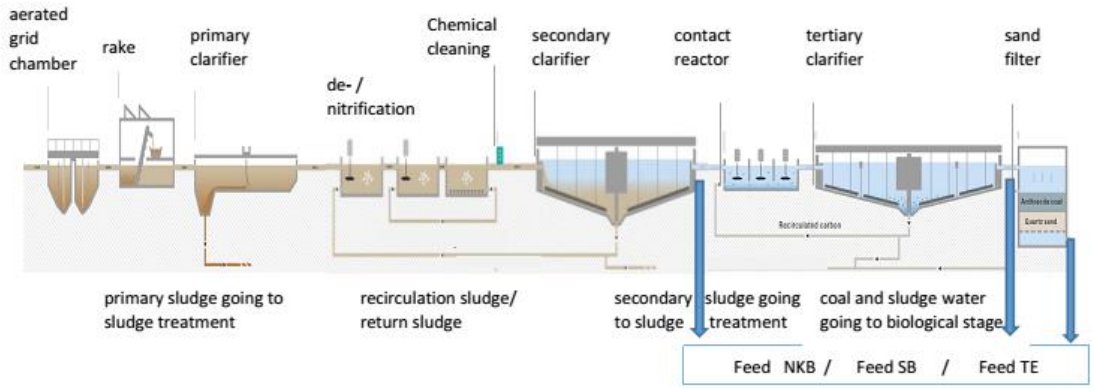

Figure 8-1: Schematic diagram of the overall wastewater treatment at WWTP Steinhäule (A). Secondary effluent (NKB), activated carbon treated secondary effluent (SB) and tertiary effluent (TE) consisting of advanced treatment of secondary effluent using activated carbon as well as sand filtration were used as feeds for membrane filtration pilot plant.

Conventional secondary effluent (NKB), activated carbon treated secondary effluent (SB), as well as tertiary effluent (TE) were used as feed water qualities for subsequent membrane filtration studies.

8.3.2 Membrane filtration pilot-scale plants

8.3.2.1 Membrane filtration pilot-plant of the inge company (Dupont Water Solutions)

AMR examinations were executed using a membrane filtration pilot plant of the inge company (Dupont Water Solutions). A conceptional flow diagram is illustrated in Figure 8-2.

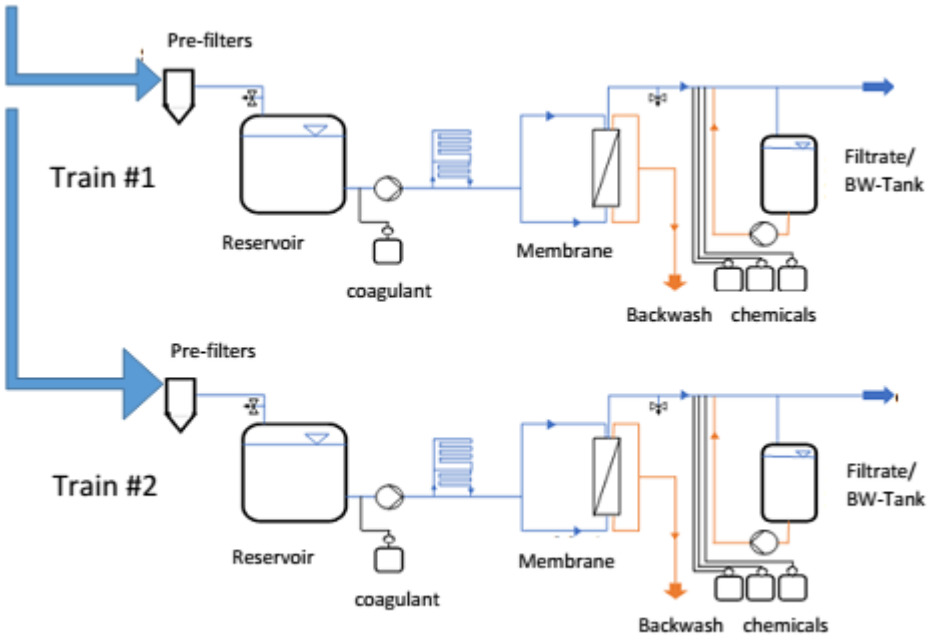

Figure 8-2: Schematic flow diagram of the inge membrane filtration pilot plant.

The pilot plant consisted of two parallel trains. Every train was comprised of two pre-filters (400 µm cut-off), feed tank (reservoir), a membrane module, and a by-pass filtrate/ backwash (BW) tank. A coagulant pump and flocculant tank enabled continuous flocculant dosing. Chemical enhanced backwash (CEB) was performed with one acid tank and pump as well as two base tanks and two backwash pumps.

UF membrane modules with pore sizes of 20 nm (UF, 80 m² surface area) and MF membrane module with pore size of 450 nm (MF, 22 m² surface area) were selected for AMR studies. Membrane modules were made of hydrophilized polyethersulfone and had a contact angle of 52°. The MF and UF membrane modules were operated in an inside-out, dead-end filtration mode in parallel. MF operated with fluxes of 40 and 70 LMH. The UF operated at fluxes of 40, 70, 80, and 90 LMH during the AMR studies.

The continuous UF filtration operation in dead-end mode resulted in a fouling layer increase at the feed side of the membrane. This fouling layer contains of particulate matter that is larger than the pores of the membrane. The increase of the fouling layer results in an increase of the transmembrane pressure (TMP) and therefore in higher power requirement of the pumps to maintain a constant flux level. That is the reason why economical UF operation requires procedures to keep the fouling layer at a lower level. Therefore, backwash mode and CEB mode are regularly applied in the membrane filtration operation especially in the application of dead-end driven capillary membranes. The filtration cycle of the membrane filtration process is described in the following section:

The standard filtration mode of the membrane filtration process was 60 minutes. In this mode, the pilot plant operated at a constant flux at 40 or 70 LMH under reversible

fouling conditions, whereas feed wastewater was pumped from the reservoir through the membrane module to the filtrate side (Figure 8-2). A final coagulant (polyaluminum chloride solution, DIN 883, PLUSPAC FD ACH, Feralco Deutschland GmbH, Germany) dose of 2 mg/L was continuously fed to the feed line directly prior to the feed side of the membrane module. Continuous coagulant dosing was applied as fouling control. Coagulation reduces the occurrence of reversible fouling and increases the filtration efficiency (Yoo, 2018). After 60 minutes, the standard filtration mode ended and both feed pumps and coagulation pumps were switched off. Backwash mode was activated, whereas hydraulic backwashing was executed by applying filtrate water from the backwash-tank to the module at an outside-in mode. The backwash mode lasted for 45 seconds at a flux of 230 LMH. After 23 backwash modes, a chemical enhanced backwash (CEB) mode was performed. The CEB mode consisted of injecting and rinsing the UF for 90 seconds with 150 ppm sodium hydroxide at an intake flux of 120 LMH. After that, the UF module was soaked for 15 min with the injected sodium hydroxide. A final (hydraulic) backwash rinsed the chemical out at a flux of 230 LMH for 70 s using filtrate water from the backwash tank. A short filtration at 70 LMH for 900 s was conducted directly after the sodium hydroxide CEB. Finally, the CEB procedure was repeated with sulfuric acid. After the CEB procedure with sulfuric acid and a backwash to rinse the chemical out of the membrane module, the standard filtration mode was initiated again.

8.3.2.2 Membrane filtration pilot plant of the Microdyn-Nadir company (Mann+Hummel)

AMR examinations were executed using a membrane filtration pilot plant of the Microdyn-Nadir company (Mann+Hummel). A conceptional flow diagram is illustrated in Figure 8-3.

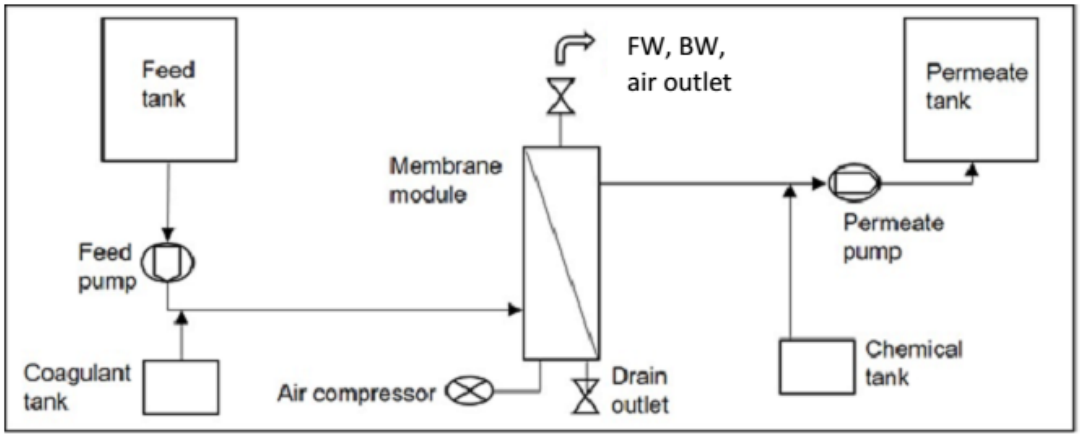

Figure 8-3: Schematic flow diagram of the Microdyn-Nadir membrane filtration pilot plant (Egner, 2017)

The pilot plant consisted of one train which was built up with a pre-filter (400 µm cut-off), feed tank (reservoir), a membrane module, and by-pass filtrate/ backwash (BW) tank. A coagulant pump and flocculant tank enabled continuous flocculant dosing.

Chemical enhanced backwash (CEB) was performed with one acid tank and pump as well as one base tank and pump. An air compressor enables air scouring during forward flush.

The membrane module was made of a hydrophilized polyethersulfone (PES) membrane. UF membrane module with a pore size of 25 nm (UF, 55 m² surface area) was selected. The UF membrane module was run in an outside-in, dead-end filtration mode. The UF operated at fluxes between 20 and 38 LMH during the AMR studies.

The standard filtration mode of Microdyn Nadir pilot plant was 30 minutes. A final coagulant (polyaluminum chloride solution, DIN 883, PLUSPAC FD ACH, Feralco Deutschland GmbH, Germany) dose between 4.5 and 9 mg/L was continuously fed into the feed line directly prior to the feed side of the membrane module. Several opportunities in membrane cleaning were available. The backwash (BW) mode can be applied whereas permeate from the filtrate tank is pumped through the membrane module from the filtrate side to the feed side. A forwardwash (FW) mode can be also applied with feed water at the feed side (Figure 8-3). Furthermore, both processes can be enhanced with air scouring. A drainage function (Drain) was available, whereas the module can be emptied. A pre-filling step (Prefill) filled the module with feed water, so that the complete membrane module was full of water. After an optimization process, the final cleaning mode was operated using the following protocol: The cleaning mode begins with an air scouring combined with a forwardwash (flux 85 LMH for 60 s) and a subsequent backwash (flux 85 LMH for 60 s). The CEB was applied with 150 ppm of sodium hydroxide and 150 ppm of hydrochloric acid. A CIP had to be applied in regular intervals operating with fluxes higher than 30 LMH. The CIP was performed with a combination of 150 ppm sodium hydroxide and 150 ppm sodium hypochlorite for 1.5 hours. The CEB chemicals were flushed out using backwash with permeate from the filtrate tank. After that, the CIP protocol consisted of an acid cleaning with hydrochloric acid for 1.5 hours. After backwash, the standard filtration mode started again.

8.4 Results and discussion

8.4.1 Studies with Inge and Microdyn-Nadir UF pilot-scale plants

The operation of the Inge and Microdyn-Nadir pilot plants was optimized according to the operational parameters sustainable flux, TMP, and coagulation demand (chapter 8.4.1.1). Furthermore, the removal efficiencies of wastewater parameters like TOC and ortho-phosphate (PO_4-P) are presented in chapter 8.4.1.2.

8.4.1.1 Operation conditions with respect to flux, TMP and coagulation demand

Operation of the Inge pilot plant

To demonstrate a long-term, secure and operationally stable operation, the UF pilot plant of the Inge company was operated with the effluent after sand filtration from 07/21/2017 to 10/19/2017 (Figure 8-4).

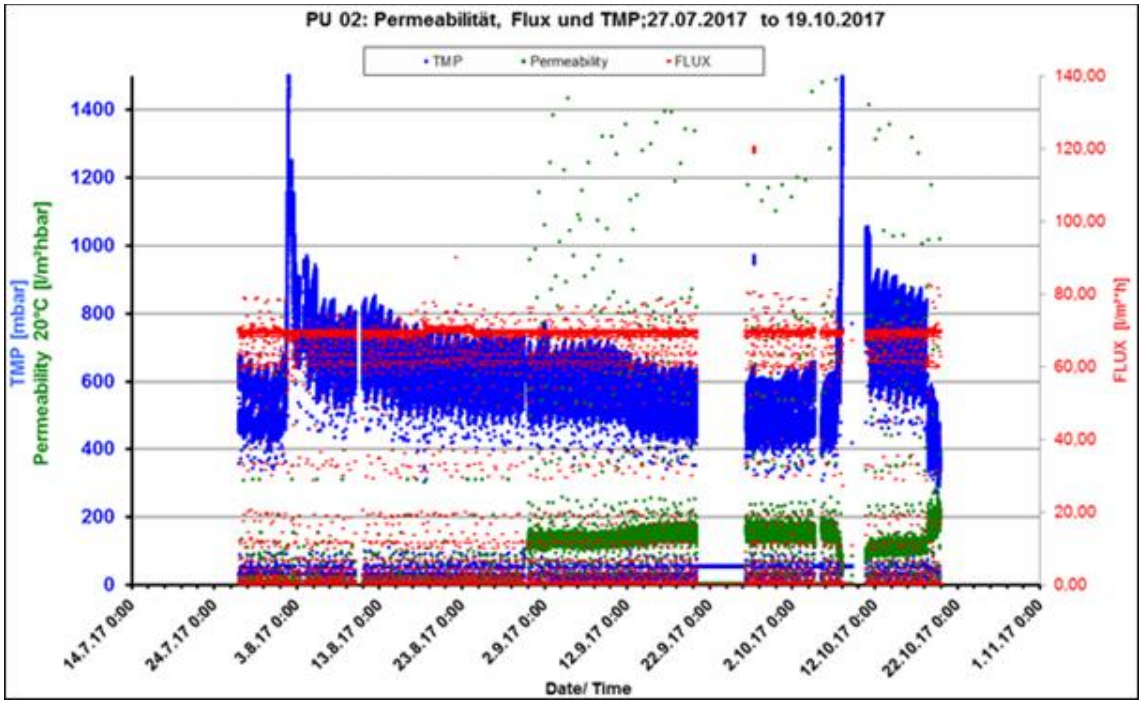

Figure 8-4: Long-term operation of the UF pilot-scale plant of the Inge company

It was observed that the operation of the UF pilot plant was improved over the period of 3 months. With a constant sustainable flux at 70 LMH and a constant dosing of 2 mg/l PAC, the TMP could be optimized from initially approximately 400 mbar - 1000 mbar to 400 - 630 mbar.

The further optimization of the operation of the UF pilot plant was executed using coagulation coating, whereas the use of coagulation was reduced step by step without disrupting the operational process. During coagulation coating a coagulation layer is formed on the membrane surface so that particulate and dissolved matter relevant to membrane clogging (foulants) accumulate on the coagulation layer. During backwash mode, the application of the coagulation layer enables a more efficient removal of the foulants. The purpose of the coagulant coating is to sufficiently coat the membrane with coagulation only at the beginning of the standard filtration mode and not over the complete standard filtration mode so that a smaller amount of coagulation is applied. Thus, during UF pilot plant operation, the constant dosing of 2 mg/L coagulation was continuously reduced from 60 minutes during standard filtration time to 50, 40, 30, 20 and 10 minutes (Figure 8-5).

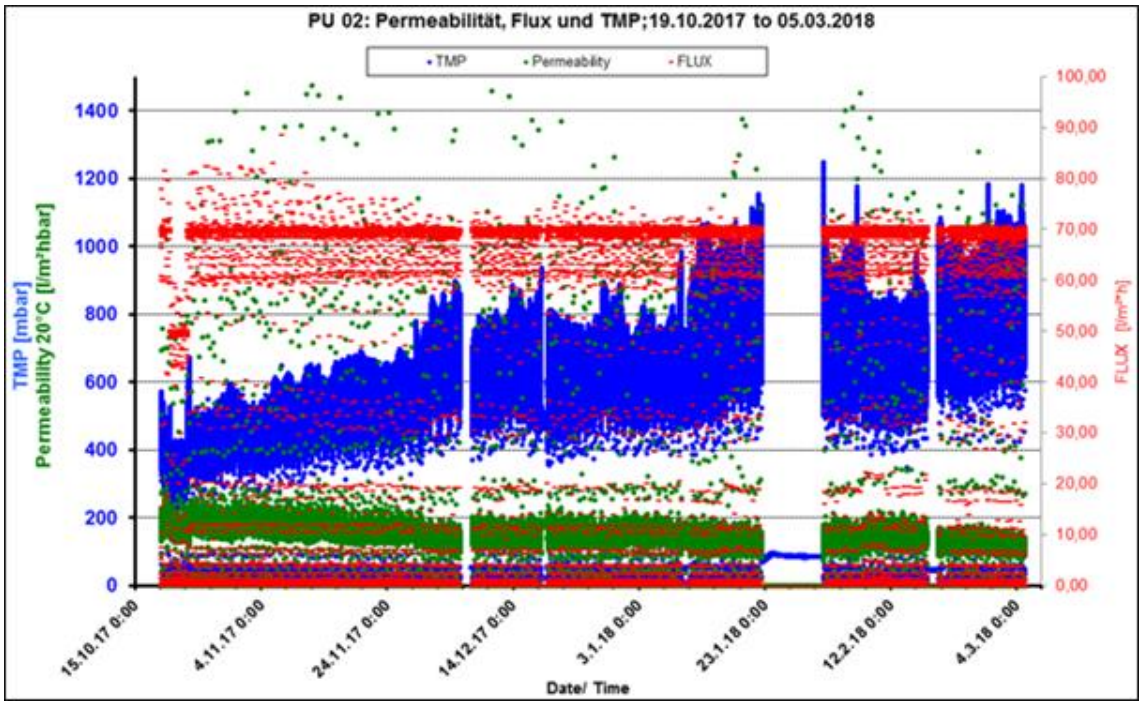

Figure 8-5: Operation of the UF pilot plant of the inge company using coagulation coating.

The coating time was reduced from 60 minutes to 50 minutes on October 2nd, 2017. On October 19th, 2017, the coating time was further decreased to 40 minutes, on October 25th, 2017 to 30 minutes and on November 7th, 2017 to 20 minutes. The coating time was even set to 10 minutes on November 29th, 2017, whereas the TMP increased significantly.

To summarize, it can be stated that the UF pilot plant operated at a sustainable flux of 70 LMH and a TMP between about 400 and 600 mbar using tertiary effluent as feed. The coating time can be reduced to 20 minutes and the consumption of coagulation can be cut in third without a significant TMP increase.

In the following section, MF and UF operation was compared under economic conditions. The operation is illustrated in Figure 8-6.

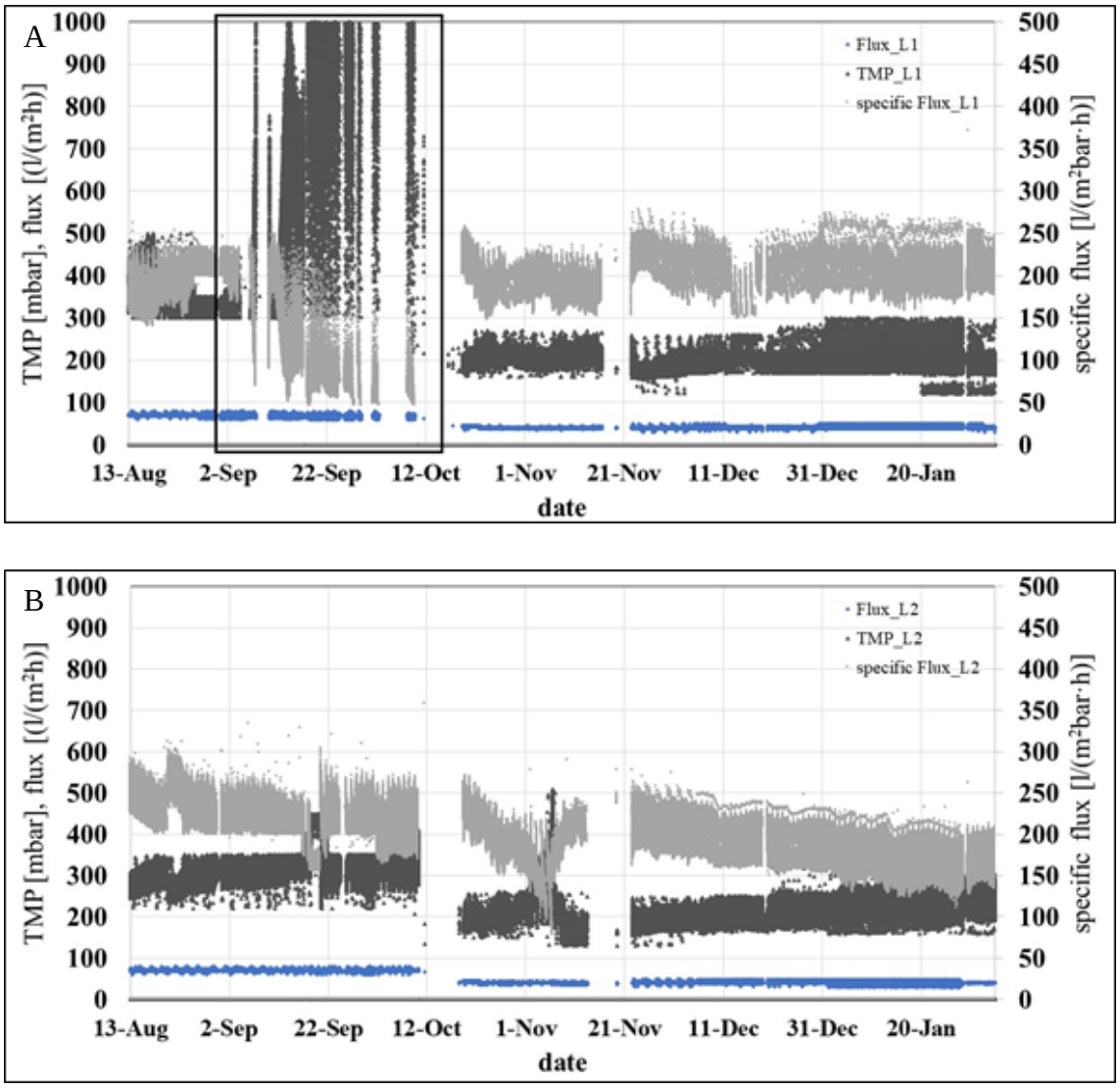

Figure 8-6: Operation of the MF train (A) and UF train (B) of the membrane filtration pilot plant of the inge company at a flux of 40 LMH and a constant PAC dosing of 2 mg/L.

The operation of the MF and UF membrane modules in parallel operation mode revealed that the two membrane modules behaved differently during continuous operation. While the ultrafiltration showed a TMP of approximately 300-600 mbar at a constant flux of 40 LMH from September 2nd, 2019 to October 12th, 2019, the TMP of the train with the MF module increased steadily from an initial average of 651 mbar to 1,171 mbar up to chemical backwashing (Figure 8-6). After several days, the terminal TMP of the membrane of 1,480 mbar was reached and the MF was automatically stopped. The same terminal TMP was reached with a different secondary effluent as feed. In order to improve the operation of the MF, an attempt was made to optimize the backwashing with filtrate water. The backwash flux was increased from 230 LMH to 600 LMH with the result that the yield of the membrane filtration deteriorated. The TMP could thus be reduced more effectively. However, this could not prevent a steady increase in TMP. The backwash effect was likely caused by the majority of the colloids apparently in the pore size range of the MF membrane module resulting in pore blockage. However, these colloids did not adversely affect the operation of the UF membrane module, which

confirmed a relatively constant TMP and constant flux. Therefore, it seemed that the colloids maintain a cake layer formation during UF, which did not result in a higher TMP.

It can be concluded that the MF is not an appropriate membrane for an efficient membrane filtration operation under economic conditions using WWTP effluents. In contrast, the operation of the UF revealed a high sustainable flux at a constant low TMP using WWTP effluents.

Operation of the Microdyn-Nadir membrane filtration pilot plant

The operation of the UF pilot plant of the Microdyn-Nadir company is presented in the Figure 8-7.

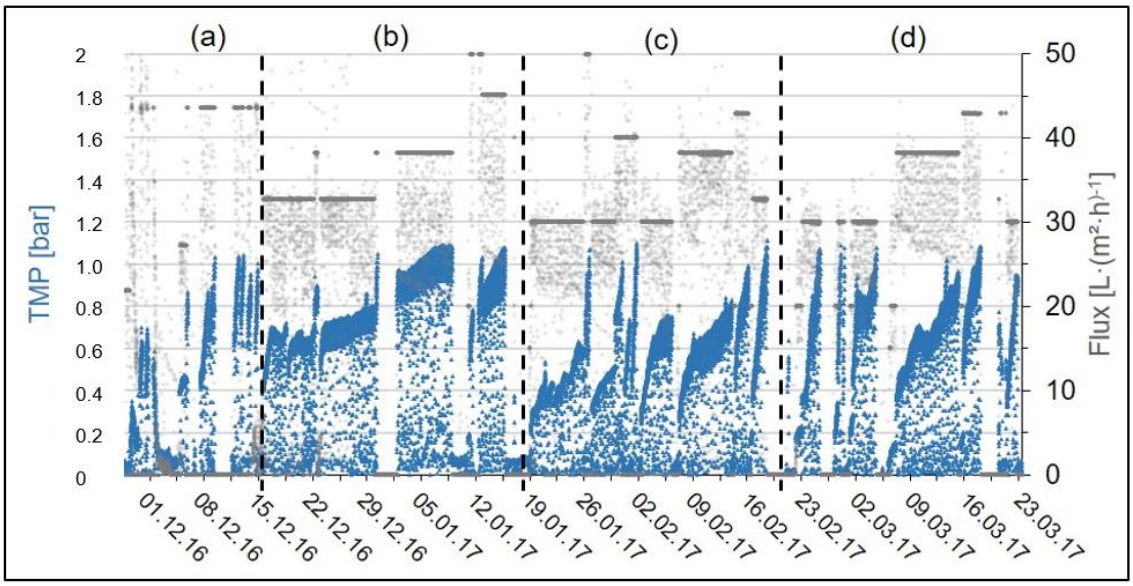

Figure 8-7: Overview of the UF operation of the Microdyn-Nadir company. The overview is separated in four testing periods using different WWTP effluents as feeds. (a) UF operation without in-line coagulation dosing and fluxes between 22 and 44 LMH. (b) UF operation with coagulation dosing and fluxes between 33 and 45 LMH using secondary effluent as feed. (c) UF operation with coagulation dosing and fluxes between 30 and 43 LMH using tertiary effluent as feed. (d) UF operation with coagulation dosing and fluxes between 30 and 43 LMH using activated carbon treated secondary effluent as feed (Egner, 2017).

The UF pilot plant of the Microdyn-Nadir company was operated with sustainable fluxes of 20-30 LMH without dosing a coagulant. The implementation of coagulation dosing enabled to increase the flux to 38 LMH. However, no sustainable flux operation could be established with fluxes higher than 30 LMH and a constant coagulant dosing between 4.5 and 9 mg/L PAC. The regular CEB was not effective due to a not significant measured TMP decrease. Therefore, a CIP was conducted in regular intervals. To summarize, the operation of the Microdyn-Nadir UF module revealed a low flux of about 38 LMH on the basis of a constant coagulation dosage of 9 mg/L PAC while using tertiary effluent as feed.

8.4.1.2 Evaluation of the filtrate quality

In addition to the removal of TOrCs and reducing antibiotic resistance in the wastewater, the aim of the WWTP Steinhäule is to reduce the parameters of the German discharge requirements as specified in the 'Abwasserabgabengesetz' with the parameter COD, N_{tot} and P_{tot}. These parameters were measured in 2017 during the pilot-scale tests using online measuring devices provided by the WWTP Steinhäule. The parameters P_{tot} and COD, measured as TOC value, were analyzed both in the feed and in the corresponding filtrate. The P_{tot} and TOC concentrations are illustrated in Figure 8-8.

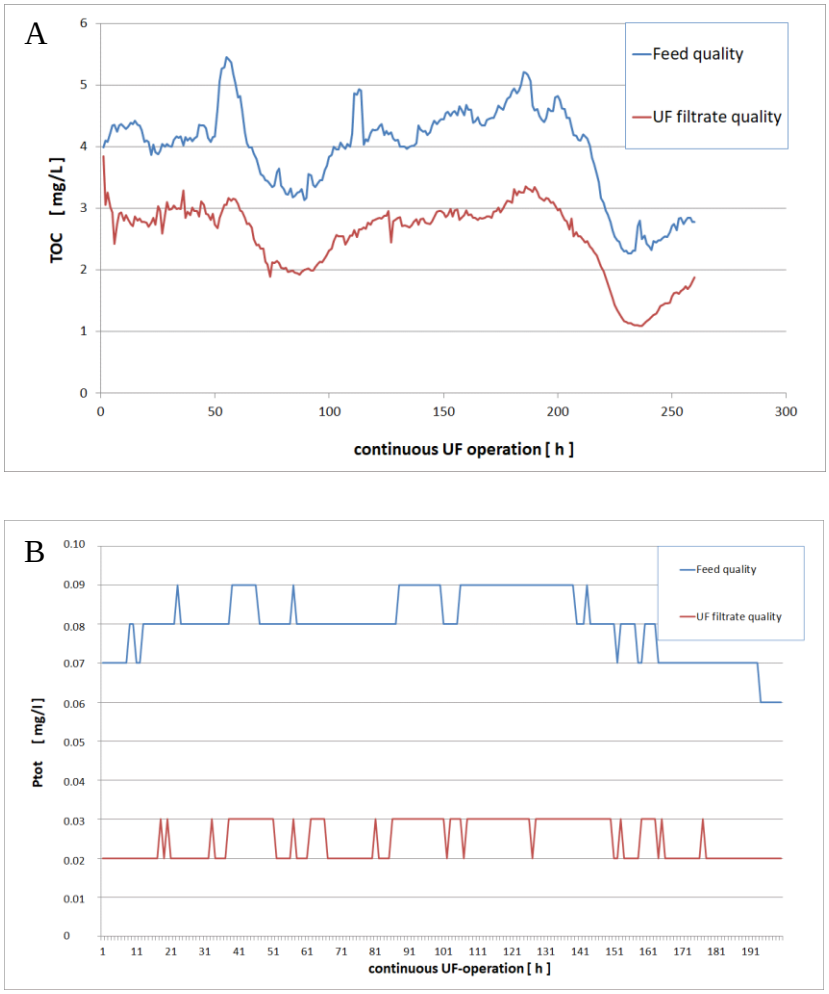

Figure 8-8: TOC (A) and Ptot (B) measurements by online measurement devices in the feed and the corresponding UF filtrate.

The phosphorus content in the wastewater consists of orthophosphates, which are chemically converted into the undissolved phase by precipitants and can thus be removed from wastewater. The other part of the phosphorus content in the wastewater is in the form of organic polyphosphates and cannot be precipitated. UF can reduce the phosphorus content by reducing the polyphosphates contained in the filterable substances and the precipitant dosage on the feed side (Miehe et al., 2013). The results of the online measuring devices revealed the additional cleaning performance of the UF technology. UF of tertiary effluent enabled to reduce the P_{tot} by approximately 43% and the TOC by

approximately 35%. The total nitrogen N_{tot} could not be reduced by UF. With these removal efficiencies, the effluent values TOC and P_{tot} can be reduced by 20 percent and the regulatory discharge fees can be offset with the invest costs of the full-scale membrane filtration plant for six years.

The ARG and the total biomass abundances in feed and corresponding UF filtrate were analyzed and ARG and total biomass removal efficiencies of the membrane filtration pilot plant were quantified. The study results are presented in chapter 5. Biofilm analyses of feed and filtrate samples were also conducted within the UF pilot scale studies. The main protagonists of the relative abundance of bacteria species in the feed of the UF pilot plant were predominantly *Actinobacteriota, Chloroflexi, Cyanobacteria, Firmicutes,* and especially *Proteobacteria.* In contrast to the biofilm samples, the wastewater samples of secondary effluent and activated carbon treated secondary effluent exhibited different abundances of *Bacteroidota* and *Acidobacteriota*. The ultrafiltration of the feeds resulted in a significant bacteria shift to *Proteobacteria, Firmicutes* and *Bacteroidota* in filtrate and biofilm samples (Figure 10-17).

The breakthrough effect of bacteria through the membrane module was already reported by Maejima et al. (2018). Maejima et al. (2018) investigated in membrane filtration and reported that 141 filterable bacteria, predominantly the phyla *Proteobacteria, Bacteriodetes, Firmicutes* and *Actinobacteria* passed through the membrane with 0.22 µm of pore size.

8.4.2 Considerations of implementing UF full-scale plant at the WWTP Steinhäule

8.4.2.1 Investigation of design values and mechanical approach

Design values for Feed pumps and Backwash pumps

The pilot-scale studies revealed that the UF could be run under sustainable flux and economic conditions. These operation parameters of the UF Inge pilot plant are the basis for the design values of a full-scale UF plant at the WWTP Steinhäule (Table 8-1). The full-scale UF plant is proposed for the implementation at the effluent of the sand filtration process.

Table 8-1: Design values for the full-scale UF plant at WWTP Steinhäule.

Parameter	Q_{TW}	Q_{MW}	Q_{MAX}
Q_{TW} [m³/h]	5000		
Q_{MW} [m³/h]		9400	
Q_{MAX} [m³/h]			13000
Q_{BW} Filter [m³/h]	205	410	533
Flux [LMH]	70 LMH	70 LMH	70 LMH
surface area membranes [m²]	74357	140143	193329
amount of modules à 80 m²	929	1752	2417
Flux Backwash [LMH]	230 LMH	230 LMH	230 LMH
Q_{BW} UF [m³/h]	285	537	741
Total sum Q_{TW} [m³/h]	5490		
Total sum Q_{MW} [m³/h]		10347	
Total sum Q_{MAX} [m³/h]			14274
Feed Pumps			
Pump 1 for Q_{TW} [m ³/h]	5500		
Pump 2 for Q_{MW} [m³/h]		5500	
Pump 3 for Q_{MAX} [m³/h]			5500
Pump 4 as Redundance [m³/h]			5500
delivery head of the pumps	20	20	20
(calculation Table SI1)			
Backwash Pumps for 80 modules	BW	CEB	
Pump 1 [m³/h]	2208	662,4	
Pump 2 [m³/h]	2208	662,4	
Pump 3 as Redundance [m³/h]	2208	662,4	
delivery head of the pumps	21		
(calculation Table SI2)			

The full-scale UF plant will be designed for dry weather (Q_{TW}) for 5,490 m³/h, for rainy weather (Q_{MW}) for 10,347 m³/h, and for a future final expansion of the WWTP Steinhäule (Q_{MAX}) for 14,274 m³/h (Table 8-1). Internal wastewater streams like the backwash of the sand filtration process and the backwash of the UF process was included of the aforementioned wastewater influents of the full-scale UF plant. Four feed pumps

were calculated whereas every pump has a feed rate of 5,500 m³/h and a delivery head of 20 m. The backwash pumps were designed for a backwash flux of 230 LMH. The full-scale UF plant was designed concerning the system T-Rack 3.0 with a 4-row configuration on both sides of the manifold unit which is illustrated in Figure 8-9.

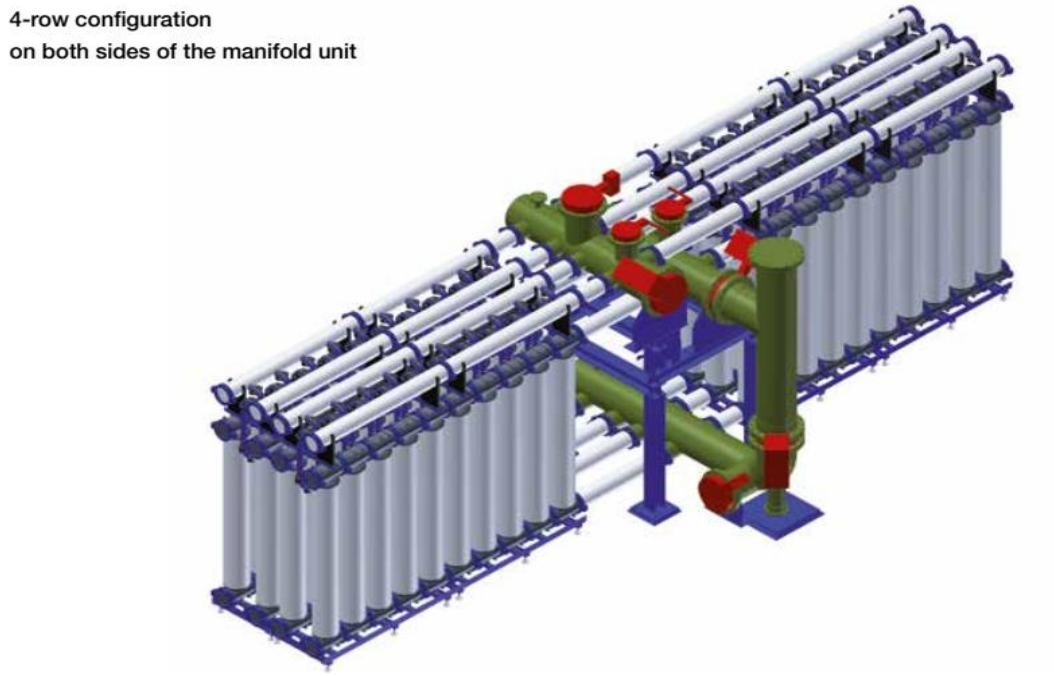

Figure 8-9: A 4-row configuration system T-Rack 3.0 of the Inge company (Inge, 2017)

If backwash mode is active, the whole T-Rack 3.0 system (Figure 8-9) is in backwash mode. Therefore, every backwash pump was calculated according to this T-Rack 3.0 system (120 membrane modules à 80 m²) with a delivery flow of 2,208 m³/h and a delivery head of 21 m. For backwash mode, 3 backwash pumps were provided for backwash mode. The calculation of the feed pumps and the backwash pumps are presented in the Table 10-17 and Table 10-18.

The standard sustainable flux is 70 LMH. However, the inge pilot plant was also operated at fluxes of 80 and 90 LMH under reversible fouling conditions (Figure 8-10).

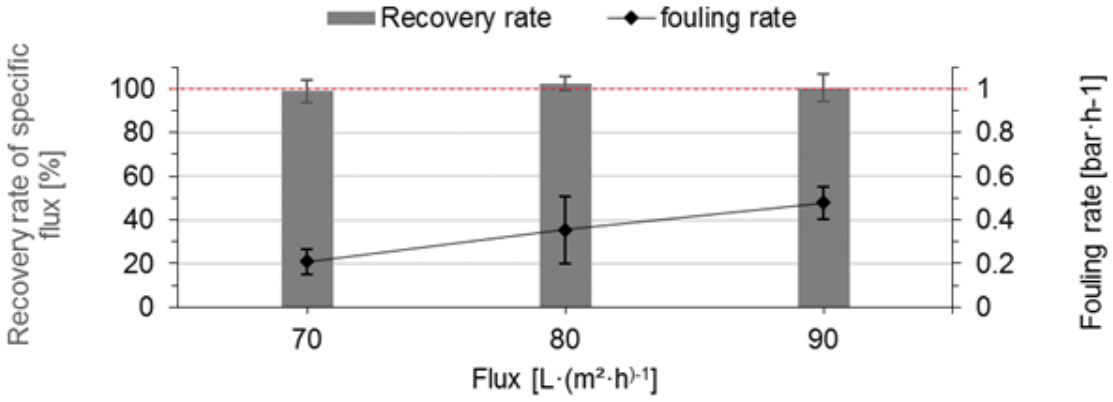

Figure 8-10: Recovery rate after CEB and fouling rate for fluxes 70, 80, 90 LMH using tertiary effluent. The error bars describe the standard deviation between 9 trials. (Egner, 2017)

In Figure 8-10, the recovery rate and the fouling rate are illustrated according to specific fluxes. The recovery rate of a specific flux after CEB mode exhibits whether the fouling is chemically irreversible (Rsp,mean < 100%) or not. The fouling rate reveals the TMP increase within one hour of standard filtration mode. It was observed that the recovery rates at fluxes of 70, 80, and 90 LMH resulted in predominantly no chemically irreversible fouling. However, operating at a flux of 90 LMH showed 4 of 9 chemical irreversible foulings so that it can be assumed that a flux of 90 LMH seemed to be the critical flux. Therefore, it can be concluded that the fluxes between 70 and 90 LMH can be provided as 'flux reserves' for emergency operation conditions, for example, when several membrane modules are defective, feed valves or filtrate valves are defective, etc.

Design values for the coagulation pumps

The pilot-scale studies revealed a coagulation demand of 2 mg Al/L (PAC). The applied coagulation of PAC had an active substance of at least 9 % and density of 1.37 g/cm³. With a wastewater average amount a day of about 100,000 m³ there is an active substance amount of 200 kg Al/d. Using an active substance of at least 8 %, it can be concluded that the daily coagulation load is 2,222 kg/d (without coagulation coating). If we consider the density of 1.37 g/cm³, the coagulation demand is 1.62 m³/d. Therefore, a coagulation amount of 67.6 L/h is necessary. Hence, every coagulation pump needs a delivery flow of about 70 L/h. The coagulation dosing takes place depending on the wastewater amount. 2 tanks for the storage of coagulation for each 30 m³ were selected so that a tank filling is always possible when 1 tank is full and the other is empty. The calculation of the three coagulation pumps is listed in the Table 10-19.

Design values for the sodium hydroxide (NaOH) pumps

The CEBs during the pilot-scale studies were executed with a sodium hydroxide demand of 150 ppm. The applied NaOH had an active substance of at least 50 % and density of 2.13 g/cm³. With a minimum delivery flow of the backwash pump of about 662.40 m³/h there is an active substance amount of 99.36 kg kg NaOH/h. Using an active substance of at least 50 %, it can be concluded that the hourly NaOH load is 198.72 kg NaOH/h. If we consider the density of 2.13 g/cm³, the NaOH delivery flow is 93.30 L/h. Hence, every NaOH pump needs a delivery flow up to 100 L/h. The NaOH dosing takes place once a day of every t-Rack 3.0 system. 2 tanks for the storage of sodium hydroxide for each 30 m³ were selected so that a tank filling is always possible when 1 tank is full and the other is empty. The calculation of the three NaOH pumps is presented in the Table 10-20.

Design values for the sulfuric acid (H2SO4) pumps

The CEBs during the pilot-scale studies were also conducted with a sulfuric acid demand of 150 ppm. The applied H_2SO_4 had an active substance of at least 30 % and density of 1.83 g/cm³. With a minimum delivery flow of the backwash pump of about

662.4 m³/h, there is an active substance amount of 99.36 kg kg H_2SO_4/h. Using an active substance of at least 30 %, it can be concluded that the hourly H_2SO_4 load is 331.20 kg H_2SO_4/h. If we consider the density of 1.83 g/cm³, the H_2SO_4 delivery flow is 180.98 L/h. Hence, every H_2SO_4 pump needs a delivery flow up to 200 L/h. The H_2SO_4 dosing takes place once a day of every t-Rack 3.0 system. 2 tanks for the storage of sulfuric acid for each 30 m³ were selected so that a tank filling is always possible when 1 tank is full and the other is empty. The calculation of the three H_2SO_4 pumps is presented in the Table 10-21.

To summarize, the layout of the full-scale UF plant is illustrated in Figure 8-11. Another overview of the WWTP Steinhäule and the implementation of the full-scale UF plant within the wastewater treatment of the WWTP Steinhäule is presented in Figure 10-16. The hydraulic calculation of the pipes between sand filtration effluent and ultrafiltration influent as well as ultrafiltration effluent and discharge in the danube is listed in the Table 10-22 and Table 10-23.

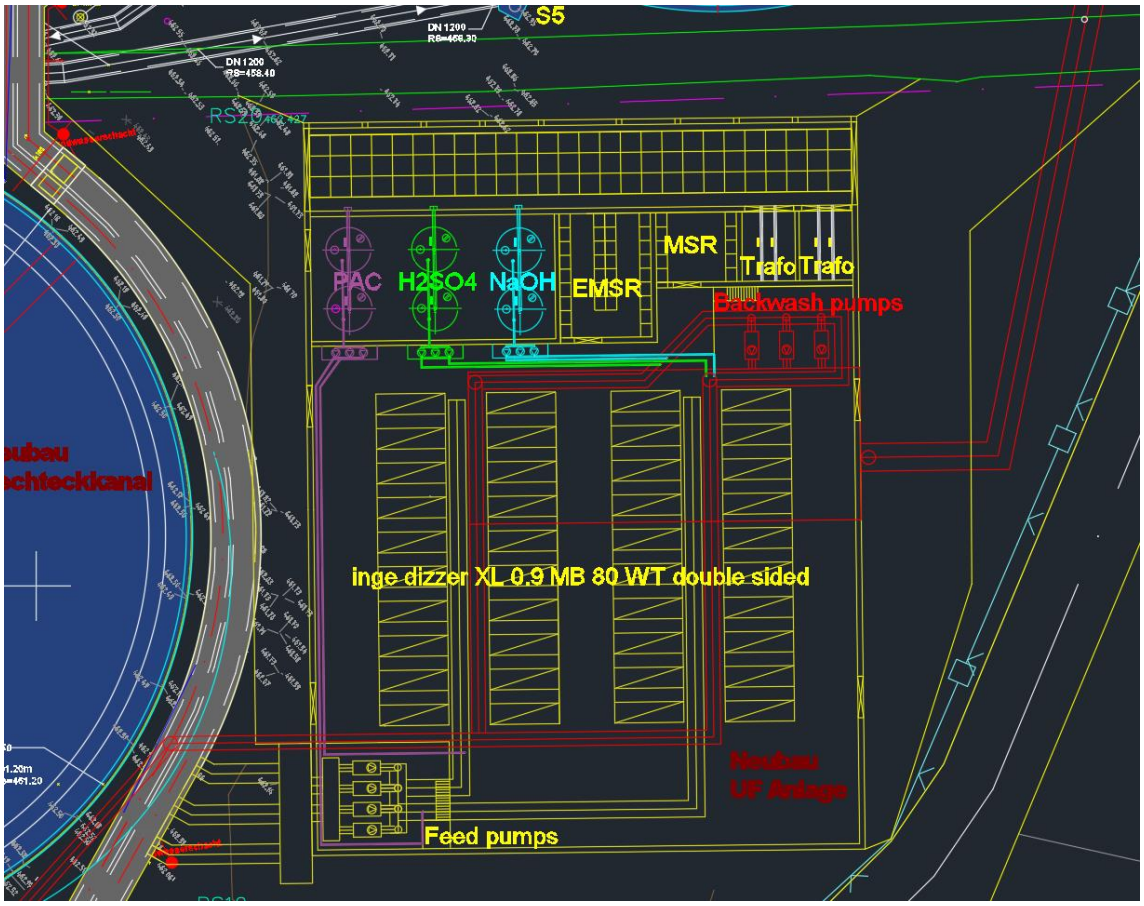

Figure 8-11: Layout of the full-scale UF plant at the WWPT Steinhäule

The feed water flows via three new DN 1200 pipes from the existing sewer effluent from the sand filtration to the feed shaft of the full-scale UF plant. 4 feed pumps enables to move up to 4 x 5,500 m³/h via 4 DN 1200 feed pipes (yellow) to the UF membrane module at the T-Rack system. The filtrate flows via the 2 x DN 1000 filtrate pipes (red)

to the backwash tank in continuous flow mode (red). The effluent of the backwash tank in continuous flow mode is discharged via a DN 2000 sewer to the danube. The backwash pumps use the filtrate from the backwash tank in continuous flow mode for backwash mode. The backwash water of the backwash mode flows via 2 x DN 1000 pipes to the membrane modules and via another 2 x DN 1000 pipes as internal wastewater to the influent sewers of the activated carbon stage. During standard filtration mode, a continuous coagulation dosing with 2 mg/L is given to the feed side in the feed pipes of the membrane modules. The daily CEB can be conducted with sodium hydroxide pumps and sulfuric acid pumps. The receiving office for chemicals is at the nord side of the UF plant. Here, the chemical transport can be unloaded and the PAC, H_2SO_4, and NaOH tanks can be filled.

8.4.2.2 Calculation of the annual operation costs and the total invest costs

The annual operation costs and the total invest costs are calculated on the basis of reference costs of similar building and machine costs that were bought or built at WWTP Steinhäule in 2022. The operation costs of a full-scale UF plant at WWTP Steinhäule based on the design values of chapter 8.4.2.1 are illustrated in Table 8-2.

The annual operation costs of the full-scale UF plant consists of the depreciation and interest for the machine technique (5 % interest and 10 years depreciation) and for the building (5 % interest and 40 years depreciation). In addition, the maintenance costs for the machine technique and the building are listed. Furthermore, costs for maintenance (building and machine technique) and for (electrical) energy predominantly for the feed and backwash pumps as well as costs for the coagulation, sodium hydroxide, sulfuric acid consumptions and the annual costs for the membrane module exchange every 10 years are presented in Table 8-2. The sum of the annual operation costs is 9,672,084.11 €/a. The inhabitants of the connected cities and villages and the connected industries of the WWTP Steinhäule have to pay the wastewater treatment via a wastewater service rate. The rate is based on the individual consumption of drinking water (annual drinking water consumption 17,000,000 m³). Therefore, the specific treatment costs for the full-scale UF plant at WWTP Steinhäule with respect to the consumption of one cubic meter of drinking water are 0.57 €/ m³. With the assumption of 40 m³ drinking water consumption per inhabitant and year, an inhabitant would have to pay additional annual costs for the implementation of the UF plant at WWTP Steinhäule of 22.76 €.

Table 8-2: Annual operation costs of the full-scale UF plant at WWTP Steinhäule.

Parameter	amount	unit	unit price	unit	full price	unit	comments/assumtions:
depreciation and interest machine technique	30437820	Euro	0.13		3941837	Euro	= 0.05 (1+0.05)^10/((1+0.05)^10-1) = 0.13
							interest with 5 % for 10 years
depreciation and interest building	39077982	Euro	0.06		2277393	Euro	= 0.05 (1+0.05)^40/((1+0.05)^40-1) = 0.06
							interest with 5 % for 40 years
maintenance costs machine technique	22098300	Euro	0.003		66295	Euro	0.3 % of invest costs per year
maintenance costs building	39077982	Euro	0.001		39078	Euro	0.1 % of invest costs per year
personnel costs	3	per.	60000	€/per.	180000	Euro	
energy costs feed pumps	332	kW	4380	€/kW	1453333	Euro	Q = 40,000,000 m³/a; 1 kWh = 0,50 €
energy costs backwash pumps	152	kW	1460	€/kW	222541	Euro	20 min operation every hour
coagulation costs	889	t	300	€/t	266667	Euro	Q = 40,000,000 m³/a; 1 t = 300 €, 2 g PAC/m³
NaOH, 50%	45260	l/a	5	€/t	226300	Euro	4 min dosage / T-Rack system x d; 1 L = 5 €
H2SO4, 30 %	87600	l/a	5	€/t	438000	Euro	4 min dosage / T-Rack system x d; 1 L = 5 €
Membrane module exchange	140160	m²	4	€/m²xa	560640	Euro	membrane module exchanging every 10 a
							1752 pieces, 40 €/m² surface area
annual operation costs					**9672084**	Euro	
operation costs per m³ drinking water					0.57	€/m³	17000000 m³ drinking water per year
operation costs per m³ wastewater					0.24	€/m³	40000000 m³ wastewater per year
additional costs for the UF plant at WWTP Steinhäule per inhabitant and year					22.76	€/Ixa	40 m³drinking water/ inhabitant and year

The total invest costs are presented in Table 8-3. The invest costs consider the costs for the building (base plate with pile foundation and the steel hall), the machine technique with membrane modules, feed pumps, backwash pumps, feed and filtrate pipes, valves, MIDs and further installations, feed and filtrate sewer, the coagulation machine technique, sodium hydroxide machine technique, sulfuric acid machine technique and EMSR technique. The invest costs are 52,641,316 €. Additional engineering costs are 10,528,263 €. Further costs are construction period interest and construction manager payment. All in all, the sum of the total invest costs of a full-scale UF plant at WWTP Steinhäule for the wastewater treatment of the recent rainy weather conditions (Q_{MW} = 10,347 m³/h) would be 111,617,284.48 €.

Table 8-3: Total invest costs of the full-scale UF plant at WWTP Steinhäule.

Parameter	amount	unit	unit price	unit	full price	unit	comments/assumtions:
building							
base plate with pile foundation	2020	m²	3000	€/m²	6060000	€	
steel hall	2000	m²	7000	€/m²	14000000	€	
machine technique							
membrane modules	140160	m²	40	€/m²	5606400	€	1752 pieces, 40 €/m² surface area
feed pumps	4	pc	100000	€/pc	400000	€	
backwash pumps	3	pc	75000	€/pc	225000	€	
feed pipes	90	m	3000	€/m	270000	€	
filtrate pipes	380	m	2500	€/m	950000	€	
valves and other installations	1	bl	10000000	€/bl	10000000	€	
feed and filtrate sewer	105	m	5000	€/m	525000	€	
coagulation machine technique	1	bl	400000	€/bl	400000	€	
NaOH machine technique	1	bl	400000	€/bl	400000	€	
H2SO4 machine technique	1	bl	400000	€/bl	400000	€	
EMSR technique	1	bl	5000000	€/bl	5000000	€	
invest costs net					44236400.00	€	
invest costs gross					52641316.00	€	
engineering costs (20 %)					10528263.20	€	
construction period interest (5 %)					2632065.80	€	
construction manager payment (3 %)					1579239.48	€	
sum total invest costs					**111617284.48**	€	

8.5 Conclusion

To prevent the spread of antibiotic resistance by implementing a full-scale UF plant was assessed for the WWTP Steinhäule. The most important design values are the sustainable flux at 70 LMH and a coagulation of 2 mg PAC/L. The annual operation costs for the full-scale UF plant at WWTP Steinhäule to treat about 40,000,000 m³/a by UF are 9,672,084 €/a. The invest costs for the full-scale UF plant at WWTP Steinhäule are 111,617,284 €. However, further investigations are necessary. The full-scale UF plant was calculated with respect to the assumption that no additional treatment of the

backwash wastewater is necessary. The backwash wastewater was planned to discharge into the influent sewers of the activated carbon stage. It is assumed that the activated carbon stage and the sand filtration process can adsorb the additional input of bacteria and ARGs so that no higher ARG and bacteria abundance in the effluent of the sand filtration process result in higher ARG and bacteria abundance in the corresponding UF filtrate. Other possibility is to give the backwash water to the biological stage whereas an additional adsorption onto sludge flocs could take place so that no ARG and bacteria increase at the secondary effluent could occur. These backwash effects require further investigations about a possible increase of bacteria and ARG due to backwash water repatriations.

8.6 Acknowledgements

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9 OVERALL DISCUSSION AND FUTURE RESEARCH NEEDS

Since several studies of AMR removal efficiencies of WWTPs with mechanical and biological treatment showing inefficient AMR retention (Alexander et al. 2015; Hembach et al. 2017), membrane filtration has been proposed as an advanced wastewater treatment technology that is able to physically remove particles like bacteria and bacteriophages. AMR removal studies of other advanced treatment processes (e.g., ozonation, UV-irradiation, chlorination) exhibited significant lower ARG removal efficiencies than low-pressure membrane filtration (Hiller et al., 2019). Membrane filtration is applied in Germany predominantly as MBR technology for industrial and municipal wastewater treatment, whereas research studies demonstrated high removal efficiencies of bacteria and ARGs (Madaeni, 1999; Munir et al., 2011a). However, MBR technology applied as advanced treatment technology is characterized by a high dry matter content, lower flux (23-31 LMH), higher energy costs due to cross flow operation, and low backwash and CEB intervals compared to capillary membrane operation (Werner et al., 2019). Therefore, capillary membrane technology was chosen for AMR studies at WWTP effluents to investigate their wastewater treatment efficiency. In this context, not only the potential of removing relevant ARB and ARGs should be accounted for but also operational stability under economic conditions. This dissertation elaborated the following aspects:

Research objective #1 was addressed by performing a literature study to reveal the actual state-of-the-art of occurrence, spread and preventive measures against antibiotic resistance in the aquatic environment with the focus on WWTP effluents. Within **chapter 4,** actual AMR research studies are critically discussed to define knowledge gaps for further research objectives. It was found that WWTP discharges result in an increase of AMR abundance in downstream surface waters compared to upstream analyses. A standardized AMR evaluation concepts are needed evaluate not only background antibiotic resistance and anthropogenic antibiotic resistance, but also AMR removal by conventional WWTPs and advanced treatment processes.

The most promising membrane filtration technology was examined in depth for AMR removal during this dissertation. Since membrane filtration is expected to be the main barrier for bacteria and viruses, factors that influence AMR removal efficiency were investigated in **research objective #2** (**chapter 5 and 6)** and **research objectives #3 and #4** (**chapter 7**). The most important findings of UF studies conducted during this dissertation were:

- The main factor for AMR removal is the pore size of the membrane.
- The increase of the fouling layer with progressive filtration time had only negligible effects on the removal of typical intrachromosomal ARGs (*sul1, ermB* gene) and a high removal effect on extrachromosomal ARGs (e.g., *vanA* gene).
- Higher ARG abundance in the feed resulted in higher ARG abundance in UF filtrate.
- Intact bacteria can breakthrough by UF membranes.
- Backwash and CEB mode can result in significantly increased *16S rRNA* and *sul1* genes abundances in the first and second minute of standard filtration mode. Higher *16S rRNA* gene and *sul1* gene abundances after backwash and CEB mode during the first and second minute of standard filtration mode are not the result of a reduced fouling layer but the result of a low backwash water quality from the backwash tank.
- Higher 16S rRNA gene abundance after CEB mode is the result of a low disinfection effect of the applied CEB chemical.
- AMR associated regrowth in UF filtrate can occur in the filtrate tank both in bypass mode and in continuous flow mode using secondary effluent as feed. This means that temporarily stagnant filtrate water and a hydraulic retention time of 3 hours of ultrafiltered secondary effluent promotes ARG associated regrowth.
- Typical erythromycin and sulfamethoxazole concentrations still present in secondary effluent will not result in bacterial adaption of ARB at the UF filtrate side.
- Pretreatment of secondary effluent as feed by activated carbon and sand filtration prevents ARG associated regrowth in UF filtrate.
- Continuously dosing 0.5 mg/L sodium hypochlorite prevents ARG associated regrowth in ultrafiltered secondary effluent.

Finally, a full-scale UF plant for the WWTP Steinhäule is planned based on the pilot-scale study results. For this, the focus was achieving maximum sustainable flux, minimum coagulation demand, and operational stability measured as TMP built-up (**research objective #5** in **chapter 8**). It was found that the inge membrane modules were able to operate at a sustainable flux of 70 LMH and a coagulation demand of 2 mg PAC/L using tertiary effluent as feed.

The next sections discuss the study results that were presented in chapters 4 to 8. For that the methodological approaches that were used in the experiments are critical discussed, alternatives or modifications are suggested, the relevance and portability of the studies to other advanced wastewater treatment applications are proposed and future research needs are presented. Finally, further research needs and requirements for the implementation of advanced treatment processes in WWTP effluents for AMR removal as well as a potential risk assessment of AMR in the environment for human health is being discussed.

9.1 Standardized Evaluation Concept for AMR analyses

The aim of applying standardized analytical techniques to assess antibiotic resistance with defined parameters should enable the comparison of AMR occurrence of background antibiotic resistance and anthropogenic antibiotic resistance. In addition, AMR removal efficiencies of different treatment processes can be verified and compared to other AMR studies. Cultivation-based methods detect living organisms with antibiotic resistance. In contrast, qPCR provide a rapid detection method for antibiotic resistance genes reporting thus an "antibiotic resistance potential", but are lacking information on the acute risk, as results are not linked to pathogens. In order to provide reliable information on treatment efficiency for different processes from qPCR analysis, underlying mechanisms for inactivation need to be well understood, considering also the gene intactness to mitigate overestimations of ARG abundances. New qPCR technologies, such as digital PCR, may furthermore help to establish new standards based on absolute gene occurrences. Evaluation of removal efficiency using qPCR requires a solid understanding of processes since different investigated gene lengths of the same ARG may render the comparison of studies difficult without further validation.

It is suggested that monitoring of fate and transport of AMR should focus on different antibiotic classes. Resistance for broad spectrum antibiotics, with occurrence in aquatic systems can be used as indicators to evaluate removal efficiencies in different processes, whereas AMR to antibiotics of last resort is more relevant to assess the associated health risks. Thus, the following indicator ARGs were recommended to monitor for AMR: resistance genes of broad-spectrum antibiotics *sul1*, *sul2* and *tet* genes (*tetA*, *tetB*, *tetO* and *tetW*) as well as resistance genes of antibiotics of last resort *vanA* and *blaVIM*. For ARB, the following suitable indicators are proposed: Fecal coliforms, *P. aeruginosa*, *Enterococci*, and *Enterobacteria* using cultivation-based methods (Hiller et al., 2019).

In comparison to the proposed standardized evaluation concept of Hiller et al. (2019), the evaluation concept of Ternes et al. (2016) focused on two main criteria that were analyzed only via DNA-based qPCR: the removal and increase of pathogens and the analyses of antibiotic resistance genes with clinical relevance. Clinically relevant antibiotic resistance genes are *vanA* gene, *blaVIM* gene, *ampC* gene, and *ermB* gene. Indicator ARGs *vanA* gene and *blaVIM* gene are also considered in the standardized evaluation concept of Hiller et al. (2019). ARGs based on broad-spectrum antibiotic class are missing in the standardized evaluation concept of Ternes et al. (2016). It is assumed that this evaluation concept only focuses on ARGs based on antibiotics of last resort. However, the ARGs *vanA* gene and *blaVIM* gene are detected in the range of about 3 log units per 100 mL in WWTP effluents (Alexander et al., 2015). With respect to a detection limit of 2 log units, AMR removal efficiencies of advanced treatment processes can only be quantified up to 1 log unit. In contrast, ARGs based on broad-spectrum antibiotics (e.g., *sul1* gene or *tet* gene) are occurring at higher concentrations in WWTP effluents (Hiller et al., 2022; Böckelmann et al., 2009). ARGs analyses based on broad-spectrum

antibiotics enables to quantify ARG removal efficiencies of advanced treatment processes by more than 3 log units.

The pathogenic bacteria of the evaluation concept of Ternes et al. (2016) are suggested to be analyzed by taxonomic gene markers considering *Enterococci*, *P. aeruginosa*, *Staphylococci* and *Enterobacteria*. In this case, the pathogens *P. aeruginosa*, *Enterococci* and *Enterobacteria* are part of both AMR evaluation concepts. However, the taxonomic gene marker quantifies individual bacterial gene sequences. Lacking information is given on the acute risk, since these gene sequences must not be linked to intact pathogens.

Sample preparation is another important issue to discuss. For the separation of ARB and ARGs from water samples, membrane filtration with pore sizes of 0.2 and 0.45 µm is commonly applied (EPA, 2002), followed by cultivation- or molecular-based methods (Alexander et al., 2015; Jäger et al., 2018; Hembach et al., 2019). However, Hiller et al. (2022) revealed that microfiltration can retain total bacteria by about 2.6 log units and extracellular ARGs (e.g., *vanA* gene) by only 1 log unit from secondary effluent (Hiller et al., 2022). Bacteria breakthrough can occur using ultrafiltration membranes (Hiller et al., 2022). Other research studies are in line with the assumption that microfiltration is insufficient in free DNA or extracellular ARG removal (Slipko et al., 2019; Wang et al., 2019). In order to gain DNA of wastewater samples for qPCR analyses, the microfiltration technique and the freeze-drying technique are presented in Figure 9-1. The two DNA accumulation techniques were executed with UF filtrate samples.

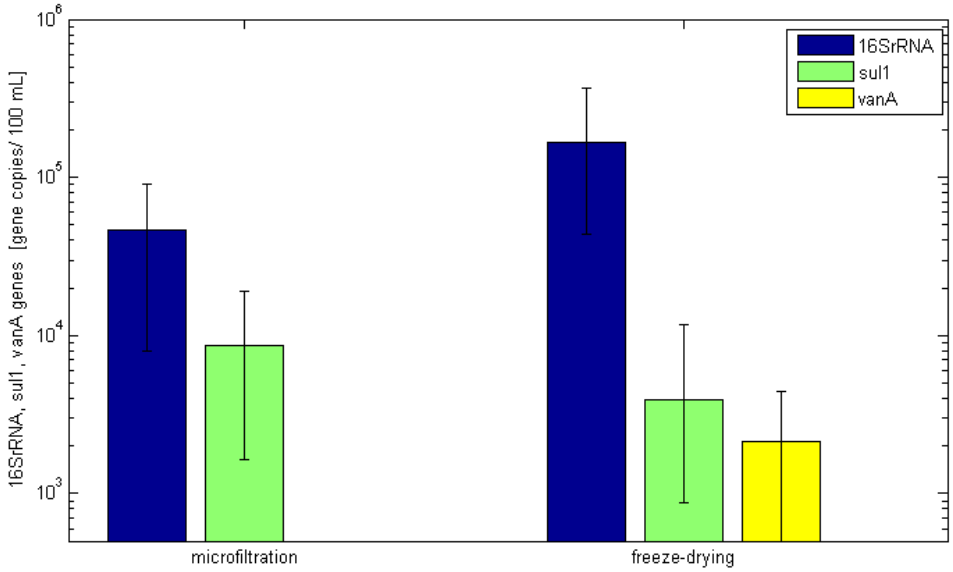


Figure 9-1: Comparison of *16S rRNA*, *sul1* and *vanA* genes analyses of ultrafiltered secondary effluent samples using microfiltration technique (n = 12 samples) and freeze-drying technique (n = 10 samples) to accumulate DNA for qPCR analyses. Error bars indicate the 95 % confidence interval.

While the *sul1* gene analyses revealed no significant difference (*sul1* gene: t-test, dF = 20; p = 0.051) between the microfiltration and freeze-drying technique, *16S rRNA* gene and *vanA* gene exhibited significant difference based on an independent samples t-test (*16S rRNA* gene: t-test, dF = 10; p = 0.010; *vanA* gene: t-test, dF = 9; p = 0.011). While *vanA* gene was measured below the limit of detection (< 1,000 gene copies/ 100 mL) in all 12 microfiltered samples, *vanA* gene was detected in 8 of 10 freeze-drying samples above the limit of detection. These study results confirm the hypotheses of paper #2 (Chapter 5) that while *sul1* gene seems to be predominantly part of intrachromosomal DNA, the *vanA* gene occurs predominantly as extrachromosomal ARG in wastewater samples. Furthermore, the significant higher bacteria concentrations in freeze-drying samples compared to microfiltration samples is the result of loosing bacteria by breaching the microfiltration membrane. That is the reason why freeze-drying is recommended for DNA accumulation for AMR analyses in the recommended standardized evaluation concept.

Before (waste-) water sampling, the standardized evaluation concept should also define key operational and site-specific parameters that were often lacking in published studies. Wastewater samples should be taken only at dry weather due to the fact that wet weather conditions cause dilutions in wastewater samples and impact ARG concentrations. Even the dry weather conditions of WWTPs should be explained to what extent WWTPs are designed. For example, dry weather sampling in the WWTP Steinhäule can vary between 400 and 1.400 l/s. Therefore, ARG concentrations can vary

by more than 3 times due to the factor wastewater flow. In addition, wet weather flow is approximately double the dry weather flow. Furthermore, the hydraulic retention time of wastewater processes should be considered during a sampling campaign. For example, the hydraulic retention time between the influent and the effluent of the wastewater treatment of the WWPT Steinhäule has an arithmetic value of about 12 hours. Not considering the hydraulic retention time would adulterate the AMR removal efficiencies. If advanced treatment processes are analyzed, factors for AMR associated regrowth effects should also be considered (e.g., photo-reactivation effect of the UV-irradiation process). Other factors that could inhibit the advanced treatment process should also be presented in the AMR studies (e.g., nitrite concentration using ozonation). If membrane filtration is applied for AMR studies sampling should be carried out at standard filtration mode (defined as constant steady flux and constant filtrate quality). Fouling layer examinations for AMR analyses has to be executed at constant TMP increase. Membrane integrity must be proved before and after AMR examinations. Treatment variability of parallel trains should be checked before AMR studies.

When AMR analyses are executed in surface waters, the water flow conditions of surface water should be listed in the study (e.g., low water flow or mean-flow conditions, AMR analyses not under high water conditions, information of wastewater amount). Furthermore, antrophogenic discharges (e.g., WWTPs or combined sewer overflows) should be presented potentially affecting the sampling sites.

To summarize, while the standardized evaluation concept of Ternes et al. (2016) considered only ARGs based on antibiotics of last resort and all ARB and ARGs analyses are analyzed based on qPCR technique, the standardized evaluation concept of Hiller et al. (2019) focuses on ARB analyzed by cultivation-based method and ARGs based on broad-spectrum antibiotics and antibiotics of last resort. ARB analyses based on the cultivation-based method can be applied for risk assessment. ARG analyses based on broad-spectrum and of last resort are both important for the evaluation concept to quantify AMR removal efficiencies and to make a statement about the spread of ARGs of clinical relevance. Furthermore, the more precise freeze-drying method is recommended for AMR analyses to accumulate DNA compared to the microfiltration method. The freeze-drying method enables to accumulate intra- and extracellular DNA so that both intra- and extrachromosomal ARGs can be analyzed. In addition, previous listed key operational and site-specific parameters for AMR analyses should be considered in a future standardized evaluation concept.

9.2 Relevant factors for AMR removal efficiency of the membrane filtration process

The membrane filtration was applied in Germany predominantly for water treatment, industrial and municipal wastewater treatment, whereas research studies of water and wastewater treatment demonstrate high removal efficiencies of bacteria and ARB (Madaeni, 1999; Munir et al., 2011a). However, low-pressure membrane filtration has not been studied in depth so far to elucidate factors and mechanisms affecting the separation of intra- and extrachromosomal ARGs. To examine these factors and mechanisms, two studies were performed.

In the first study (Chapter 5), key factors were investigated within the standard filtration mode of the UF pilot plant whereas the removal efficiencies of ARGs and total bacteria were examined. Firstly, it was proposed that '*the increase of a fouling layer with progressive filtration time will lead to a higher AMR removal efficiency*' (**research hypothesis #2.1**). The second research hypothesis proposed: '*Higher AMR feed abundance results in significantly higher AMR abundance in UF filtrate*' (**research hypothesis #2.2**) and thirdly it was hypothesized that '*Comparing AMR removal efficiencies of membranes with large pore size differences (UF = 20 nm vs. MF = 450 nm) will result in significantly different AMR removal*' (**research hypothesis #2.3**). **Research hypotheses #2.2** and **#2.3** were **accepted.** We observed higher ARG abundance in the filtrate and higher ARG abundance in the feed using secondary effluent as feed. Lower ARG abundance in the filtrate was measured using tertiary effluent as feed with lower ARG abundance than secondary effluent. Furthermore, the main factor for AMR removal was the pore size of the membrane. Thus, AMR removal efficiency of MF membrane was significantly lower to UF membrane. **Research hypothesis #2.1** was only **partially confirmed**, since the increase of the fouling layer enables a significant removal of assumed extrachromosomal ARGs (e.g., *vanA* gene) whereas removal of predominantly assumed intrachromosomal ARGs (e.g., *ermB* and *sul1* genes) was negligible. It was also observed that intact bacteria breaking through the UF membrane. These penetrated bacteria exhibited high correlations to *sul1* genes.

The **core hypothesis I** *'ARGs removal efficiency of micro- and ultrafiltration of WWTP effluent is higher than 90 percent during standard filtration mode'* was only **partially confirmed**, since the individual ARG removal efficiency of micro- and ultrafiltration strongly depends on the relation of intra- and extrachromosomal ARG abundance of the feed wastewater. ARG with high amount of intrachromosomal ARG abundance in the feed wastewater will result in higher MF and UF removal efficiencies than 1 log unit. However, if feed wastewater would have predominantly

extrachromosomal ARG (e. g. *vanA* gene), the ARG removal efficiency of MF and UF could be lower than 1 log unit.

In Chapter 5, the research hypotheses #2.1 and #2.3 focused on the ARG removal efficiencies with respect to an increasing fouling layer and different pore sizes of the membranes. The study results revealed that the fouling layer and the pore size has significant different ARGs removal efficiencies depending on specific ARG. While no significant different *sul1* and *ermB* gene abundance was analyzed between the fifth and fiftyfifth minute of standard filtration mode, a significant different *vanA* gene abundance of about 1 log unit was detected between the fifth and fiftyfifth minute of standard filtration mode. Furthermore, the removal efficiencies of *sul1* gene and *ermB* gene were measured by 2.7 and 2.5 log units using a UF membrane with 20 nm of pore size. In contrast, *vanA* gene removal efficiency of the same UF membrane was low with 1.1 log units. Therefore, the question arises what is the reason for these significant different ARGs removal efficiencies. The reason for the low *vanA* gene removal efficiency of UF membranes was explained by the predominantly extrachromosomal occurrence of the *vanA* gene in the wastewater samples. Other research studies are in line with this study. For example, the first vanA-mediated vancomycin resistant *Staphylococcus aureus* was isolated in 2002. This isolate harbored a 57.9 kilobase conjugative plasmid Tn1546 with the *vanA* gene (Weigel et al., 2002). Flannagan et al. (2003) isolated two further conjugative plasmids namely pAM830 (45 kb) and pAM831 (95 kb) of vancomycin-resistant *enterococcus faecalis*. Concerning the occurrence of vancomycin resistant *enterococci* (VRE), Kühn et al. (2005) analyzed VRE in the environment in different European regions. In raw and treated urban sewage samples VRE was detected in 71 % and 36 %, respectively. The study of Liu et al. (2018) and Che et al. (2019) examined chromosomal and mobile ARGs in wastewater. The study results support the hypothesis that *vanA* gene is a representative of predominantly mobile DNA. Liu et al. (2018) studied intra- and extracellular ARGs and *16S rRNA* gene as surrogate for bacterial quantification. While *vanA* gene had the lowest correlation to *16S rRNA* gene, *sul1*, *sul2*, *tetM* and *ermB* genes had the highest correlations to *16S rRNA* gene. Che et al. (2019) investigated in a metagenomic sequencing study the occurrence of intra- and extrachromosomal ARGs in wastewater and reported that *vanA* gene had higher extrachromosomal abundances (sum of plasmid as well as integrative and conjugative elements) than intrachromosomal abundances compared to resistance genes *ermB* and *sul1*.

The extrachromosomal *vanA* gene on plasmids and its transfer and spread into the environment is discussed in this section. The plasmids with ARGs can be transferred to other bacteria by direct cell to cell contact (conjugation). In contrast, Keen et al. (2017)

reported that bacteriophages induce cell lysis and liberate intact plasmids into the environment (transformation). The study of Foladori et al. (2015) focused on cell analysis of viable, dead and lysed bacteria in wastewater treatment plants. Batch tests revealed in activated sludge about 12 log units of viable bacterial cells per g TSS. In contrast, about 11 log units of dead bacterial cells per g TSS were analyzed. Significant increase of dead cells in biological treatment was the result of bacteria exposition in anaerobic phases like secondary clarifier and compartments for biological phosphorous removal (Foladori et al., 2015). Cell lysis by bacteriophages and the natural lysis of bacterial cells result in liberated plasmids in wastewater.

The effect of mobile DNA with its representatives of various ARGs (e. g. *vanA* gene) breaking through the membrane in a higher degree than intracellular DNA is reported in several peer-reviewed published studies. Liu et al. (2020) investigated in an UF study intracellular and extracellular DNA transmissions through UF membrane. The UF membrane had a molecular weight cut off (MWCO) of 30 kDa. The hollow fiber UF membrane had intracellular DNA recovery efficiencies of about 96.5 %. For extracellular DNA analyses, DNA fragments with different length (10.0, 4.0, 1.0, and 0.5 kbp) were applied. DNA fragments with 10.0 and 4.0 kbp could be recovered by about 62.2-62.9 %, and 38.8-44.5 % recovery for 1.0 and 0.5 kbp. Not recovered extracellular DNA at the membrane surface breached the UF membrane. The study results demonstrate that UF membrane had a significant lower recovery efficiency of extracellular DNA than of intracellular DNA. The lab-scale study of Slipko et al. (2019) is in line with the study of Liu et al. (2020). Slipko et al. (2019) applied a pure supercoiled plasmid pNORM1 with a length of 3,342 bp and a radius of plasmid of 70 nm containing *sul1* gene. The plasmid removal efficiency was analyzed by qPCR analysis of the *sul1* gene. Plasmid retention of UF membrane (nominal pore size 40 nm) was low with 23 %. The author concluded that the plasmid was able to pass through all tested UF membranes due to plasmid detection in all analyzed UF filtrates, although plasmid size was significantly larger than the nominal pore size of the membrane. In comparison, the nominal pore size of the UF membrane in this pilot-scale study was 20 nm. Breazeal et al. (2013) investigated plasmid removal containing *vanA* gene using a 100 kDa MWCO membrane at lab-scale with a 10 nm nominal pore size. The molecular weight of the spiked plasmid was about 2,600 kDa. The study results demonstrated plasmid removal containing *vanA* gene between 1.2 and 2.3 log units. The UF membrane used in this dissertation resulted in a similar *vanA* gene removal efficiency of 1.1 log units. Several further membrane studies confirm plasmid transmission through UF membranes (Arkhangelsky et al., 2011; Latulippe et al., 2007). Bacteria breakthrough UF membranes was analyzed in the study of Ren et al. (2018).

Nevertheless, it is recommended to repeat the examinations to test research hypotheses #2.1 and #2.3 whereas DNA extraction should be adapted to quantify intra-

and extrachromosomal ARGs. While intrachromosomal ARGs could be retained by 0.2 µm microfiltration and analyzed by qPCR technique, the MF filtrate should be freeze-dried and subsequently analyzed for extrachromosomal ARGs by qPCR technique.

In the second study (Chapter 6), key factors were examined within the first minute of standard filtration mode after backwash mode and CEB mode of a UF pilot plant whereas the ARG and total bacteria abundance in UF filtrate were studied. Firstly, it was expected that *'CEB will result in higher total biomass and ARGs abundance in UF filtrate within the first minute of standard filtration mode than backwash'* (**research hypothesis #3.1**). Furthermore, it was assumed that '*lower cake layer after backwash and CEB mode will result in higher AMR abundances in UF filtrate*' (**research hypothesis #3.2**). The third research hypothesis was proposed: '*Backwash mode and CEB mode will result in higher AMR abundance in UF filtrate due to contaminated filtrate from the filtrate tank*' (**research hypothesis #3.3**) and fourthly it was hypothesized that *'CEB event using sodium hypochlorite instead of sodium hydroxide will result in significantly lower biomass and ARG abundances in UF filtrate'* (**research hypothesis #3.4**). **Research hypothesis #3.3** was **accepted.** We measured higher ARG abundance in the UF filtrate after backwash and CEB mode within the first minute of standard filtration mode whereas contaminated filtrate from the filtrate tank was responsible for low filtrate quality. **Research hypothesis #3.4** was **partially confirmed**, since the application of sodium hypochlorite as CEB chemical instead of sodium hydroxide resulted in significant lower bacteria abundance within the first minute of standard filtration mode. However, an additional ARG removal in UF filtrate due to the application of sodium hypochlorite as CEB chemical instead of sodium hydroxide was not analyzed. The **research hypotheses #3.1 and #3.2** were not confirmed. The comparison of UF filtrate quality after backwash mode and CEB mode resulted in no significant different ARG abundance. The operation of a virgin membrane module with the same pore size than a fouled membrane module had no significant different ARG abundance in UF filtrate after backwash and CEB mode.

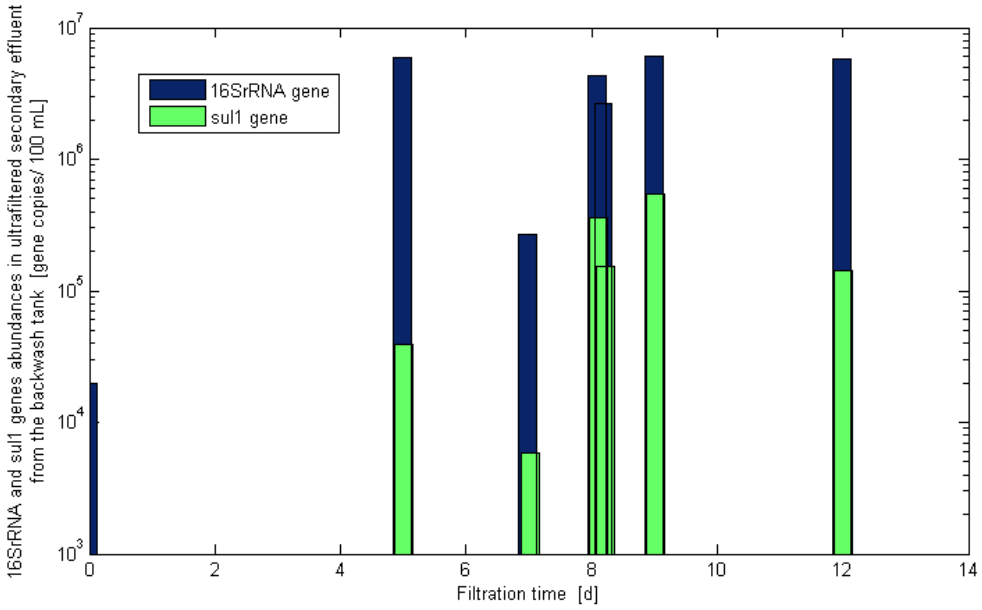
The **core hypothesis II** '*Chemical enhanced backwash results in significant higher ARGs levels in the UF filtrate than during regular backwash*' **was not confirmed**. The contaminated filtrate water of the bypass filtrate tank that is applied during backwash mode and CEB mode, was responsible for observing higher *sul1* gene abundances in UF filtrate within the first and second minute of standard filtration mode. An additional disinfection effect of the applied CEB chemicals during CEB mode towards ARG removal was not observed.

The findings of the two studies (Chapters 5 and 6) revealed that UF exhibited a successful application of membrane filtration for wastewater treatment to prevent the spread of AMR in the urban water cycle. Membrane filtration produces high filtrate

quality with low bacteria and ARGs abundances during 3 and 60 minutes of standard filtration mode. However, the filtrate quality of the first and second minute of standard filtration mode is strongly affected by the backwash and CEB mode. It was found that temporarily stagnant water conditions in the backwash tank promotes *sul1* gene associated regrowth. In Chapter 5, the pilot-scale studies and syringe filter tests revealed that bacteria up to 6 log units can break through UF membranes. Furthermore, bacteria and *sul1* gene analyses in UF filtrate exhibited high correlations. Therefore, it was concluded that *sul1* gene resistant bacteria in UF backwash tank continuously penetrated through UF membranes during every filling of the backwash tank. Within stagnant water phase of about 58 minutes, *sul1* gene associated regrowth can increase in UF backwash tanks.

Several measures were discussed to increase the filtrate quality of the first and second minute of standard filtration mode. This would mean to change the filtrate quality of the backwash tank to a higher filtrate quality. The filter-to-waste protocol of the first and second minute of standard filtration mode could be avoided by regular treatment of the filtrate tank with sodium hypochlorite (variant I), pretreatment of the feed secondary effluent with activated carbon and sand filtration (variant II), or change the tank from bypass mode to continuous flow mode (variant III). While variants II and III were studied in Chapter 7, variant I was examined in a further study. The study applied 150 ppm sodium hypochlorite application in backwash tank (variant I) and results are illustrated in Figure 9-2.

A



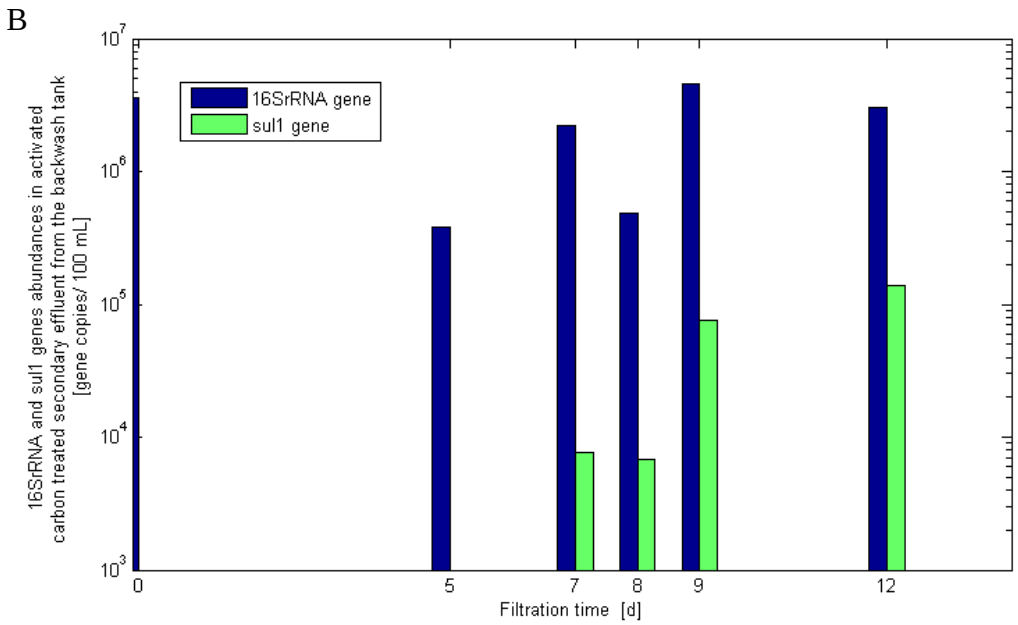


Figure 9-2: Comparison of *16S rRNA* and *sul1* genes analyses of ultrafiltered secondary effluent samples (A) and ultrafiltered activated carbon treated secondary effluent samples (B) from the backwash tanks with daily 150 ppm sodium hypochlorite dosages in the backwash tanks

Before this AMR study, the backwash tanks of both trains had a clean-in-place (CIP) for 24 hours with 150 ppm sodium hypochlorite. After 8 days of continuous UF operation, the *16SrRNA* gene as well as the *sul1* genes increased in the backwash tanks. Within the eight day of continuous operation, the backwash tanks were given 150 ppm of sodium hypochlorite every day with a contact time of 58 minutes before and after a backwash mode. It was observed that in both backwash tanks no significant *16S rRNA* gene and *sul1* gene decrease occurred. Therefore, it was concluded that a daily sodium hypochlorite of 150 ppm was not effective enough to prevent a *sul1* gene associated regrowth in the backwash tanks.

If we think about modifications of the executed UF studies to remove AMR abundances, another interesting investigation would be to repeat the pilot-scale studies whereas instead of using a polymeric UF membrane the application of a ceramic UF membrane is suggested. Ceramic membranes have a lower pore size distribution than polymeric membranes (Zhang et al., 2009). Due to this different pore size distribution Jeong et al. (2018) concluded that the ceramic membrane (nominal pore size 0.1 µm) had a higher COD removal than the polymeric membrane (pore size 0.08 µm). Repeating the AMR studies with a ceramic membrane with the same nominal pore size of 20 nm and same operational conditions, it is expected that UF ceramic membranes will have higher AMR removal efficiencies than polymeric membranes. Furthermore, operation with ceramic membranes can be conducted with significant higher sustainable flux than

polymeric membranes (Miehe et al., 2013). Schwaller et al. (2022b) investigated the retention of MS2 phages with ceramic UF membrane and concluded that with increasing fluxes/TMPs, MS2 phage removal significantly increased. This study is in line with Latulippe et al. (2007). Therefore, the question is raised whether higher flux also resulted in higher AMR removal. These hypotheses require further investigations.

To summarize, it can be concluded that the low reduced *vanA* genes by UF and its low correlation to the *16S rRNA* gene in wastewater samples are indeed confirming that *vanA* gene is predominantly representative in the mobile DNA fraction. On the other hand, *sul1* and *ermB* genes are rather part of intrachromosomal DNA. Therefore, it can be concluded that UF technology have high intrachromosomal ARG removal efficiencies by more than 2.5 log units. In contrast, extrachromosomal ARG removal efficiency of the UF process is low by about 1 log unit. The low extrachromosomal ARG removal efficiency of the UF technology is further discussed in Chapter 9.4 '*Research Needs.*'

Otherwise, ultrafiltration is an appropriate advanced treatment technology producing high filtrate quality between 3 and 60 minutes of standard filtration mode. In order to prevent filter-to-waste applications with the first and second minute filtrate of standard filtration mode, the filtrate quality of the backwash tank has to be optimized. Since daily sodium hypochlorite dosages of the backwash tank was ineffective to significantly reduce AMR abundance in the backwash tank, variants II and III are recommended measures to reduce AMR associated regrowth effects in the backwash tank. These study results are discussed in Chapter 9.3.

9.3 Relevant factors for AMR associated regrowth at UF filtrate side and consequences to the planning of a full-scale UF plant

Since UF treatment of secondary effluent revealed detectable *sul1* gene in UF filtrate (Hiller et al., 2022), a *sul1* gene associated regrowth effect at UF filtrate side was observed, as well. The strong correlation of *sul1* gene and *16S rRNA* gene in UF filtrate side using secondary effluent as feed suggested that *sul1* gene resistant bacteria breach UF membranes and thus promote ARG associated regrowth at the UF filtrate side (Hiller et al., 2023).

In the UF study in Chapter 7, relevant factors for ARG associated regrowth at UF filtrate side were examined. Firstly, it was proposed that '*AMR associated regrowth in UF filtrate can occur using secondary effluent as feed*' (**research hypothesis #4.1**). The second research hypothesis was defined: '*Advanced wastewater treatment of the feed wastewater prior to ultrafiltration will result in no ARG associated regrowth in the filtrate water in the bypass filtrate tank*' (**research hypothesis #4.2**). Thirdly, it was hypothesized that '*typical erythromycin and sulfamethoxazole concentrations of*

secondary effluent will not result in bacterial adaption on to antibiotic resistant bacteria at UF filtrate side' (**research hypothesis #4.3**). In the fourth hypothesis, it was expected that '*the filtrate tank in continuous flow mode can reduce ARG associated regrowth effect compared to bypass mode*' (**research hypothesis #4.4**). '*Continuously dosing 0.5 mg/L sodium hypochlorite in UF filtrate tank can successfully prevent ARG associated regrowth in UF filtrate tank in continuous flow mode using secondary effluent as feed*' was tested as **research hypothesis #4.5**. All **research hypotheses #4.1, #4.2, #4.3, #4.4** and **#4.5** were **confirmed.** We measured a *sul1* gene associated regrowth both in bypass filtrate tank and in filtrate tank in continuous flow mode. The physical separation due to the pore size of the membrane module resulted in a bacterial shift, which led to an increase of penetrated bacteria of the phyla *Proteobacteria*, *Firmicutes* and *Bacteroidota* at the filtrate side (chapter 8). It seems that the increase of penetrated bacteria phyla was accompanied with the *sul1* and *intl1* gene increases at the filtrate side with progressive filtration time. However, pretreating the secondary effluent as feed with activated carbon treatment and sand filtration resulted in additional *sul1* gene removal of the feed so that no *sul1* gene associated regrowth was observed at the filtrate side in the bypass filtrate tank. Typical antibiotic concentrations measured in secondary effluent in the range of human PNEC values revealed no selection pressure to bacterial adaption of antibiotic resistant bacteria at filtrate side. The factor of temporarily stagnant filtrate water and the hydraulic retention time concerning ARG associated regrowth was examined applying the bypass filtrate tank and the filtrate tank in continuous flow mode with a hydraulic retention time of 3 hours. While 3 hours of hydraulic retention time in filtrate tank resulted in about 1 log unit higher *sul1* gene resistant bacteria, temporarily stagnant filtrate water strongly promoted this *sul1* gene resistant bacteria to increase by more than 3 log units. In order to prevent ARG associated regrowth and thus to keep this high ARG removal efficiency measured directly after UF membrane module, continuously dosing 0.5 mg/L sodium hypochlorite at the filtrate side can successfully prevent ARG associated regrowth using secondary effluent as feed. Alternatively, pretreatment of the feed wastewater by advanced treatment processes (e.g., activated carbon treatment and sand filtration) was also examined as a successful measure to prevent ARG associated regrowth at UF filtrate side.

The **core hypothesis III** '*AMR associated regrowth in UF filtrate can be prevented by activated carbon pretreatment of feed water*,' and the **core hypothesis IV** '*Presence of antibiotics in secondary effluent can cause bacterial adaption to antibiotic resistance in UF filtrate*' **were not confirmed**. The study results of Chapter 7 revealed that the PAC treatment of secondary effluent cannot reduce *sul1* gene abundance enough so that no *sul1* gene associated regrowth could be inhibited in the filtrate tank in continuous flow mode. Furthermore, pilot-scale UF studies examining bacterial adaption due to spiked

sulfamethoxazole and erythromycin concentrations in the filtrate resulted in no ARG associated regrowth in UF filtrate.

The **core hypothesis V** '*AMR regrowth in the UF filtrate of ultrafiltered secondary effluent can be prevented by continuous chlorine dosing*' **was confirmed**. Continuously dosing 0.5 mg/L NaOCL in the filtrate tank in continuous flow mode resulted in no ARG associated regrowth in UF filtrate.

Chapter 7 also focused on the application of the bypass filtrate tanks for temporarily stagnant water analyses and separate filtrate tanks in continuous flow mode for the hydraulic retention time analyses for ARG associated regrowth studies. However, the rebuild of the bypass filtrate tanks to filtrate tanks in continuous flow mode was not possible in the pilot plant to examine the backwash mode on the filtrate quality of the first and second minute of standard filtration mode. Due to the significant reduction of ARG associated regrowth from 3 log units to 1 log unit using filtrate tanks in continuous flow mode, it is expected that the filtrate quality between the first and the fifth minute of standard filtration mode has no significant difference. Furthermore, the hydraulic retention time of a filtrate/backwash tank in continuous flow mode is rather about 1 hour. Therefore, it is hypothesized that '*the application of backwash mode based on filtrate tanks in continuous flow mode with 1 hour of hydraulic retention time at dry weather will have no significant different filtrate quality between the first and fifth minute of standard filtration mode'*. This hypothesis requires further investigations and repetitions of the conducted examinations of Chapter 6.

The ARG associated regrowth studies with respect to the 3-hour hydraulic retention time should be repeated using the feeds activated carbon treated secondary effluent and tertiary effluent. The reason for the use of secondary effluent as feed was the fact that the ARG associated regrowth results of ultrafiltered secondary effluent can be transferred to other conventional WWTPs without an advanced treatment step for trace substances removal. The application of activated carbon treated secondary effluent as feed was the intention to demonstrate a lower ARG associated regrowth effect due to the activated carbon treatment of the feed prior ultrafiltration. The ARG associated regrowth examinations at UF filtrate side using activated carbon treated secondary effluent and tertiary effluent should be executed at the WWTP Steinhäule whereas the activated carbon stage and the sand filtration process is now expanded for wet weather conditions. The second sedimentation basin was taken into operation for wet weather conditions in 2023. With this sedimentation basin, the wastewater is treated with activated carbon and sand filtration during dry and rainy weather. Thus, no secondary effluent of the train 2 will be directly given to the sand filtration process during wet weather what was the fact during the ARG associated regrowth studies executed in Chapter 7. It is expected that '*no*

ARG associated regrowth will occur with a hydraulic retention time of 3 hours at UF filtrate side using tertiary effluent as feed'. This assumption requires, as described, further investigations.

In Chapter 8, design considerations for implementing a full-scale UF plant at the WWTP Steinhäule were elaborated. Two parallel UF pilot plants were operated to quantify design values for full-scale applications. The following aspects were considered: Where is the best position for the implementation of a membrane filtration process in an existing conventional treatment train? What kind of pore size of the membrane is needed to achieve a high AMR removal efficiency under economic conditions? What operational conditions are required to assure a high AMR removal efficiency while maintaining economic conditions? What strategies are needed to prevent AMR associated regrowth? The study results revealed that the effluent of the sand filtration process (tertiary effluent) was the best location for the implementation of a full-scale membrane filtration plant. In this case, *sul1* gene resistant bacteria were significantly removed within the activated carbon stage with subsequent sand filtration so that no ARG associated regrowth at filtrate side occurred. The application of an inge MF module had a pore blockage with significant higher TMP during continuous MF process whereas a higher backwash flux could not solve the problem. The operation of a Microdyn-Nadir UF module revealed a low flux of about 38 LMH using a constant coagulation dosis of 9 mg/L PAC. The application of the Inge UF module revealed the highest sustainable flux at 70 LMH on the basis of a constant 2 mg/L PAC dosing. Further coagulation coating studies revealed that the coating time can be reduced to 20 minutes and the consumption of coagulation can be cut in third without a significant TMP increase. The Inge UF module was further investigated in the planning of the full-scale UF plant at WWTP Steinhäule.

However, the full-scale UF plant was calculated with respect to the assumption that no additional treatment of the backwash wastewater is necessary. The backwash wastewater was planned to discharge into the influent sewers of the activated carbon stage. It is assumed that the activated carbon stage and the sand filtration process can adsorb the additional input of bacteria and ARGs so that no higher ARG and bacteria abundance in the effluent of the sand filtration process result in higher ARG and bacteria abundance in the corresponding UF filtrate. Therefore, these backwash effects require further investigations about a possible increase of bacteria and ARG due to backwash water repatriations, which are further elaborated in chapter 9.4.2.

The findings of the two studies (Chapters 7 and 8) revealed that ultrafiltered secondary effluent can result in ARG associated regrowth. Pretreatment of the feed with advanced treatment processes (e.g., activated carbon treatment and sand filtration) or continuously dosing 0.5 mg/L sodium hypochlorite in UF filtrate can prevent ARG associated regrowth at UF filtrate side. This observation raises the question how could be the implementation of advanced treatment processes to reduce AMR for other municipal WWTPs?

To make a statement about the appropriate advanced treatment processes to reduce AMR, it is important to be aware of the main requirements of advanced treatment processes need to reduce AMR.

Ultrafiltration exhibited the best AMR removal efficiency under economic conditions and as result of the literature study to remove AMR from wastewater. Furthermore, pilot-scale studies suggest that ultrafiltration of wastewater under economic conditions should be executed with UF capillary membranes. In order to safely operate capillary membranes, several membrane filtration companies recommend pre-filters with at least 200 µm are suggested to protect the membranes. An example for suitable pre-filters for wastewater streams are the Disc Filter RoDISC (Huber SE, Germany). This pre-filter has a mesh size between 10 and 100 µm and a throughput capacity per unit of 2,000 m^3/h. Otherwise, the sand filtration process is an already established technology in municipal WWTPs.

In order to prevent ARG associated regrowth at UF filtrate side pretreatment of the feed is recommended. The new European Urban Wastewater Treatment Directive (2022) requests for all WWTPs with 100,000 population equivalents and higher the implementation of advanced treatment processes to reduce trace organic chemicals. Therefore, these WWTPs will expand their wastewater treatment either with activated carbon treatment or with an ozonation step. Both technologies were realized so far with a downstream physical treatment step predominantly applied with a sand filter or a granular activated carbon (GAC) filter. The implementation of an activated carbon treatment or an ozonation step depends on the expansion area of the WWTP, required discharge standards to surface water, the wastewater amount, and potential transformation products (e.g., bromide, nitrosamines and chrome (III) concentrations).

Therefore, a possible advanced treatment processes should include the removal of trace organic chemicals and where required also AMR. It is suggested that a treatment step for TOrCs (activated carbon treatment or ozonation) followed by a pre-filter and an ultrafiltration step should be implemented in WWTPs. Three variants were elaborated in Figure 9-3. While the variant I and II is built up with an already established treatment process for TOrCs, the variant III is only for municipal WWTPs whereas treatment processes to reduce TOrCs was not realized so far. In the variant III, it is recommended to prove that ARG associated regrowth will not occur, since the sand filtration had the highest *sul1* gene resistant bacteria removal efficiency in the AMR studies of Chapter 7. Furthermore, required aforementioned discharge standards of the WWTP should be adhered.

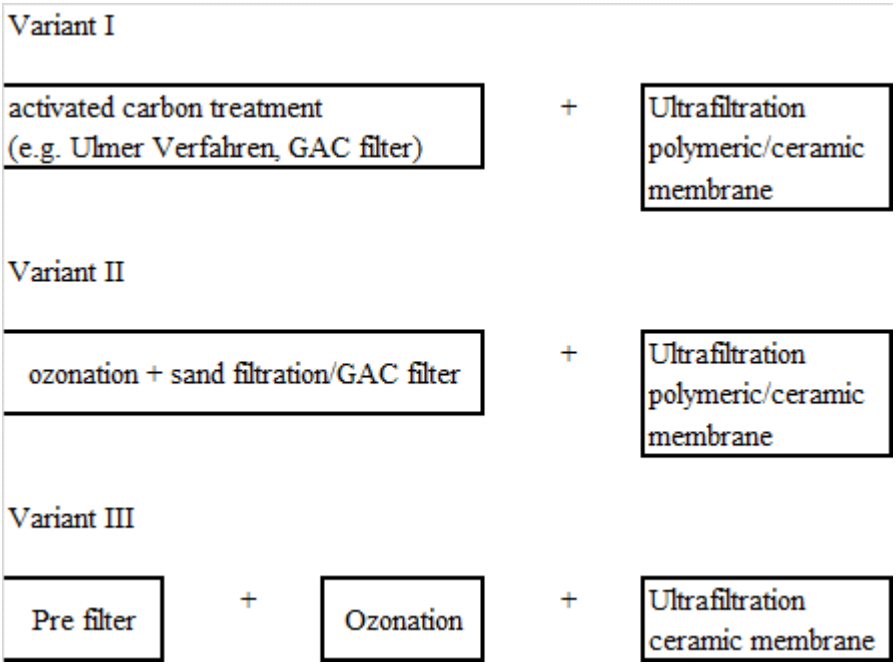


Figure 9-3: Proposed variants I, II and III of advanced treatment processes to reduce trace substances and AMR and prevent ARG associated regrowth in the effluent of municipal WWTPs

At present, membrane companies also elaborate on membrane filtration studies whereas flat sheet membranes were applied with an activated carbon pretreatment without a sedimentation basin and sand filtration (Werner et al., 2019). However, the activated carbon is very abrasive in nature and no long-term experiences for a safe operation of membranes in activated carbon concentrations of 5 g/L and higher exists so far. Flat sheet studies achieved very low flux rates within the UF study of Werner et al. (2019). Furthermore, it is assumed that an ARG associated regrowth at UF filtrate side can only be prevented with this wastewater treatment system by continuously dosing 0.5 mg/L sodium hypochlorite. Therefore, this system of advanced treatment processes is not recommended for TOrC and AMR removal.

The ARG associated regrowth was examined in Chapter 7, whereas the relevant factors were the temporarily stagnant water in a bypass tank and the hydraulic retention time in tanks in continuous flow mode. The ARG associated regrowth was proved either with temporarily stagnant water or with a long hydraulic retention time of 3 hours. If wastewater is purified with the aforementioned advanced treatment processes and after that it is directly discharged into the surface water, an ARG associated regrowth would not occur due to a short hydraulic retention time. However, this filtrated wastewater is too good and too expensive to discharge into surface water. In terms of the climate change that also occurs in Germany, water resources even recedes today (Frey, 2023) and in the future. Water reuse will be a part of a municipal WWTP process in Germany and Europe (Schwaller et al., 2021).

In order to use the wastewater of the WWTP Steinhäule for reuse purposes, the purified wastewater has to be pumped via pipes to the customers. In this case, the ARG associated regrowth studies can be transferred from pilot-scale to full-scale observations. Within a pipe system and a longer hydraulic retention time an ARG associated regrowth can occur as examined in the AMR study of Chapter 7. Therefore, measures to prevent ARG associated regrowth are recommended. The possible water reuse in different facilities is illustrated according to the example of the WWTP Steinhäule (Figure 9-4).

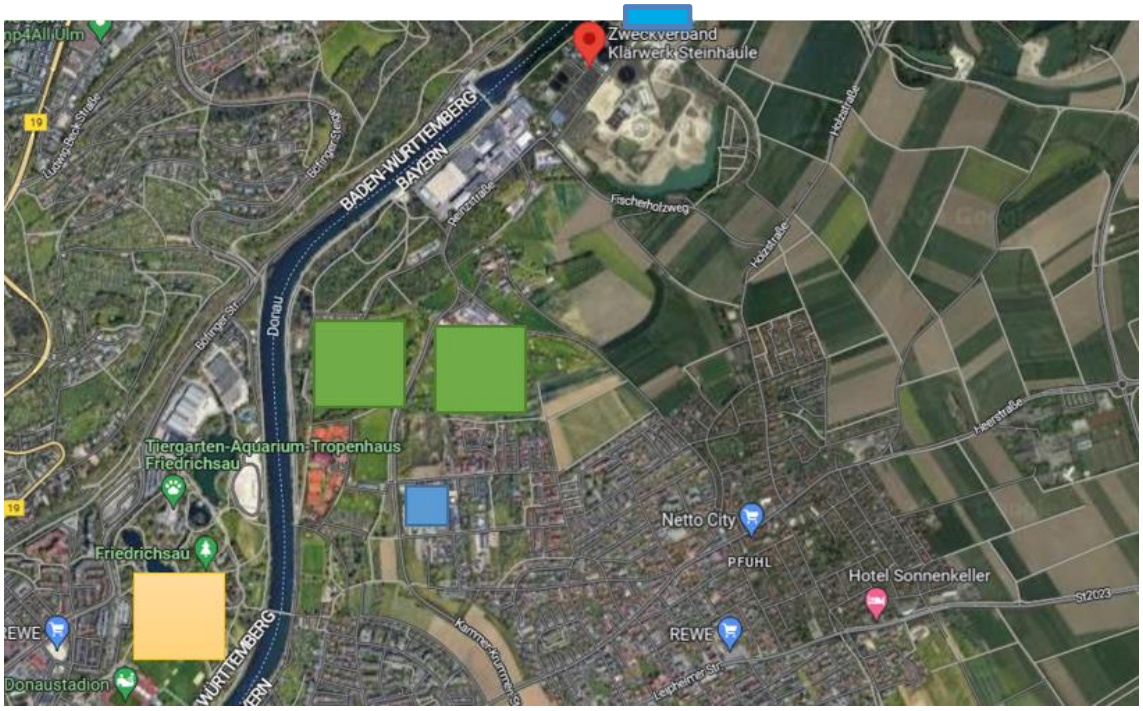


Figure 9-4: Map of the WWPT Steinhäule and enclosed customers for water reuse purposes of purified wastewater, blue rectangle: concrete factory, green rectangles: golf course, yellow rectangle: city park (google maps, 2023)

Possible customers for water reuse applications are within a radius of 3 kilometers of the WWTP Steinhäule, including two concrete factories, a golf course with more than 30 hectares, and the city park ‘Friedrichsau’ with about 30 hectares. Furthermore, east of the WWTP Steinhäule are large areas used for agriculture.

About 10 kilometers upstream the Danube River from the WWTP Steinhäule the biggest industrial area of Ulm ‘Donautal’ can be also supplied with reclaimed water. In this industrial area of about 350 hectares, 20,000 people work every day in 300 different companies. Water intensive companies of the industrial area ‘Donautal’ are the companies of metal processing (Wieland) whereas water is applied for cooling and surface treatment, concrete factory (Schwenk), chemical industries (Brenntag and Microchemicals GmbH), and pharmaceutical companies (Teva Biotech GmBH, ratiopharm, AbZ-Pharma, Merckle, CT Arzneimittel).

9.4 Research Needs

9.4.1 Intra- and extrachromosomal ARG removal efficiency of the UF process and intra- and extrachromosomal ARG associated regrowth in UF filtrate

The UF pilot-scale studies revealed that membrane filtration is an appropriate advanced treatment technology to reduce the spread of AMR. However, the pores of the applied ultrafiltration membrane are characterized by a pore size distribution with even significant larger pores than the nominal pore size of 20 nm. This is the reason why active bacterial cells breakthrough the UF membrane and could be detected by flow cytometry at the UF filtrate side. A bacterial species analyses demonstrated that the penetrated bacteria were the phyla of *Proteobacteria, Firmicutes* and *Bacteroidota*. The breakthrough of these bacteria is a promoter for the ARG associated regrowth effect at UF filtrate side. This means that ultrafiltered secondary effluent alone cannot be sufficient for efficient retention of ARB and an ARG associated regrowth can occur at UF filtrate side. This ARB breakthrough effect and ARG associated regrowth effect at UF filtrate side were successfully prevented by pretreating the influent water of the UF pilot plant using the activated carbon stage and the sand filtration process.

However, the ARG associated regrowth studies considered the analyses of the ARGs *sul1, ermB, vanA* and *blaOXA58* genes. While (intrachromosomal) *sul1* gene increased with increasing *16S rRNA* gene at UF filtrate side, *ermB* gene, predominantly part of intrachromosomal DNA, did not increase at UF filtrate side (Hiller et al., 2022). The question arises if the intrachromosomal ARG breakthrough depends on specific bacteria with intrinsic antibiotic resistance or whether these ARGs are incorporated by horizontal gene transfer? Therefore, pilot-scale UF studies should be performed in WWTP effluents whereas further intrachromosomal ARGs analyses were conducted at UF filtrate side concerning ARG associated regrowth.

Furthermore, it was observed that also extrachromosomal ARGs like *vanA* gene could be detected at UF filtrate above the limit of detection. Within the pilot-scale UF studies, it was noticed that the physical separation due to the pore size of the ultrafiltration module was not able to effectively reduce extrachromosomal *vanA* genes. The studies to analyze the ARG removal efficiency of the fouling layer resulting significantly reduction of the *vanA* gene with the increase of the fouling layer (Hiller et al., 2022). This extrachromosomal ARG removal effect of the fouling layer requires further investigations about the factors of separation predominantly extrachromosomal ARGs. Factors for extrachromosomal ARG removal of the fouling layer could be the clogging of greater pores and a reduction of the overall pore size of the membrane and electrostatic charge

effects of the fouling layer. Furthermore, extrachromosomal ARGs can have a high tendency to interact with negatively charged extracellular polymeric substances (EPS) and soluble microbial products (SMP) in the presence of divalent cations like Ca^{2+} und Mg^{2+}. Extrachromosomal ARG removal efficiency of the fouling layer of the ultrafiltration process was not studied in depth so far. That is the reason why further investigations are required to examine the factors for extrachromsomal ARGs removal of the UF process.

Beside of extrachromosomal ARGs removal studies within the UF process, it was observed that no extrachromosomal *vanA* gene associated regrowth occurred at the UF filtrate side. Therefore, the question must be addressed to what extent intrachromosomal ARG forces ARG associated regrowth at UF filtrate side or whether extrachromosomal ARG abundance in UF filtrate also play a part in the observed ARG associated regrowth studies. Which biotic and abiotic factors are responsible for extrachromosomal ARG incorporation of bacteria at UF filtrate side. There are still knowledge gaps concerning extrachromosomal ARG associated regrowth at UF filtrate side. These knowledge gaps require further investigations about possible extrachromosomal ARG associated regrowth.

9.4.2 Treating AMR contaminated backwash water

The UF pilot-scale studies as well as ozonation and UV-irradiation studies at WWTP Steinhäule have shown that ultrafiltration is the most effective method for reducing antibiotic resistance (Hiller et al., 2022; Hembach et al., 2019; Jäger et al., 2018). The scientific studies exhibited the effectiveness of the reduction of antibiotic resistance genes (ARGs) by ultrafiltration (ARGs reduction 2.6 - 7 log units). As a result of the HyReKA research project are high reduction rates of ARGs measured over a period of 3 years (Hembach et al., 2019, accepted in Nature Scientific Research). However, open points concerning the operation of ultrafiltration were raised in the research project, which need to be further clarified.

Ultrafiltration retains ARGs via filtration with pore sizes smaller than 100 nm. The ‘particles’ are retained on the membrane surface via the filter cake using capillary membranes in dead-end mode. The backwash contains the predominant load of ARGs retained on the membrane surface during the filtration phase. This raises the question of what should be done with the backwash water, which contains a very high abundance of ARGs. Backwash analyses revealed a concentration of facultative pathogenic bacteria and antibiotic resistances up to 2 log units higher than in the ultrafiltration feed (Hembach et al., 2019). Thus, the backwash shows a significantly increased microbiological load of critical antibiotic-resistant bacteria and the question arises for further treatments of these

critical backwash water. In the following sections 3 measures are proposed for backwash water treatment:

The backwash water can be returned to the activated carbon stage and sand filtration process (variant A) or to the biological stage (variant A) (Figure 9-5). If these measures result in ARG increase within the wastewater treatment processes, the ARG abundance of the backwash water could be enriched with another two ultrafiltration steps (variant C) and after that the concentrated backwash should be given to the sludge incineration process.

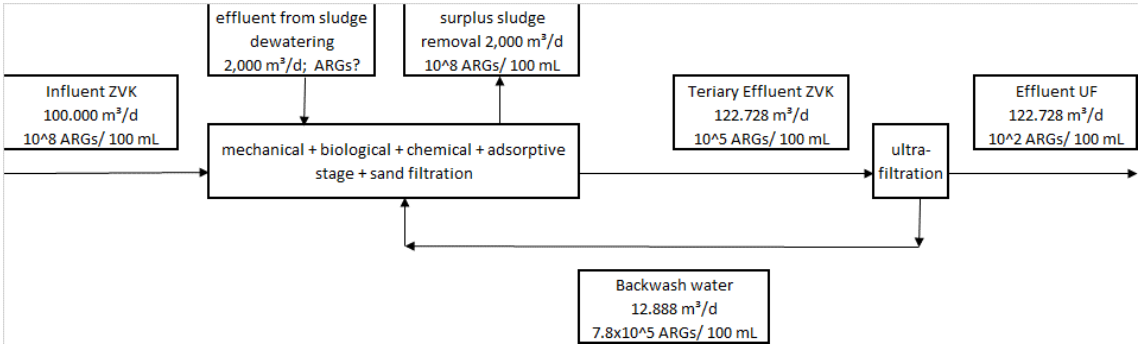


Figure 9-5: Return backwash water to the influent of the activated carbon stage and sand filtration or to the biological stage

A) Discharge of the backwash water in the influent of the activated carbon stage with sand filtration

One possibility for backwash utilization is the recirculation into the influent of the activated carbon stage and sand filtration. However, this continuous recirculation with high loads of ARGs could result in an increase of ARG abundance in the effluent of the sand filtration process and an ARG increase in UF filtrate. Further studies should investigate whether the physical process of the activated carbon stage and the sand filtration process can reduce this additional ARG load due to the continuously return of the backwash water. The additional ARG abundance adsorbed at the activated carbon and enriched in the cake layer of the sand filtration process would be returned with the backwash of the sand filtration and the surplus activated carbon outlet to the biological stage and with the surplus sludge removal would be given to the sludge incineration process.

B) Discharge of the backwash water in the biological stage

Another possibility for backwash utilization is the recirculation into the biological treatment stage. On the one hand, this continuous recirculation with high loads of ARGs could have a selection pressure effect for the adaption on to antibiotic resistant bacteria on the total population of bacteria in the biological treatment stage. On the

other hand, it could also lead to a clustered accumulation of bacteria onto sludge flocs, which can thus be removed from the biological treatment stage system via the surplus sludge removal into the sludge incineration process.

C) Utilization of the backwash water via two ultrafiltration steps in the incineration

If variant A and B with backwash utilization were not possible for a suitable ARG removal within the WWTP Steinhäule, the backwash water could be treated with additional two ultrafiltration steps (Figure 9-6). Using another two ultrafiltration steps the load of ARGs could be concentrated in a smaller volume of wastewater, with the aim of being able to give the backwash water of the last ultrafiltration step continuously directly to the sludge incineration process.

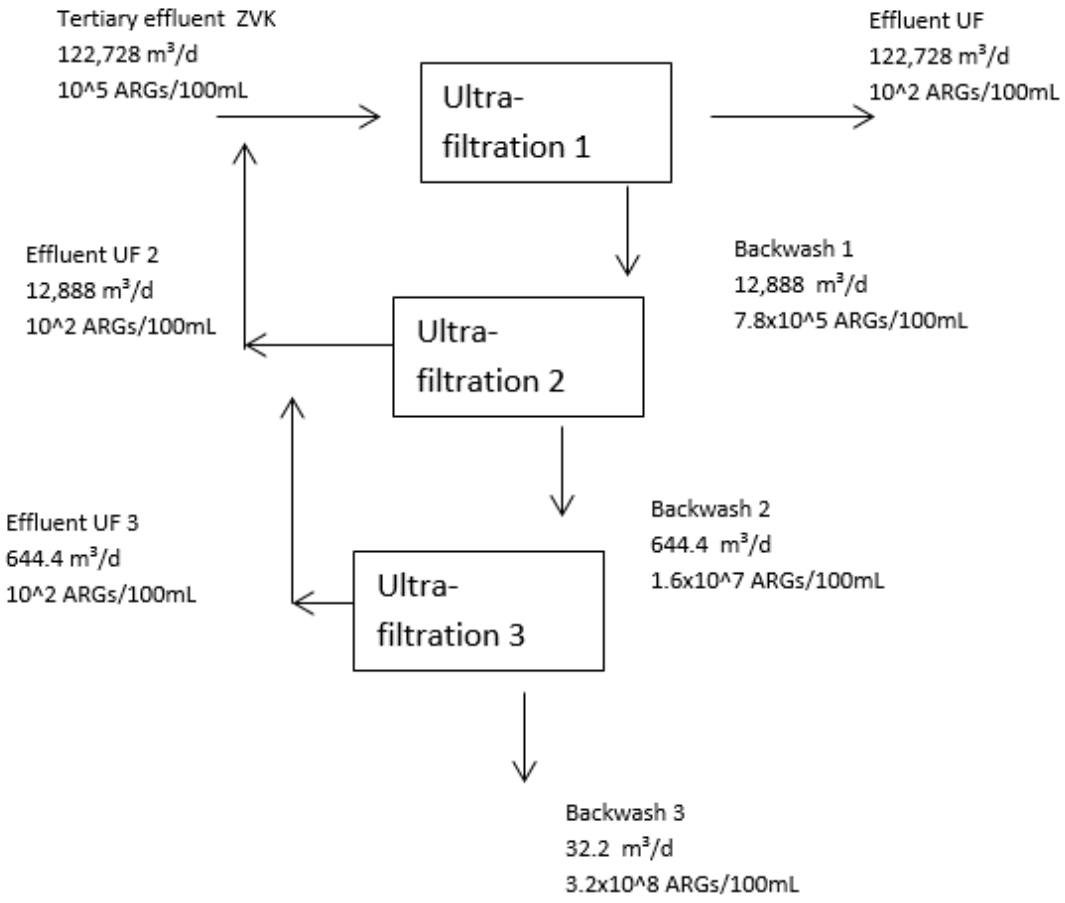


Figure 9-6: Treatment of the backwash water with two additional ultrafiltration steps for utilization of the last backwash water with very high ARG abundance in the sludge incineration process.

In this case, further examinations are required whether the highly loaded backwash from the first ultrafiltration plant can be retained so effectively with further ultrafiltration steps so that the ARGs can be significantly increased in the backwashes.

9.4.3 Measures for AMR removal, risk of AMR discharges in the aquatic environment and the link to human health

Antibiotic resistance does not only occur in hospital wastewater but also in residential wastewater and municipal WWTPs (Sigala, Unc, 2012; Li et al., 2015; Rizzo et al., 2013). Elevated ARB and ARGs abundances as well as antibiotics were also detected in effluents from a slaughterhouse (Um et al., 2016; Moura et al., 2007) and antibiotic manufacturing facilities in China (Yang et al., 2008) and India (Larsson et al., 2007). Besides WWTPs, storm water overflows are components in the urban drainage systems in order to reduce the flow to the WWTPs during heavy rain events whereas high ARG and antibiotic loads find the way into the urban water cycle (Alexander et al., 2015). Another pathway of anthropogenically derived resistances is agricultural manure and land-applied biosolids from WWTPs, which is still being practiced in Germany and worldwide (Munir, Xagoraraki, 2011).

On the other hand, Kohanski et al. (2010) also reported that antibiotic resistance can increase through selection pressure of naturally arising resistant mutants and through horizontal gene transfer. The presence of naturally-occurring resistance background was examined in several scientific studies (Pei et al., 2006; Kristiansson et al., 2011; Makowska et al., 2016). These studies revealed the increase of AMR due to anthropogenic influences in the aquatic environment. Several researchers support the thesis that advanced treatment processes at WWTPs should be established in terms of precautionary approach to reduce the spread of ARB and ARGs into the aquatic environment (Rizzo et al., 2013; Czekalski et al., 2016; Pruden et al., 2013). However, many researchers require further studies to better explain the effect of advanced treatment processes (e.g., ozonation and UV-irradiation) on ARB selection (Czekalski et al., 2016; McKinney and Pruden, 2012; Rizzo et al., 2013). Researchers of the research project ‘Hyreka’ analyzed critical ARGs and ARB, especially multi drug resistant bacteria to 4 resistant antibiotics of last resort (4 MRGN) in hospital wastewater and in WWTP effluents. The researchers refer to the German Infection Protection Law: Those responsible for wastewater treatment must ensure that wastewater is disposed of in this way that any threat to human health from pathogens do not arise. Therefore, it was concluded that advanced treatment processes in WWTP effluents are recommended for large WWTPs with more than 100,000 population equivalents, when the wastewater amount of the surface waters are more than 50 percent, downstream of the WWTP discharges the surface water is applied as raw water for drinking water purposes or as bathing water and when the influent of WWTPs have a high wastewater amount of hospital wastewater or slaughterhouse wastewater without wastewater pretreatment processes. If advanced wastewater treatment is necessary, a combination of technologies for trace substances removal (e. g., activated carbon treatment or ozonation) and of technologies for AMR removal (e. g., UV-irradiation plus ozonation or membrane filtration) are recommended.

However, the acute and long-term risk resulting from the release of resistance genes and antibiotics into the aquatic environment but also the presence of naturally-occurring resistance background, requires further investigations. Naturally-occurring resistance and background antibiotic resistance is not defined so far. Several researchers suggest that study data for background antibiotic resistance and antibiotics in natural ecosystems has to be analyzed to normalize baseline antibiotic concentrations and antibiotic resistance of natural ecosystems (Rothrock et al., 2016; Aminov, 2009; Czekalski et al., 2015).

Further research is necessary concerning survivability of pathogenic antibiotic resistant bacteria in aquatic environment and the selection pressure to environmental bacteria. What are the biotic and abiotic factors for the increase of pathogenic antibiotic resistant bacteria in the aquatic environment? Which factors facilitate a horizontal gene transfer in the aquatic environment? What is the dose-response relationship between pathogenic antibiotic resistant bacteria and infection diseases? How can extracellular antibiotic resistance genes affect human health, even if they are not linked to living pathogenic bacteria (Berglund, 2015; Dong et al., 2019; Sharma et al., 2003)?

If the previous aspects are studied in depth, a risk assessment of antibiotic resistance for human health could be elaborated.

10 APPENDIX

10.1 List of publications

Research articles (peer-reviewed)

1. Hiller, Christian X., Hübner Uwe, Fajnorova Sona, Schwartz Thomas, Drewes Jörg E. (2019). Antibiotic microbial resistance (AMR) removal efficiencies by conventional and advanced wastewater treatment processes: A review. *Science of the Total Environment* **685**, 596-608. DOI: 10.1016/j.scitotenv.2019.05.315

   This publication is included in Chapter 4.

2. Hiller Christian X., Schwaller Christoph, Wurzbacher Christian, Drewes Jörg E. (2022). Removal of antibiotic microbial resistance by micro-and ultrafiltration of secondary wastewater effluents at pilot scale. *Science of the Total Environment*, 838, 156052.
   DOI: https://doi.org/10.1016/j.scitotenv.2022.156052

   This publication is included in Chapter 5.

Research articles (in review)

1. Hiller Christian X., Drewes Jörg E. (in review). Impact of backwash and CEB modes on retention of biomass and antibiotic resistance genes during ultrafiltration of WWTP effluents. *Membranes*

   This publication is included in Chapter 6.

2. Hiller Christian X., Wurzbacher Christian, Drewes Jörg E. (in review). Factors affecting antibiotic resistance gene associated regrowth in WWTP effluents after membrane filtration. *Water Research*

   This publication is included in Chapter 7.

Additional research articles (peer-reviewed) in other research areas

1. Schwaller Christoph, Hoffmann Grit, Hiller Christian X., Helmreich Brigitte, Drewes Jörg E., 2021, Inline dosing of powdered activated carbon and coagulant prior to ultrafiltration at pilot-scale – Effects on trace organic chemical removal and operational stability. *Chemical Engineering Journal* 414, 128801

2. N., Schmid F., Alexander J., Hiller C., Rogall E.T., Schwartz T., 2017, Occurrence of the mcr 1 colistin resistance gene and other clinically relevant antibiotic resistance genes in microbial populations at different municipal wastewater treatment plants in Germany, *Frontiers in Microbiology*, 8, 1–11

3. Hembach N., Alexander J., Hiller C., Wieland A., Schwartz T., 2019, Dissemination prevention of antibiotic resistant and facultative pathogenic bacteria by ultrafiltation and ozone treatment at an urban wastewater treatment plant, *Scientific Reports*, 9, 12843, 1-12

4. Jäger T., Alexander J., Kirchen S., Dötsch A., Wieland A., Hiller C., Schwartz T., 2018a, Live-dead discrimination analysis, qPCR assessment for opportunistic pathogens, and population analysis at ozone wastewater treatment plants. *Environ. Pollut.* 232, 571–579

5. Jäger T., Hembach N., Elpers C., Wieland A., Alexander J., Hiller C., Krauter G., Schwartz T., 2018b, Reduction of Antibiotic Resistant Bacteria During Conventional and Advanced Wastewater Treatment, and the Disseminated Loads Released to the Environment. *Frontiers in Microbiology*, 9, 2599, 1-16

Research articles (non-peer-reviewed)

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Conference talks

1. *Hiller Christian X., Alexander Johannes, Schwartz Thomas, 2019, 'Weitergehende Abwasserbehandlungsverfahren und Kostenbetrachtung' at final event of the BMBF Project Hyreka in Berlin, Germany, 4.04.2019*

10.2 List of supervised student theses

Master theses

1. Egner Sebastian: Operation of two Ultrafiltation Pilot Plants at three different Wastewater Effluent Stages, submitted to the Faculty of Engineering Science, Chair of Mechanical Process Engineering/ Water Technology, university of Duisburg-Essen

2. Rehm Kevin: Optimierung des Antibiotikaresistenzrückhaltes der Mikrofiltration in der Abwasserreinigung, submitted to the Faculty of environment and process engeneering, university of applied sciences Ravensburg-Weingarten

3. Wehrheim Carolin, Investigation and Optimization of Antibiotic Resistance Retainment in Membrane Technology for Waste Water Treatment with Flow Cytometry and Biofilm Measurement, submitted to Faculty of the Department of Civil, Geo and Environmental Engineering, TUM

4. Kott Sinclair, Optimization of the Retention of Antibiotic Resistance Genes using sand filtration, submitted to Facutly of the Pharmaceutical Bioprocess Engineering, TUM

10.3 Supplementary information for Chapter 4

The following studies have been considered to compile Figure 2-1: Makowska et al. (2016) studied total ARB and ARGs abundances in the WWTP (secondary mechanical-biological treatment plant with 55,000 m^3/d) of Lezyca (120,000 population equivalent) in Poland and its influence on the Zimny Potok River. Young and Fellow (2012) examined ARB abundances in WWTP effluents and the receiving Hudson River, New York, USA. In the study of Guyomard-Rabenirina et al. (2017) ARB analyses were investigated in WWTP effluent and the receiving Rivière Salée, Gouadeloupe, French. Koczura et al. (2012) studied ARB in municipal WWTP effluent samples of Poznan (city, 550,000 population) and from receiving Warta River, Poland. Li et al. (2010) examined ARB analyses in WWTP effluent and the receiving river in Hebei Province, China. Alexander et al. (2015) examined total ARGs abundances in a region in South Germany sampling at four WWTPs (2,500, 16,000, 16,600 and 445,000 population equivalent) and three different surface waters (Danube and two tributaries). Böckelmann et al. (2009) studied the WWTP of the city Sabadell (200,000 population equivalent, Spain) and its effect on the Ripoll River. Koczura et al. (2016) examined the Warta River in Poland with a sampling site under relatively low anthropogenic influence upstream of a WWTP discharge as well as river samples at the WWTP discharge (200,000 m^3/d) point in the city of Poznan (550,000 population equivalent) and five kilometers downstream of the WWTP discharge. In the study of Rodriguez-Mozaz et al. (2015) the antibiotic resistance level was investigated in the municipal effluent of the WWTP of Girona (45,000 – 55,000 m^3/d) and the wastewater receiving Ter River. Marti et al. (2013) studied ARGs analyses in effluent samples from Ripoll WWTP (8,000 m^3/d) and receiving Ter River in Catalonia, Spain.

The following studies have been considered in Figure 3-1: Pei et al. (2006) examined relative ARGs abundances of the La Poudre River in northern Colorado, USA. The sample near the origin of the river is investigated as the pristine site of the river without anthropogenic influence. Downstream sampling considered urban river areas. Kristiansson et al. (2011) studied the relative ARGs abundances upstream, WWTP effluent and downstream of a Swedish River. The upstream site of the Swedish River was evaluated as the pristine site because of very low measured ARGs abundances (no detected sul2 genes, strA and strB genes). The study of Xu et al. (2014) was about relative ARGs abundance analyses upstream, WWTP effluent and downstream of a river in Beijing, China. Marti et al. (2013) examined the relative ARGs abundances of the Ter River in Catalonia, Spain. Pruden et al. (2006) investigated the La Poudre River in northern Colorado, USA. Urban areas downstream of the river are analyzed as urban river samples. The origin of the river is sampled as pristine river sample.

Table 10-1: ARB removal efficiencies of different treatment processes. The colors in the table represent the changes of the total ARB abundances (green color = ARB decrease, red color = ARB increase). The abbreviations FC, HB and ENT are short for Fecal Coliforms, Heterotroph Bacteria and Enterococci. NR is the shortening for 'no results.'

Tetracycline Resistance Bacteria Removal Treatment Processes:	Water Purification (WP): Wastewater Treatment (WT)	Technical Resources	Total Abundace Change [log10 CFU/ 100 mL]			Relative abundance Change [%]			References
			FC	HB	ENT	FC	HB	ENT	
Conventional wastewater treatment									
(mechan.+biolog. Stage)	WT	activated sludge	≈1.8	≈ 1.1	≈1.1	≈ 77	≈ 88	≈ 97	Novo, Manaia (2010); Galvin et al. (2010)
	WT	trickling filter	≈ 0.6	≈ 0.4	≈0.9	NR	≈ 50	≈ 34	Novo, Manaia (2010); Galvin et al. (2010)
	WT	submerged aerated filter	≈ 2.5	≈ 2.2	≈2.2	NR	≈ 35	0	Novo, Manaia (2010); Galvin et al. (2010)
	WT	mechan.+biolog. Stage+microfiltration	NR	≈ 3	NR	NR	NR	NR	Munir et al. (2011)
	WT	mechan.+biolog. Stage+microfiltration	≈ 2.7 - 5.4	NR	NR	NR	NR	NR	Le et al. (2018)
Physical separation processes									
sand filtration	WP	not mentioned	≈0.28	≈ 0.24-0.45	NR	≈ 61	≈ 2.4	NR	El-Zanfaly (2015)
	WT	sand filtr as a ozone postreatment	NR	NR	NR	≈ 17	NR	NR	Lüddeke et al. (2015)
	WT	sand filtr as a ozone postreatment	NR	≈ 1.1	NR	NR	NR	NR	Czekalski et al. (2016)
GAC	WP	not mentioned	NR	≈ 0.5-0.59	NR	NR	≈0.1-6	NR	El-Zanfaly (2015)
	WT	gac filtr as a ozone postreatment	NR	NR	NR	≈ 12	NR	NR	Lüddeke et al. (2015)
micro-/ ultrafiltration	WT	micro/ultrafiltration, pore size 200 kDa	NR	NR	NR	≈ 100	NR	≈ 100	Luczkiewicz et al. (2011)
mechan.+biolog. Stage+microfiltration	WT	MBR with pore size 0.1 to 0.4 µm	NR	≈ 3	NR	NR	NR	NR	Munir et al. (2011)
ultrafiltration	WT	ultrafiltration, pore size 10 kDa, lab scale	≈0.9 (<LOD)	NR	NR	NR	NR	NR	Schwermer et al. (2017)
nanofiltration	WT	nanofiltration, pore size 150-400 Da, lab scale	≈0.9 (<LOD)	NR	NR	NR	NR	NR	Schwermer et al. (2017)
Oxidation and disinfection processes									
UV irradiation	WT	UV dose 3.2-110 mJ/cm²,	NR	NR	NR	≈ 97	NR	≈ 97	Luczkiewicz et al. (2011)
	WT	UV dose 45 mJ/ cm²	≈2.7	NR	NR	≈ 83	NR	NR	Meckes (1982)
	WT	UV dose 0, 5, 10, 20 mJ/ cm²	NR	NR	NR	NR	≈ 50	NR	Guo et al. (2013)
	WT	UV dose not mentioned	NR	≈ 0.2-0.4	NR	NR	NR	NR	Munir et al. (2011)
	WT	UV dose < 10 mJ/cm²	≈ 4	NR	NR	NR	NR	NR	McKinney and Pruden (2012)
ozonation	WT	Ozone dose 0.5 - 11 mg O3/l	NR	NR	NR	≈ 36	NR	≈37	Luczkiewicz et al. (2011)
	WT	ozone dose 3 mg/l	≈1.5	NR	NR	NR	NR	NR	Oh et al. (2014)
	WT	ozone dose 5 mg/l	≈2.5	NR	NR	NR	NR	NR	Oh et al. (2014)
	WT	ozone dose 7 mg/l	≈5.5	NR	NR	NR	NR	NR	Oh et al. (2014)
	WT	0.73 g O3 g DOC−1	NR	NR	NR	≈7	NR	NR	Lüddeke et al. (2015)
	WT	0.55 g O3 g DOC−1	NR	1.4-1.8	NR	NR	NR	NR	Czekalski et al. (2016)
chlorination	WT	chlorine dose 6-10 mg/l	≈0.5	NR	NR	NR	NR	NR	Oh et al. (2014)
	WT	chlorine dose 30 mg/l	≈1	NR	NR	NR	NR	NR	Oh et al. (2014)
	WT	sod. Hypochlorid 2 mg/l	NR	≈ 1.5	NR	NR	0 ≈ 10	NR	Huang et al. (2011)
	WT	sod. Hypochlorid 5-10 mg/l	NR	≈ 3.5	NR	NR	0	NR	Huang et al. (2011)
	WT	chlorine dose 25 mg Cl2 / l x 2 min	NR	≈ 4	NR	NR	NR	NR	Huang et al. (2011)
	WT	chlorine dose 5 mg Cl2 / l x 10 min	NR	≈ 3.5	NR	NR	NR	NR	Huang et al. (2011)
	WT	chlorine dose 2 mg Cl2 / l x 25 min	NR	≈ 3	NR	NR	NR	NR	Huang et al. (2011)
	WT	chlorine dose not mentioned	NR	≈ 0.2-0.8	NR	NR	NR	NR	Munir et al. (2011)

Table 10-2: ARGs removal efficiencies of different treatment processes. The colors in the table represent the changes of the total ARGs abundances (green color = ARG decrease, red color = ARG increase). The abbreviations tet, sul and bla genes are short for tetracycline, sulphonamide and beta-lactamase resistance genes. NR is the shortening for 'no results.'

Treatment Processes:	Water Purification (WP); Wastewater Treatment (WT)	Technical Resources	Tet Genes Removal [log10 genes/ 100 mL]	Sul Genes Removal [log10 genes/ 100 mL]	Bla Genes Removal [log10 genes/ 100 mL]	References
Conventional wastewater treatment						
(mechan.+ activated Stage) CAS	WT	not mentioned	≈ 2 - 4.5	≈1.5 - 3	≈1 - 3	Zhang et al. (1998); Laht et al. (2014); Munir et al. (2011); Gao et al. (2012); Alexander et al. (2015); McConnell et al.
mechan.+biolog. Stage + microfiltration	WT	MBR, pore size 0.1 to 0.4 µm	≈ 5 - 6	≈ 7	NR	Du et al. (2015)
mechan.+biolog. Stage + ultrafiltration	WT	MBR, pore size 0.04 µm	≈ 6 - 7	≈ 2.6	NR	Munir et al. (2011)
fluidized bed reactor+ ultrafiltration	WT	anMBR, pore size 100 kDa	≈ 4	≈ 3.6	NR	Kappell et al. (2018)
biolog. Stage + microfiltration	WT	anMBR, pore size 0.3 µm, lab scale	NR	NR	2.76 to 3.84	Cheng and Hong (2017)
biolog. Stage + microfiltration	WT	anMBR, pore size 0.4 µm	≈ 4 - 4.5	≈ 2.5 - 4	≈ 3 - 5	Le et al. (2018)
Biological treatment processes						
Biological Pre-Treatment	WP	not mentioned	≈ 0 - 1	≈ 0 - 0.5	≈ 0 - 0.5	Zhang et al. (2016)
BAC	WP	not mentioned	≈ 0	≈ 0.2	NR	Xu et al. (2016)
activated sludge	WT	not mentioned	≈ 3 - 3.5	≈ 2.5	NR	Gao et al. (2012)
Physical treatment processes						
flocculation + sedimentation	WP	not mentioned	≈ 1	≈ 0.8	≈ 0.5	Zhang et al. (2016)
Grease trap	WT	not mentioned	≈ 0.2 - 0.4	≈ 0.6	NR	Gao et al. (2012)
Primary Settlement	WT	not mentioned	≈ 1.3	≈ 0.1	NR	Gao et al. (2012)
sand filtration	WP	not mentioned	≈ 0.1	≈ 0.1	NR	Xu et al. (2016)
	WP	not mentioned	≈ 0.5 - 2	≈ 0.5 - 2.5	≈ 1 ≈ 1.5	Zhang et al. (2016)
	WT	ozone post treatment	NR	≈ 0.4	NR	Czekalski et al. (2016)
	WT	ozone post treatment	NR	NR	≈ 0.2	Alexander et al. (2016)
		EBCT = 20 - 30 min				
microfiltration	WT	pore size 0.1-0.45 µm	NR	NR	≈ 1	Breazeal et al. (2013)
ultrafiltration	WT	pore size 40 nm	≈ 7	NR	NR	Böckelmann et al. (2009)
	WT	pore size 100 kDa	NR	NR	≈ 1.7	Breazeal et al. (2013)
	WT	pore size 10 kDa	NR	NR	≈ 4.9	Breazeal et al. (2013)
nanofiltration	WT	pore size 1 kDa	NR	NR	> 5.9	Breazeal et al. (2013)
Oxidation and disinfection processes						
UV irradiation	WT	UV dose 25 - 400 mJ/ cm²	≈ 0.5 - 3	NR	NR	McKinney and Pruden (2012)
	WT	UV dose 62.4 - 249.5 mJ/cm²	≈ 0.1 - 0.58	≈ 0.1 - 0.4	NR	Zhang et al. (2015)
	WT	0 - 12,477 mJ/ cm², lab scale	≈ 2.48	≈ 2.70	NR	Zhuang et al. (2014)
ozonation	WP	pre-ozonation 0,3-0,6 mg O3/ L	≈ 0	≈ 0.2	NR	Xu et al. (2016)
	WP	ozonation 1,5-1,7 mg O3/ L	≈ 0.5	≈ 0.1	NR	Xu et al. (2016)
	WT	ozone dose 0.55 g O3/ g DOC	NR	≈ 0.1	NR	Czekalski et al. (2016)
	WT	ozone dose 0.5-0.9 g O3/ g DOC	NR	NR	≈ 0.16	Alexander et al. (2016)
	WT	ozone dose 1.5 mg O3 s/ L; lab scale	NR	≈ 1.4	NR	Czekalski et al. (2016)
	WT	0.55 mg O3/ mg DOC; full scale	≈ 0.1	≈ 0.1	NR	Czekalski et al. (2016)
	WT	ozone dose 177.6 mg O3/ L, lab scale	≈ 2.5	≈ 1.75	NR	Zhuang et al. (2014)
chlorination	WP	chlorine dose not mentioned	≈ 1.4	≈ 1.7	NR	Xu et al. (2016)
	WT	chlorine dose not mentioned	≈ 0.1 ≈ 0.3	≈ 0.1 ≈ 0.3	NR	Fahrenfeld et al. (2013)
	WT	NaClO dose 5-20 mg/ L, t = 30 min	≈ 0.3	≈ 0.3	NR	Zhang et al. (2015)
	WT	NaClO dose 25-30 mg/ L, t = 30 min	≈ 0.95-1.5	≈ 0.8-1.2	NR	Zhang et al. (2015)
	WT	NaClO dose 160 mg/l, lab scale	≈ 3.24	≈ 3.16	NR	Zhuang et al. (2014)
	WT	NaClO dose 20 mg/ L, 120 min CT, lab scale	≈ 1.2	≈ 0.7	NR	Zhuang et al. (2014)
	WT	NaClO dose 40 mg/ L, 60 min CT, lab scale	≈ 2.25	≈ 1.78	NR	Zhuang et al. (2014)
	WT	NaClO dose 80 mg/ L, 30 min CT, lab scale	≈ 3.00	≈ 2.9	NR	Zhuang et al. (2014)
	WT	chlorine dose 60 mg Cl2 min/ L	≈ 0.3	NR	NR	Yuan et al. (2015)
	WT	chlorine dose 300 mg Cl2 min/ L	≈ 0.4	NR	NR	Yuan et al. (2015)
	WT	chlorine dose not mentioned	NR	NR	≈ 0.01 - 0.17	Yang et al. (2016)

10.4 Supplementary information for Chapter 5

10.4.1 Wastewater parameter of WWTP Steinhäule

During MF and UF studies, wastewater parameters of the secondary and tertiary effluents of the full-scale WWTP Steinhäule were measured by online measurement devices. The range of measured dissolved organic fractions (UVA_{254}), total nitrogen and ortho-phosphate during the studies are illustrated in the Table 10-3.

Table 10-3: Secondary effluent (SE) and tertiary effluent (SE+PAC+SF) with arithmetic average of wastewater parameters measured during MF and UF studies.

values	TE	SE
UVA_{254} [m^{-1}]	4.73 ± 1.20	9.30 ± 1.14
N_{total} [mg/L]	4.47 ± 0.98	4.02 ± 0.92
PO_4-P [mg/L]	0.08 ± 0.05	0.37 ± 0.16

10.4.2 Role of the fouling layer for additional AMR removal

The data series of the Figure 5-4 were proved for significance mean values using pair samples T-tests (Table 10-4). The study results revealed that the correlation values for the pair samples T tests are moderate to significant at 0.57 to 1.00. The data series of 55 minutes' samples had lower but not significant lower *sul1* gene, *ermB* gene and TCC mean values than the data series of 5 minutes' samples. In contrast, *16S rRNA* gene and HNAC value had almost the same mean values comparing data series of 5 and 55 minutes' samples. Remarkable was the fact that *vanA* gene had significant lower abundances in the data series of 55 minutes' samples than the data series of 5 minutes samples.

Table 10-4: Results of pair samples T-Tests of sul1, ermB, vanA, 16S rRNA genes and HNAC, TCC values (experiment V

Parameter	sul1 [gene copies/100 mL]	ermB [gene copies/100 mL]	vanA [gene copies/100 mL]	16SrRNA [gene copies/100 mL]	HNAC [1/100 mL]	TCC [1/100 mL]
Mean value 5 min	4490	737	3844	113264	221600	6496467
Mean value 55 min	3384	500	500	133542	226833	4082933
Correlation	0.95	-	-	0.57	1	1
P-Value	0.166	0.356	0.004	0.548	0.629	0.187

10.4.3 Role of feed water quality for UF filtrate water quality

For statistical data analyses of the analyzed values of Figure 5 independent samples T-tests were conducted (Table 10-5). The data analyses demonstrated that *sul1*, *ermB*, *vanA* and *16S rRNA* genes as well as HNAC and TCC had significant different mean values comparing the feeds secondary and tertiary effluent. While all parameters of secondary effluent had significant higher mean values than tertiary effluent, mean values of *sul1*genes and TCC value were significant higher in ultrafiltered secondary effluent compared to ultrafiltered tertiary effluent ($p < 0.05$). Despite of significant HNAC and *16S rRNA* gene values in the feeds, the filtrates had almost the same mean values of HNAC and *16S rRNA* gene.

Table 10-5: Results of independent samples T-tests of sul1, ermB, vanA, 16S rRNA genes and HNAC, TCC values (experiment VI)

Parameter	sul1 [gene copies/100 mL]	ermB [gene copies/100 mL]	vanA [gene copies/100 mL]	16SrRNA [gene copies/100 mL]	HNAC [1/100 mL]	TCC [1/100 mL]
Mean value SE	1044646	149452.7	20012.2	330000000	410000000	1300000000
Mean value TE	215132	50258.4	6799.3	48846771	30816783	180000000
P-value	0.001	0.001	0.000	0.005	0.010	0.000
Mean value UF SE	3554	603.5	2244	128940	332900	4728115
Mean value UF TE	927	564.8	1285	162223	325800	940250
P-value	0.029	0.744	0.164	0.194	0.947	0.007

10.4.4 Absolute removal efficiencies of different parameters of the MF and UF process using secondary effluent as feed

To analyze the effect of different pore sizes leading to different AMR removal efficiencies, MF and UF with different pore sizes were employed. AMR analyzes were performed in the SE feed water as well as in the MF and UF filtrates, while MF and UF modules were operated in parallel using the same feed. The results are illustrated in Figure 10-1. While slightly higher TCC removals were analyzed by UF compared to MF, significantly higher HNAC and *16S rRNA* gene removals were observed using UF compared to MF. While MF and UF reduced *ermB* gene by about 2.7 and 2.8 log units, *sul1* gene removal was higher using UF (2.9 log units) compared to MF (2.1 log units). Significant lower *vanA* gene removal by MF (1.1 log units) and UF (1.2 log unit) were observed.

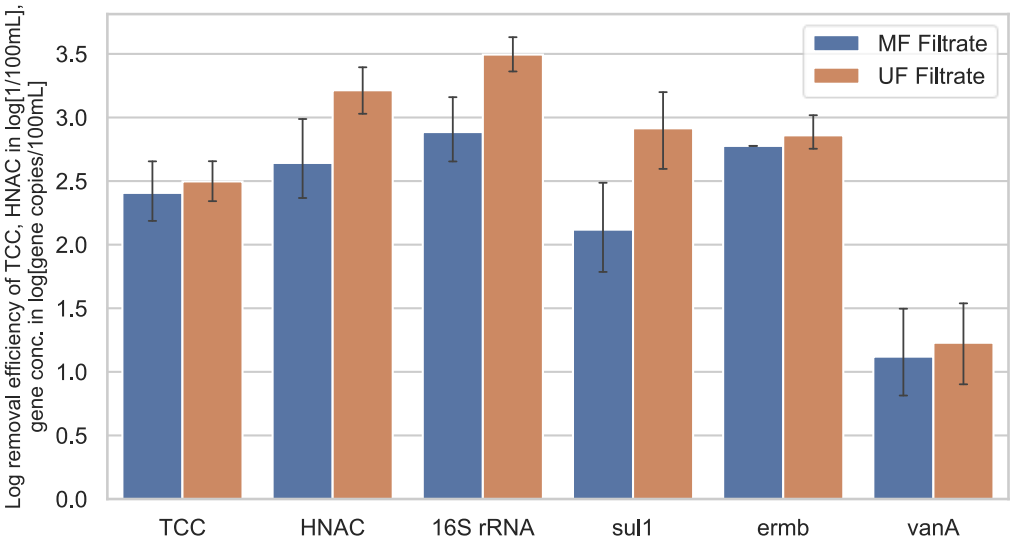


Figure 10-1: The absolute removal differentials of different parameters of the MF and UF process are illustrated while using secondary effluent from WWTP Steinhäule as feed water.

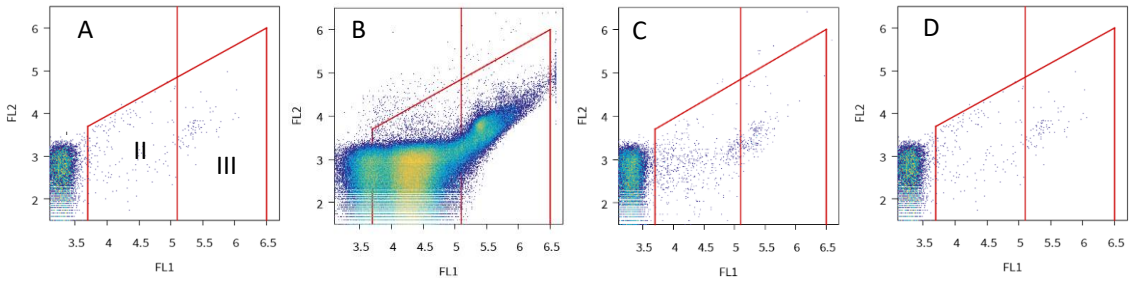
Independent samples T-tests of the analyzed values of Figure 6 were performed for significant mean value evaluation (Table 10-5). The data analyses in Table 10-6 illustrated that *ermB* and *vanA* genes had no significant different mean values. Indeed, *ermB* and *vanA* genes had almost the same abundances comparing MF and UF samples. In contrast, a clear trend in TCC mean values were observed whereas UF samples had about 37 % lower TCC values than MF samples. However, significant different mean values could be confirmed according to *sul1* gene, *16S rRNA* gene and HNAC value comparing MF and UF samples ($p < 0.05$).

Table 10-6: Results of independent samples T-tests of sul1, ermB, vanA, 16S rRNA genes and HNAC, TCC values (experiment VII)

Parameter	sul1 [gene copies/100 mL]	ermB [gene copies/100 mL]	vanA [gene copies/100 mL]	16SrRNA [gene copies/100 mL]	HNAC [1/100 mL]	TCC [1/100 mL]
Mean value MF SE	15136.8	500	2417.7	567215.9	1494257	7465050
Mean value UF SE	3605.3	603.5	1932.5	128939.9	332900	4728115
P-value	0.036	0.492	0.521	0.010	0.026	0.175

10.4.5 Comparison of TCC and HNAC abundance in Secondary effluent as well as in MF and UF filtrate

Flow cytometry measurements were performed in the SE feed water as well as the MF and UF filtrates, while MF and UF modules were operated in parallel using the same feed in order to compare retention of bacteria measured as HNAC. The results are illustrated in Figure 10-2. While MF reduced TCC values by about 2.6 log units, UF could reduce TCC values by about 3 log units.



Example graph and description	Secondary effluent (SE) 1:3 dilution	MF effluent	UF effluent
TCC [1/100mL]	355,502,000	1,071,100	286,900
HNAC [%]	11.50	19.39	28.69

Figure 10-2: A: Structure of the flow cytometry diagram: x-axis: Fluorescence signal (FL1); y-axis: Fluorescence signal (FL2). Section I is the background signal; section II is the LNAC abundance and section III is the HNAC abundance. TCC is the sum of LNAC and HNAC. B: TCC and HNAC values of feed SE. C: TCC and HNAC values of corresponding MF filtrate. D: TCC and HNAC values of corresponding UF filtrate.

10.4.6 Live/Dead bacteria analyses

Live/dead bacteria analyses were undertaken to show living bacteria breakthrough the UF membrane. While in Figure 10-3A, the living bacteria are illustrated, the dead bacteria are presented in Figure 10-3B. The overall living and dead cells as well as 0.2 µm beads are shown in the graphical Figure 10-3C.

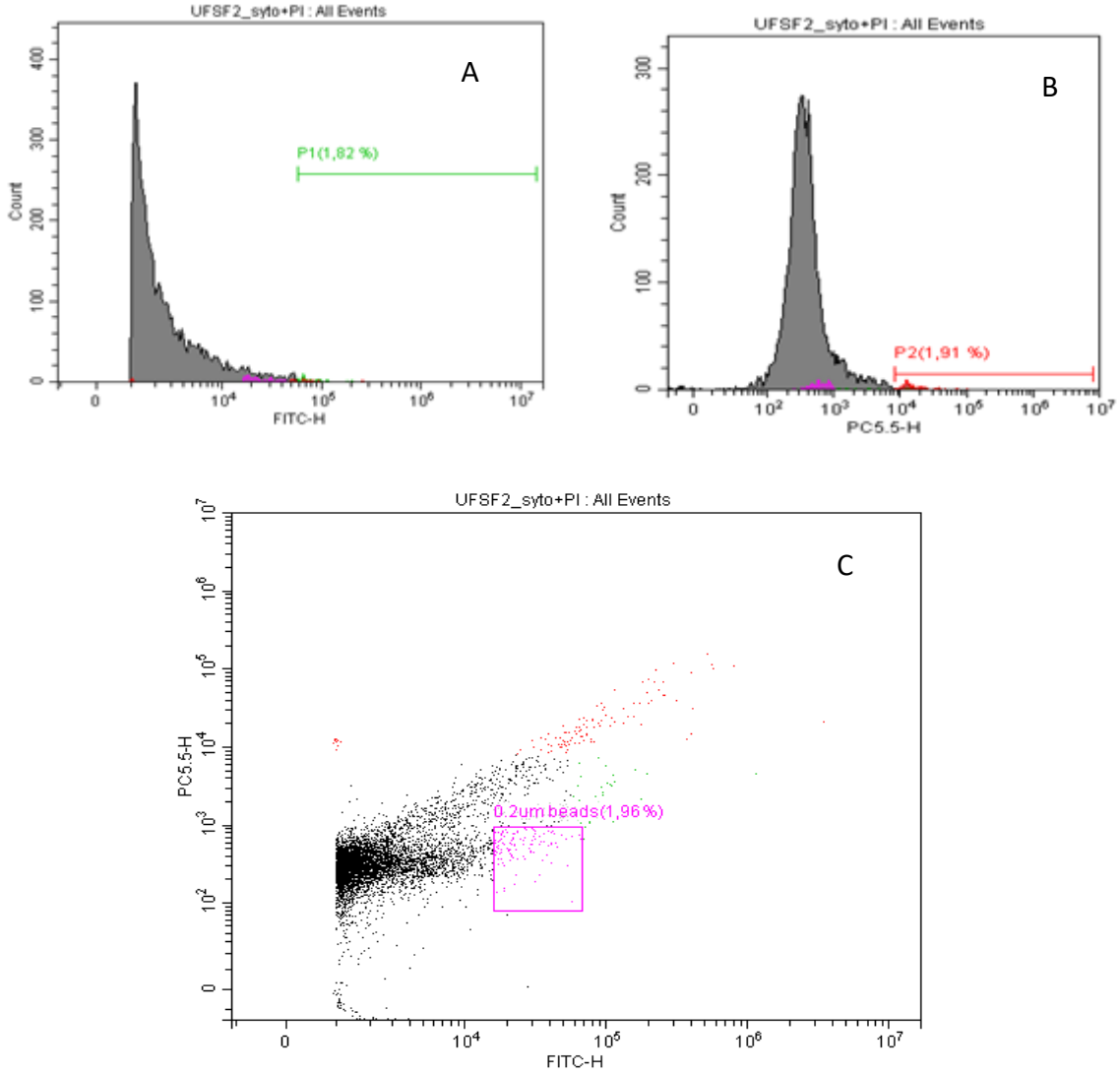


Figure 10-3: A: Living bacteria analysis of sample UF filtrate using feed tertiary effluent. Fluorescent channel FITC was applied. Sample was stained with Syto9. B: Dead bacteria analysis of sample UF filtrate using feed tertiary effluent. Fluorescent channel PC 5.5 was applied. Sample was stained with Propidiumiodide. C: graphical illustration of all detected DNA. Red dots are dead bacterial cells. Green dots are living bacterial cells. Purple dots are added 0.2 µm beads. Black dots are either LNAC or background noise.

10.4.7 Quantitative PCR standard curves

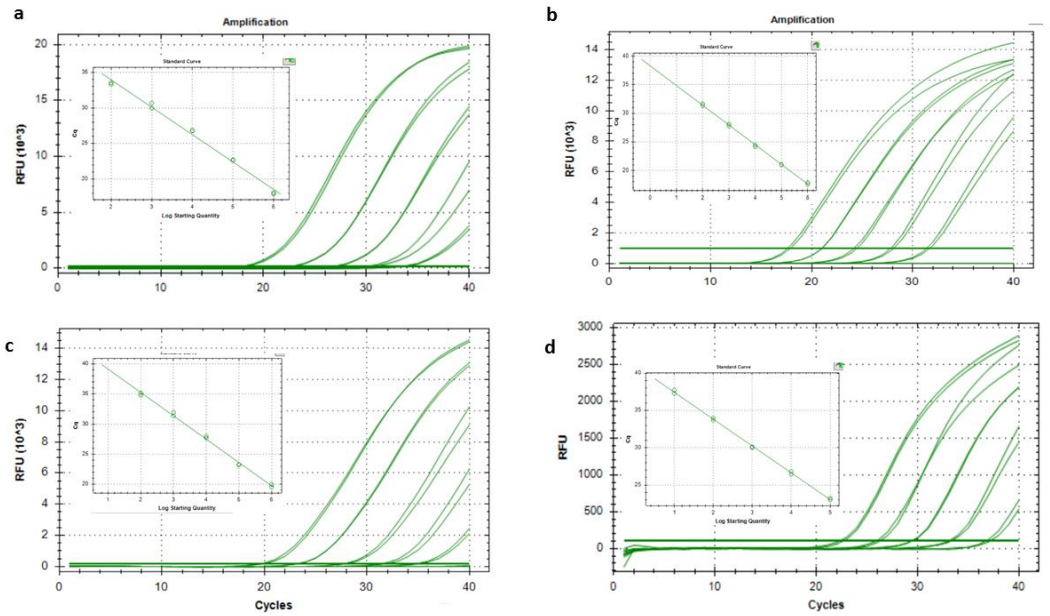


Figure 10-4: Standard curves for calibration of the quantitative PCR assays for the quantification of microbial genes. The above examples are extracted from the Bio-Rad CFX Manager (version 3.1) software for analyzing the qPCR results for a) 16S, b) ermB, c) sul1 and d) vanA gene copies. Inlets are showing the standard curves that resulted from the respective dilution series.

10.5 Supplementary information for Chapter 6

10.5.1 Wastewater parameter of WWTP Steinhäule

During UF studies wastewater parameter of the secondary and tertiary effluent of the full-scale WWTP Steinhäule were measured by online measurement devices. The range of measured dissolved organic compartments (UVA_{254}), total nitrogen and ortho-phosphate during the studies are illustrated in the Table 10-7.

Table 10-7: Secondary effluent (SE) and tertiary effluent (TE) with arithmetic average of wastewater parameters measured during UF studies.

values	TE	SE
UVA_{254} [m^{-1}]	4.73 ± 1.20	9.30 ± 1.14
N_{total} [mg/L]	4.47 ± 0.98	4.02 ± 0.92
PO_4-P [mg/L]	0.08 ± 0.05	0.37 ± 0.16

10.5.2 Role of the backwash and CEB mode on ARGs and total biomass increase in UF filtrate

The data series of the Figure 3 were proved for significance mean values using independent samples T-tests (Table 10-8). The data series of CEB samples had no significant higher TCC, HNAC, *16S rRNA* as well as ARGs mean values than the data series of backwash samples ($P > 0.05$).

Table 10-8 Results of the independent samples T-Test between first minute samples during standard filtration mode after backwash (BW) and CEB mode

Parameter	sul1	ermB	16SrRNA	HNAC	TCC
	gene copies/1	gene copies/1	gene copies/1	1/100 mL	1/100 mL
Mean value BW	162458	500	1597037	12540740	24535040
Mean value CEB	549800	500	18512405	100000000	140000000
P-value	0.433	-	0.203	0.061	0.061

Backwash mode was focused in the following investigations. Here, *16S rRNA* and *sul1* genes were measured within the first 5 minutes of standard filtration mode (Table 10-9). The study results exhibited that first and second minutes samples had higher 16S rRNA and sul1 gene abundances compared to third, fourth and fifth minute samples.

Table 10-9 Results of the independant samples T-Test of sul1 gene and 16S rRNA gene parameter of the first and second minute filtrate samples compared to the third minute filtrate samples

Parameter	sul1	16S rRNA
	gene copies/100 mL	gene copies/100 mL
Mean value first min	217239.2	6942092
Mean value third min	1536.7	17638.3
P-value	0.116	0.171
Mean value second min	3314	42812.5
Mean value third min	1536.7	17638.3
P-value	0.271	0.038

The comparison of TMP decrease after backwash and CEB mode demonstrated that CEB mode was more effective in fouling layer decrease (measured as TMP) than backwash mode (Figure 10-5).

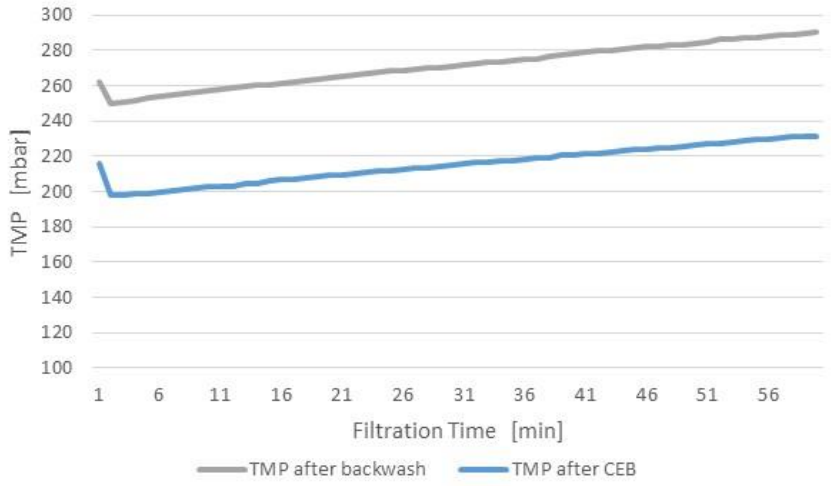
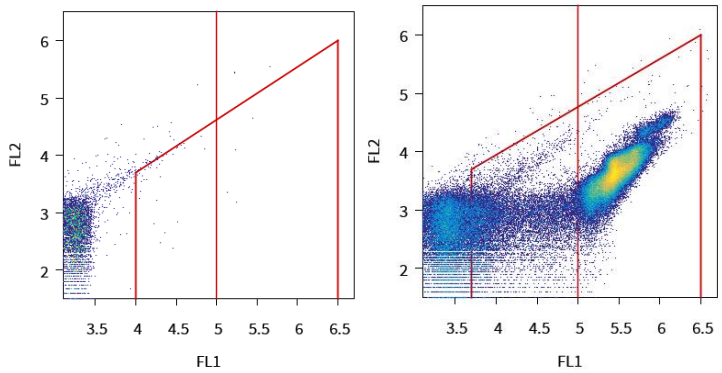


Figure 10-5: Typical TMP increase within 60 minutes of a standard filtration mode after backwash mode (grey line) and CEB mode (blue line) using secondary effluent as feed

10.5.3 Role of the water quality of the filtrate tank for the backwash and CEB mode and consequences on ARGs and total biomass increase in UF filtrate

TCC and HNAC analyzes were executed in filtrate water of the UF filtrate tank directly after 24 hour CIP and after 14 days of continuous UF operation. The study results of TCC and HNAC analyzes by flow cytometry are illustrated as graphical plots in Figure 10-6.



UF filtrate: example graph	UF filtrate tank (blank sample)	UF filtrate after 14 days
TCC [1/mL]:	546	3,164,170
HNAC [%]:	8.06	62.67

Figure 10-6 Graphical plots of flow cytometry analyses measuring TCC and HNAC values of filtrate samples of the filtrate tank (blank sample) directly after CIP and after 14 days of continuous UF operation

For statistical data analyses of the analyzed values of Figure 6-7 independent samples T-tests were conducted (Table 10-10). The data analyses demonstrated that sul1 and 16S rRNA genes had significant different mean values comparing first minute samples taken within 1 week and 3 weeks as well as fifth minute samples taken during continuous UF operation.

Table 10-10 Results of independent samples T-Test of sul1 gene and 16S rRNA gene parameter of the first miunte filtrate samples measured within the first week and within three weeks as well as 5 minute filtrate samples of continuous UF operation

Parameter	sul1	16SrRNA
	gene copies/100 mL	gene copies/100 mL
Mean value 1 min week 1	2249	466192
Mean value 1 min week 3	338817.5	5950500
P-value	0.012	0.035
Mean value 5 min	875	43741
Mean value 1 min week 3	338817.5	5950500
P-value	0.012	0.029

10.5.4 Role of the CEB chemicals within CEB mode on ARGs and total biomass increase in UF filtrate

To analyze the effect of different CEB chemicals on the reduction of total biomass and ARGs abundances, sodium hydroxide and sodium hypochlorite were employed at train #1 and train #2 as CEB chemical. The study results revealed that AMR abundance had no significant difference. In contrast, TCC and HNAC values had significant

difference mean values sampling within the first minute, fifth minute and fifty-fifth minute of standard filtration mode (Table 10-11, Figure 10-7) and the TMP was significantly reduced by CEB using sodium hypochlorite instead of sodium hydroxide (Figure 10-8).

Table 10-11 Results of independent samples T-Test of TCC und HNAC parameter of the first, fifth and fifty-fifth filtrate samples measured after CEB mode using CEB chemical NaOH in Line 1 and NaOCl in Line 2

Parameter	TCC	HNAC
	1 /100 mL	1 /100 mL
1 min samples NaOH v. NaOCl		
Mean value NaOH	200000000	150000000
Mean value NaOCl	1311433	10700
P-value	0.000	0.012
5 min samples NaOH v. NaOCl		
Mean value NaOH	6653800	4447700
Mean value NaOCl	88466.67	2200
P-value	0.008	0.029
55 min samples NaOH v. NaOCl		
Mean value NaOH	1090233	715700
Mean value NaOCl	104400	4800
P-value	0.024	0.002

CEB NaOH 1 min TCC 189,283,300/ 100 mL

CEB NaOH 5 min TCC 9,226,600/ 100 m

CEB NaOCl 1 min TCC 1,362,200/ 100 mL

CEB NaOCl 5 min TCC 136,600/ 100 mL

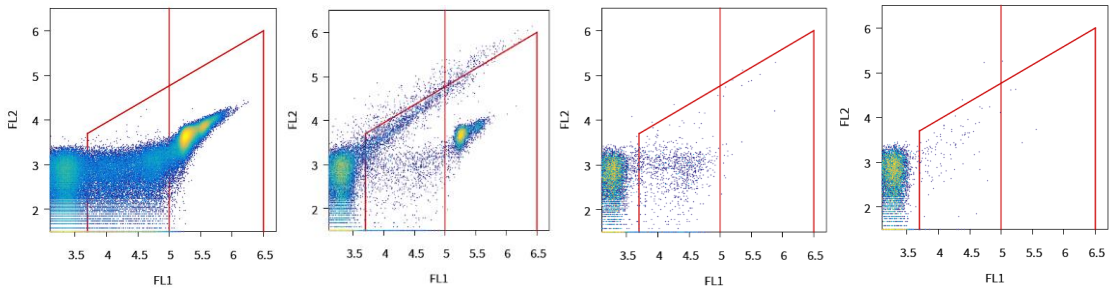


Figure 10-7 Graphical plots of flow cytometry analyses measuring TCC values of sample CEB with NaOH after 1 min, sample CEB with NaOH after 5 min, sample CEB with NaOCl after 1 min and sample CEB with CaOCl after 5 min

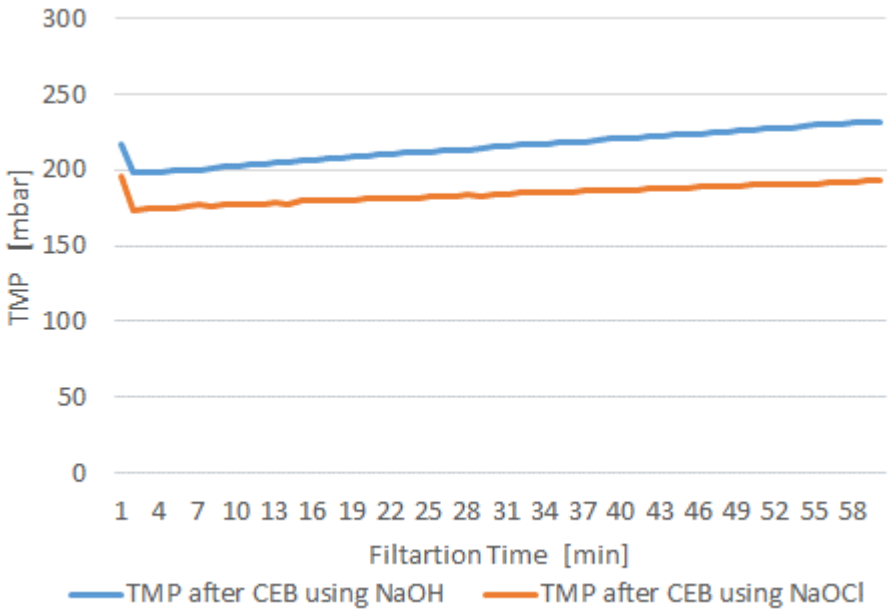


Figure 10-8 Typical TMP increase within 60 minutes of a standard filtration mode after CEB mode with CEB chemical NaOH (blue line) and CEB mode with CEB chemical NaOCl (orange line) using tertiary effluent as feed

10.6 Supplementary information for Chapter 7

10.6.1 Wastewater parameter of WWTP Steinhäule

During UF studies wastewater parameter of the secondary and tertiary effluent of the full-scale WWTP Steinhäule were measured by online measurement devices. The range of measured dissolved organic compartments (UVA_{254}), total nitrogen and ortho-phosphate during the studies of the chapters 7.4.1 and 7.4.2 are illustrated in the Table 10-12.

Table 10-12: Secondary effluent (NKB) and tertiary effluent (TE) with arithmetic average of wastewater parameters measured during UF studies.

values	NKB	TE
UVA_{254}	9.30	4.73
$[m^{-1}]$	± 1.14	± 1.20
N_{total}	4.02	4.47
[mg/L]	± 0.92	± 0.98
PO_4-P	0.37	0.08
[mg/L]	± 0.16	± 0.05

10.6.2 Role of amtibiotic concentrations in UF filtrate as factor for possibly promoting ARG associated regrowth

The antibiotic spike trial was executed whereas sulfamethoxazole and erythromycin were continuously given to the filtrate tank of train #2 whereas train #1 of the UF pilot plant was operated without antibiotic spiking in the filtrate tank of train #1. Grab samples were regularly taken from filtrate tank #1 and #2 in continuous flow mode during continuous UF operation. Grab samples were analyzed for *16 SrRNA*, *ermB* and *sul1* genes. The study results of sample analyses of train #1 are illustrated in Figure 10-9.

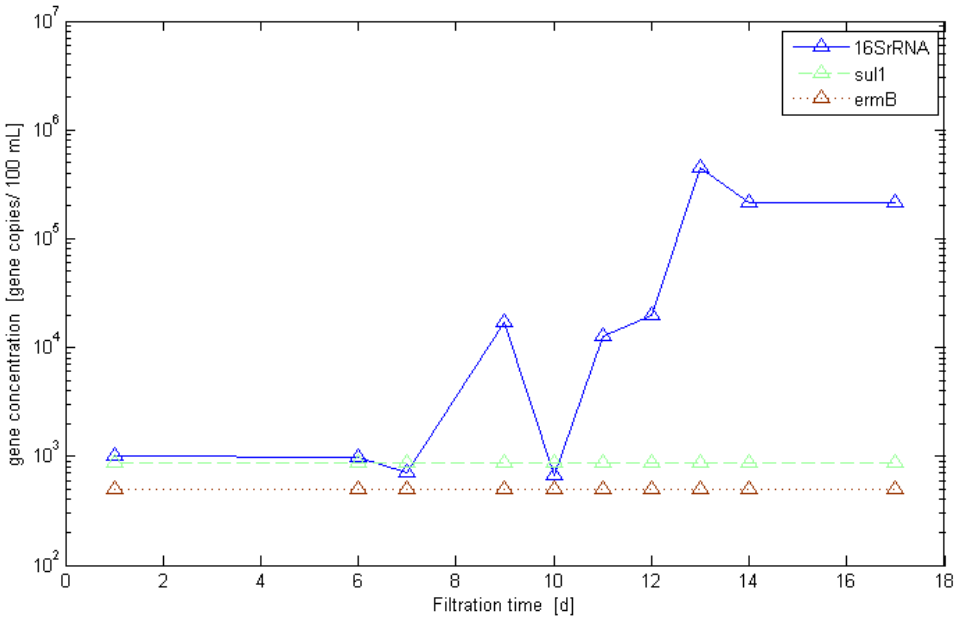


Figure 10-9 Abundances of *16S rRNA*, *ermB* and *sul1* genes in the UF filtrate from the filtrate tank of train #1 without antibiotic dosage using tertiary effluent ($AWT_{PAC\text{-}Filtration}$) as feed

UF studies were executed using AWT_{PAC} effluent as feed whereas powdered activated carbon already reduced sulfamethoxazole and erythromycin concentrations to the range of the limit of detection (LOD = 0.00005 mg/L) (Table 10-13).

Table 10-13: Sulfamethoxazole analyses of influent, secondary effluent (SE) and tertiary effluent ($AWT_{PAC\text{-}Filtration}$) of the WWTP Steinhäule

Sample	trace substances	concentration	unit
Sampling 23.7.-24.7.2019			
influent WWTP	sulfamethoxazole	0.00042	mg/L
seconday effluent	sulfamethoxazole	0.00027	mg/L
tertiary effluent ($AWT_{PAC\text{-}Filtration}$)	sulfamethoxazole	0.00015	mg/L
influent WWTP	erythromycin A	0.00061	mg/L
seconday effluent	erythromycin A	0.0006	mg/L
tertiary effluent ($AWT_{PAC\text{-}Filtration}$)	erythromycin A	< 0.00005	mg/L

A stock solution of sulfamethoxazole and erythromycin was executed for the antibiotic spike trials. After 0, 24, 48 and 120 hours samples were taken from the stock solution to analyze the sulfamethoxazole concentration in the samples (Table 10-14). The analyses should demonstrate that the stock solution concentration had no decrease with progressive filtration time.

Table 10-14: Sulfamethoxazole analyses of the stock solution after 0, 24, 48 and 120 hours to reveal that the stock solution had a constant sulfamethoxazole concentration of about 1 mg/L

Sample	Sulfamethoxazole concentration	unit
0 hour	0.97	mg/L
24 hours	1.2	mg/L
48 hours	0.94	mg/L
120 hours	1.1	mg/L

10.6.3 Role of the hydraulic retention time of the UF filtrate on ARG associated regrowth

The bacterial regrowth measured in the filtrate tanks #1 and #3 in continuous flow mode is accompanied with a reduced *16S rRNA* gene removal efficiency of the ultrafiltration process with progressive filtration time (Figure 10-10).

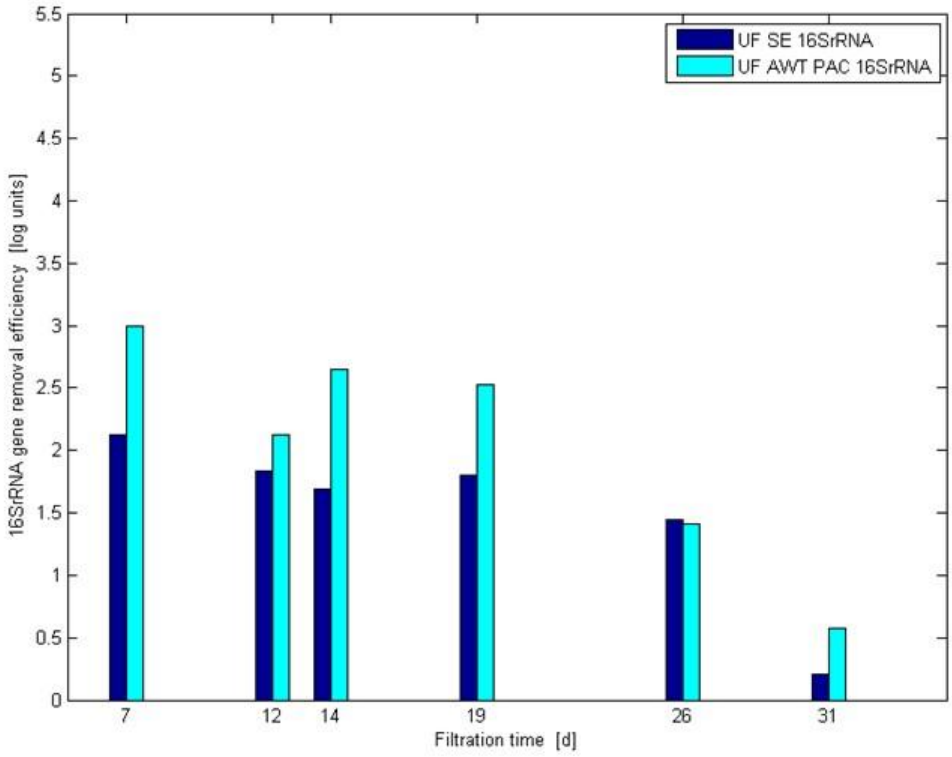


Figure 10-10 Ultrafiltration removal efficiency of *16S rRNA* genes is illustrated with progressive filtration time based on feed and corresponding filtrate samples taken in the effluent of the filtrate tank in continuous flow mode.

Dissolved organic carbon as well as total phosphorus and nitrate were measured during the ultrafiltration studies in the different filtrate samples in order to quantify the nutrient supply for the microbiome in the filtrate tanks (Table 10-15).

Table 10-15: Measured dissolved organic carbon (DOC), total phosphorus (Ptotal) and nitrate (NO3) in secondary effluent (SE) and activated carbon treated secondary effluent (AWTPAC) during the 31 days of ARG associated regrowth study.

Sample	UF SE			UF AWTPAC			unit
	DOC	Ptotal	NO3	DOC	Ptotal	NO3	
day 5	5.20	0.008	4.94	3.40	0,00	4.6	mg/L
day 7	6.28	0.036	4.47	4.80	0.021	4.94	mg/L
day 10	5.46	0.045	5.78	2.77	0.002	5.36	mg/L
day 12	5.85	0.13	4.99	3.59	0.053	5.43	mg/L
day 14	5.69	0.062	5.37	4.02	0.031	5.67	mg/L
day 19	6.04	0.042	2.82	4.5	0.016	2.97	mg/L
day 21	6.37	0.037	5.42	3.94	0.025	5.31	mg/L
day 24	3.27	0.15	2.52	3.04	0.009	2.57	mg/L
day 26	4.74	0.029	4.49	3.74	0.013	4.47	mg/L
day 28	3.99	0.025	4.42	3.21	0.016	3.55	mg/L
day 31	4.74	0.029	4.49	3.74	0.013	4.47	mg/L

Sewage flow of the WWTP Steinhäule is illustrated in the Figure 10-11 in order to show the beginning of the rainy weather events. The first rainy weather between day 6 and day 8 was really short and therefore negligible, since the activated carbon stage can treat wastewater up to the flow of 1,600 l/s. Furthermore, the DOC values between day 6 and 8 had no reduced concentrations (Table 10-15). The rainy weather event between day 22 until day 31 was characterized with 3 days with more than 2000 l/s whereas dilution effects occurred in the days 22-24, and days 26-27. In the days 24 and 28, the DOC values were measured in secondary effluent in the range of the effluent of the activated carbon stage (AWTPAC). In this case, the feed activated carbon treated secondary effluent was a mixture of activated carbon treated secondary effluent and secondary effluent.

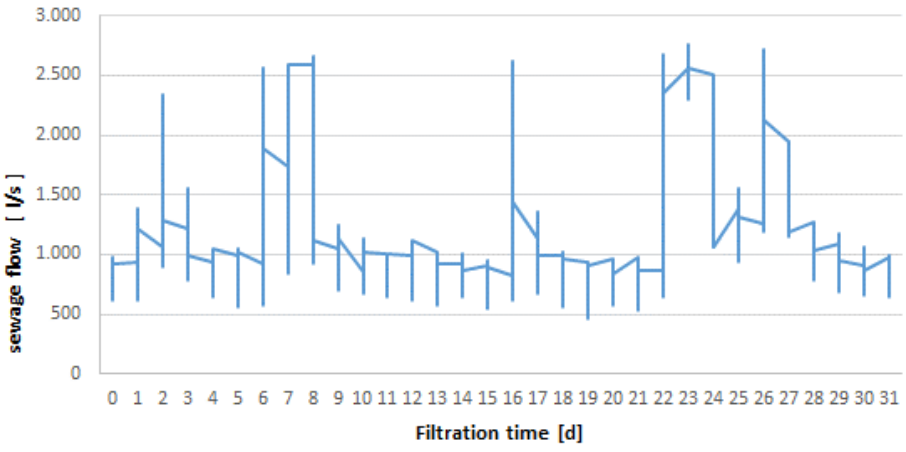


Figure 10-11 sewage flow of WWTP Steinhäule within 31 days of ARG associated regrowth studies in filtrate tanks #1, #2 and #3 in continuous flow modes

To quantify the removal efficiencies of *16S rRNA* gene, *intl1* gene and *sul1* gene of the ultrafiltration process using secondary effluent (SE) and activated carbon treated secondary effluent (AWT_{PAC}) as feeds, the filtrate water samples were sampled directly at the filtrate side of the membrane module (Figure 10-12). Therefore, the removal efficiencies do not include the 3 hours effect of hydraulic retention time or the effect of stagnant water.

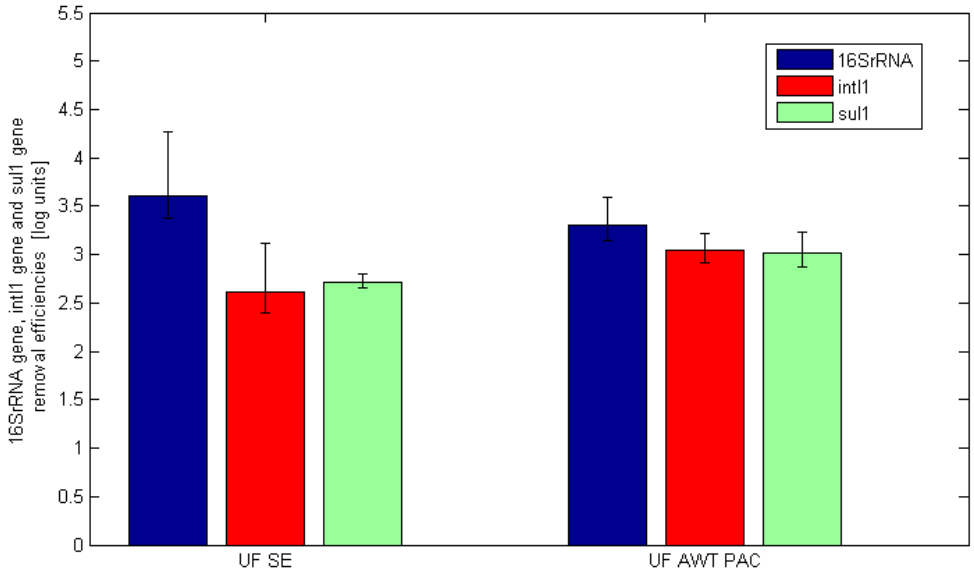


Figure 10-12 The absolute removal differentials of *16SrRNA* gene, *intl1* gene and *sul1* gene of the ultrafiltration process using secondary effluent (UF SE) and activated carbon treated secondary effluent (UF AWT_{PAC}) as feeds from the WWTP Steinhäule. 6 secondary effluent samples and 3 filtrate samples as well as 6 activated carbon treated secondary effluent samples and 3 filtrate samples were considered in the data analyses.

The data series of the Figure 7-4, 7-5 and 10-14 were proved for significance mean values using pair samples T-tests (Table 10-16). The data series of ultrafiltered secondary effluent samples had between day 0 and day 21 significant higher *16SrRNA* gene as well as *sul1* and *intl1* genes mean values than the data series of ultrafiltered activated carbon treated secondary effluent (AWT_{PAC}) samples ($P < 0.05$). The data series of ultrafiltered secondary effluent samples had between day 0 and day 21 significant higher *16SrRNA* gene as well as *sul1* and *intl1* genes mean values than the data series of ultrafiltered secondary effluent + 0.5 mg/l sodium hypochlorite samples ($P < 0.05$).

Table 10-16: Results of pair samples T-Test of *16SrRNA* gene, *sul1* gene and *intl1* gene of the filtrate samples taken from filtrate tank #1, #2 and #3 using samples of day 0 until day 21. Samples were abbreviated with ultrafiltered secondary effluent (UF SE), ultrafiltered activated carbon treated secondary effluent (UF AWTPAC) and ultrafiltered secondary effluent plus 0.5 mg/l sodium hypochlorite dosage (UF SE + 0.5 mg/L NaOCl). Samples between day 22 and day 31 were not considered for statistical evaluation due to rainy weather conditions.

Parameter	*sul1*	*intl1*	*16S rRNA*
statistical evaluation day 0-21			
Mean Value UF SE	11190411	4129290	22934860
Mean Value UF AWT$_{PAC}$	321788.3	105326.1	5261863
P-value	0.010	0.009	0.003
Mean Value UF SE	11190411	4129290	22934860
Mean Value UF SE + 0.5 mg/L NaOCl	16337.2	1952.2	21559.5
P-value	0.009	0.009	0.002

The *intl1* and *sul1* genes removal efficiencies after a hydraulic retention time of 3 hours, decreased to about 0.68 and 0.77 log units (secondary effluent as feed) as well as 2.09 log units (activated carbon treated secondary effluent as feed) after 19 days by almost dry weather conditions of continuous UF operation (Figure 10-13).

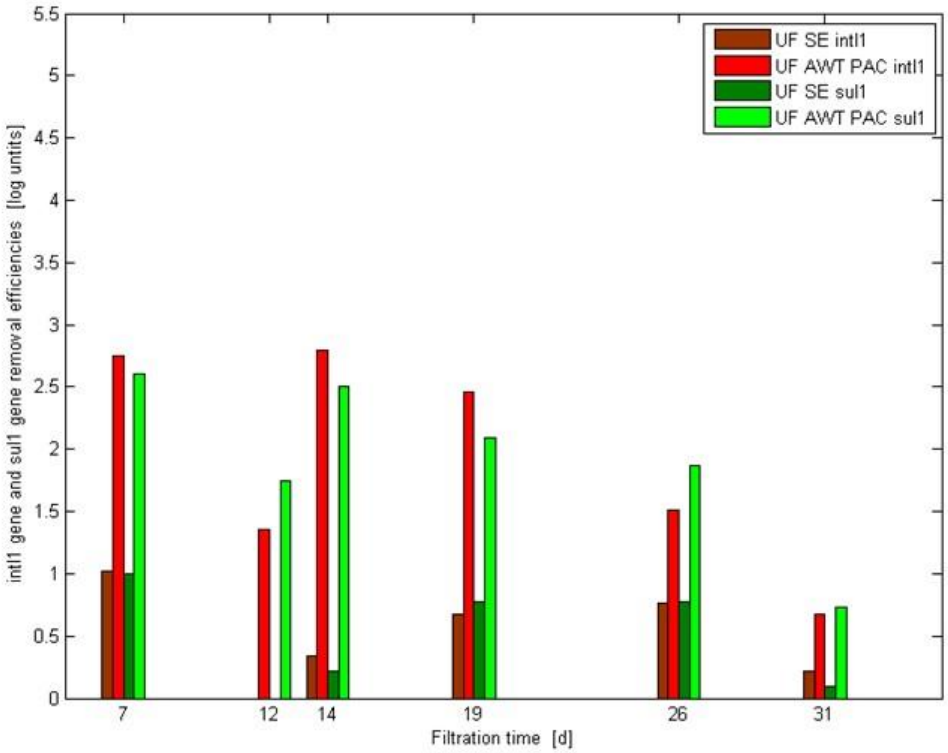


Figure 10-13 Ultrafiltration removal efficiency of *sul1* and *intl1* genes is illustrated with progressive filtration time based on feed and corresponding filtrate samples taken from the effluent of the filtrate tank in continuous flow mode.

Continuous chlorination as an additional treatment processes in the filtrate tank #2 in continuous flow mode using secondary effluent as feed was examined in order to prevent ARG associated regrowth at UF filtrate side. The total biomass measured as *16SrRNA* gene, *sul1* and *intl1* gene analyses of this study is presented in Figure 10-14 and 10-15. The sodium hypochlorite dosage resulted in significant decrease of *sul1* and *intl1* genes close to or below the limit of detection during the 31 days of continuous UF operation (Figure 10-14) and the *16SrRNA* gene, *sul1* and *intl1* genes removal efficiencies could be kept in this high level since the beginning of the UF study (Figure 10-15).

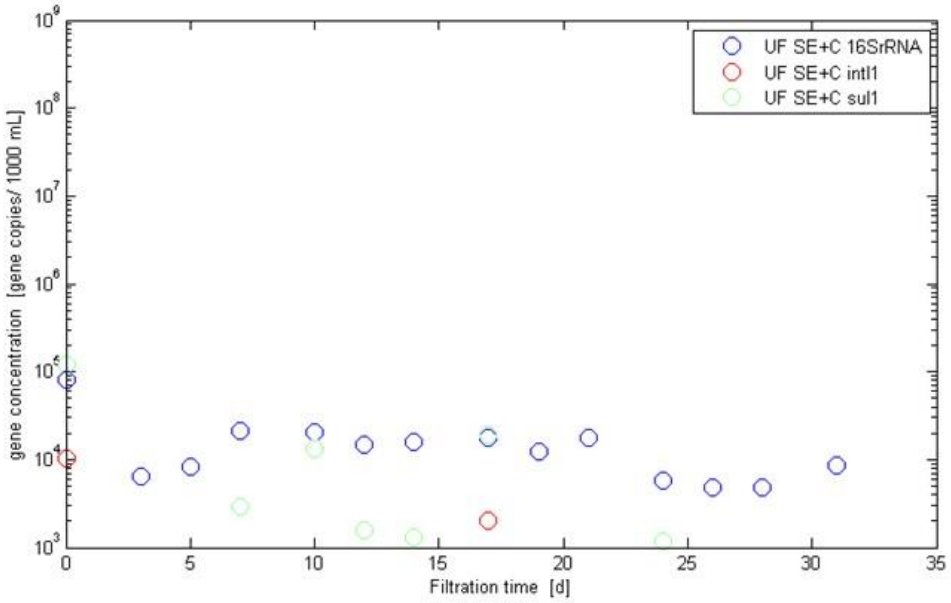


Figure 10-14 Abundances of *16SrNRA*, *sul1* and *intl1* genes in samples taken from UF filtrate tank during continuous dosing of 0.5 mg/L sodium hypochlorite using secondary effluent as feed (UF SE+C).

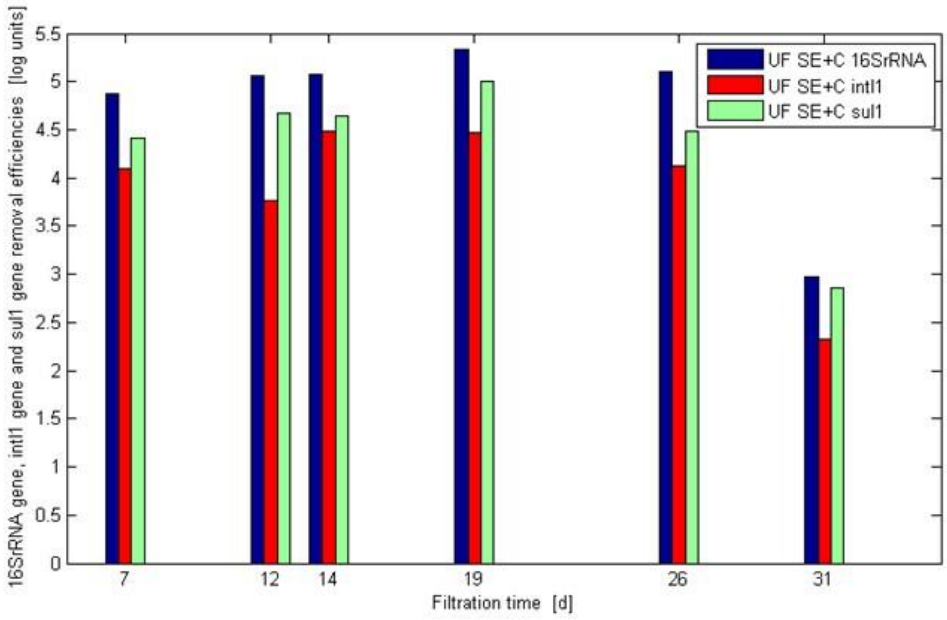


Figure 10-15 Removal efficiencies of *16SrNRA*, *sul1* and *intl1* genes in samples taken from UF filtrate tank during continuous dosing of 0.5 mg/L sodium hypochlorite using secondary effluent as feed (UF SE+C).

This study is the first to report the prevention of ARG associated regrowth in UF filtrate using sodium hypochlorite as advanced treatment process. The bacterial regrowth study of Lin et al. (2017) is in line with the study results of this study. Lin et al. (2017) reported the effect of sodium hypochlorite on typical biofilms formed in drinking water distribution systems. 0.5 – 1 mg/L sodium hypochlorite with 30 min. contact time was an appropriate dose to prevent bacterial regrowth.

10.7 Supplementary information for Chapter 8

10.7.1 Calculations for the UF full-scale plant at WWTP Steinhäule

Table 10-17: Calculation of the feed pumps

			variant 1	variant 2			variant 1	variant 2
pipe with equivalent sand roughness **ks = 0,035 mm**						ks		
diameter			0,15	1,2	m	d	2,33E-04	2,92E-05
Length of pipe			10	50	m	v =	2,40633403	4,42737489
local losses		ξ	sum ξ			Re =	2,E+05	2,E+05
90° manifolds		0,14	2,8	1,96				
Input losses		1,5	1,5	1,5		**Moody Diagram**		
Output losses		1	1	1				
			5	4		=> λ =	0,019	0,011
			Filtrate pipe	Feed pipe				
Water level Effluent Pipe				460,5	müNN			
Water level Backwash Pipe				468	müNN			
further losses			TMP	12	mWS			
			rate of flow					
Pump calculation			67,2 m3/h	5500 m3/h				
		δ=	1000	1000				
		Q =	67200	5500000				
		v =	1,06	1,35				
		hv =	0,37	0,46				
		h ges =	0,37	19,96				
		η =	0,85	0,85				
		P =	81	351905				
		Sum discharge head		**20,33**	**mWs**			
		Medium	Tertiary Effluent					

Table 10-18: Calculation of the backwash pumps

			variant 1	variant 2			variant 1	variant 2
pipe with equivalent sand roughness ks = 0,035 mm						ks		
diameter			0,15	1	m	d	2,33E-04	3,50E-05
Length of pipe			10	40	m	v =	8,48371534	2,09568277
local losses		ξ	Summe ξ			Re =	5,E+05	1,E+05
90° manifolds		0,14	2,8	1,96				
Input losses		1,5	1,5	1,5		**Moody Diagram**		
Output losses		1	1	1				
			5	4		=> λ =	0,016	0,018
			Filtrate pipe	Filtrate pipe				
Water level Effluent UF plant				463	müNN			
Water level Backwash pipe				468	müNN			
further losses			TMP	12	mWS			
			rate of flow					
Pump calculation			220,8 m3/h	2208 m3/h				
		δ=	1000	1000				
		Q =	220800	2208000				
		v =	3,47	0,78				
		hv =	3,91	0,16				
		h ges =	3,91	17,16				
		η =	0,85	0,85				
		P =	2770	121477				
		Sum discharge head		**21,07**	**mWs**			
		Medium	Tertiary Effluent					

Table 10-19: Calculation of the coagulation pumps

coagulation dosing		
average wastewater amount	100000,00	m³/d
PAC concentration	2,00	g/m³
active substance	9,00	%
density	1,37	g/cm³
active substance amount	200,00	kg Al/d
coagulatioin demand	2222,22	kg PAC/d
coagulation delivery flow	1,62	m³/d
coagulation delivery flow	67,59	l/h
Design value pumps		
Pump1	35-70	l/h
Pump2	35-70	l/h
Pump3	35-70	l/h

Table 10-20: Calculation of the NaOH pumps

CEB mode for sodium hydroxide		
QCEB Pumps	662,40	m³/h
Lenght pipe	40,00	m
volume pipe	31,40	m³
volume 120 modules	10,00	m³
sum volume	41,40	m³
dosing time	3,75	min
dosing time approx.	4,00	min
NaOH concentration	150,00	g/m³
active substance	50,00	%
density	2,13	g/cm³
active substance amount	99,36	kg/h
NaOH load	198,72	kg NaOH/h
NaOH delivery flow	93,30	l/h
Design value pumps		
Pump1	50-100	l/h
Pump2	50-100	l/h
Pump3	50-100	l/h

Table 10-21: Calculation of the H_2SO_4 pumps

CEB mode for sulfuric acid		
QCEB Pumps	662,40	m³/h
Lenght pipe	40,00	m
volume pipe	31,40	m³
volume 120 modules	10,00	m³
sum volume	41,40	m³
dosing time	3,75	min
dosing time approx.	4,00	min
H2SO4 concentration	150,00	g/m³
active substance	30,00	%
density	1,83	g/cm³
active substance amount	99,36	kg/h
H2SO4 load	331,20	kg H2SO4/h
H2SO4 delivery flow	180,98	l/h
Design value pumps		
Pump1	100-200	l/h
Pump2	100-200	l/h
Pump3	100-200	l/h

Table 10-22: Hydraulic calculation of the pipes from the discharge to the danube to the backwash tank in continuous flow mode in case of dry weather flow (5490 m³/h) and maximum flow (14274 m³/h)

Q dry weather = 1525 l/s (5490 m³/h)

Effluent Danube to backwash tank ultrafiltration

Station	comments	Q_d / Q_m	pipe-roughness	pipe size	A	v	Js	L		hr = Jp * L	$v^2/2g$	inlet losses	outlet losses	other losses	ΣHe	sole station X	sole station X-1	WL station X	WL station X-1	comments
	1	[l/s]			[m²]	[m/s]	[‰]	[m]		[m]	[m]	[m]	[m]	[m]	[m]	[m ü. NN]	[m ü. NN]	[m ü. NN]	[m ü. NN]	
Rückstaufaktor:																			460,4	
Water Level Danube																				
pipe	**pipes fullfilled**	1525	k_b = 1,0mm	DN 2000	3,14	0,37	0,11	30		0,003	0,007	0,5	1	0,5	0,017	458,35	458,40	460,40	460,42	
pit	**pipes fullfilled**	1525	k_b = 1,0mm		3,14	0,49				0,000	0,012	0,5	1	0	0,018	458,40	458,42	460,42	460,44	
pipe	**pipes fullfilled**	1525	k_b = 1,0mm	DN 2000	3,14	0,49	0,11	90		0,010	0,012	1	1	1	0,046	457,80	457,70	460,44	460,48	
spillway		1525	bride of water fall [m] =		3	c =	0,67	μ =	0,64								461,00	completely water fall		
									hü =	0,544									461,54	
																		top ground surface = 463,00 m ü NN		

Q max = 3965 l/s (14274 m³/h)

Effluent Danube to backwash tank ultrafiltration

Station	comments	Q_t / Q_m	pipe-roughness	pipe size	A	v	Js	L		hr = Jp * L	$v^2/2g$	inlet losses	outlet losses	other losses	ΣHe	sole station X	sole station X-1	WL station X	WL station X-1	comments
	1	[l/s]			[m²]	[m/s]	[‰]	[m]		[m]	[m]	[m]	[m]	[m]	[m]	[m ü. NN]	[m ü. NN]	[m ü. NN]	[m ü. NN]	
Rückstaufaktor:																			460,4	
Water level danube																				
pipe	**pipes fullfilled**	3965	k_b = 1,0mm	DN 2000	3,14	0,95	0,7	30		0,021	0,046	0,5	1	0,5	0,114	458,35	459,27	460,40	460,51	
pit	**pipes fullfilled**	3965	k_b = 1,0mm		3,14	1,26				0,000	0,081	0,5	0,2	0	0,057		459,45	460,51	460,57	
pipe	**pipes fullfilled**	3965	k_b = 1,0mm	DN 2000	3,14	1,26	0,7	90		0,063	0,081	1	1	1	0,307	457,80	457,70	460,57	460,88	
spillway		3965	bride of water fall [m] =		3	c =	0,67	μ =	0,64								461,00	completely water fall		
									hü =	1,029									462,03	
																		top ground surface = 463,00 m ü NN		

Table 10-23: Hydraulic calculation of the pipes from the influent of the ultrafiltration to the filter chamber of the sand filtration in case of dry weather flow (5490 m³/h) and maximum flow (14274 m³/h)

Q dry weather = 1525 l/s (5490 m³/h)

Influent ultrafiltration to influent sand filtration chamber

Station	comments	Qt Qm	pipe- roughness	pipe size	A	v	Js	L	h	hr = Jp * L	v²/2g	inlet losses	outlet losses	other losses	ΣHz	sole station X	sole station X-1	WL station X	WL station X-1	comments
Rückstaufaktor:	1	[l/s]		[m²]	[m²]	[m/s]	[‰]	[m]	[m]	[m]	[m]	[m]	[m]	[m]	[m]	[m]	[m ü. NN]	[m ü. NN]	[m ü. NN]	[m ü. NN]
Pit Influent ultrafiltration		1525																	460,50	
pipe DN 1200	**pipes fullfilled**	1525	ks = 1,0mm	3,3912	3,2	0,48	0,2	5		0,001	0,012	1	1	1	0,036	457,80	457,70	460,50	460,54	
rectangular 3 m² pipe	**pipes fullfilled**	1525	ks = 1,0mm	3 m² pipe	3	0,51	0,13	50		0,007	0,013	1	1	1	0,046	457,70	457,70	460,54	460,58	
DN 2000 pipe	**pipes fullfilled**	1525	ks = 1,0mm	DN 2000	3,14	0,49	0,12	246		0,030	0,012	0,5	0,3	1,5	0,057	457,70	458,60	460,58	460,64	
spillway of filtrate tank in continuous flow mode		1525	bride of water fall [m] =		3	c =	0,67	µ =	0,64								461,00	completely water fall		
									hü =	0,544									461,54	
DN 1200 pipe of 1 train	**pipes fullfilled**	762,5	ks = 0,5 mm	DN 1200	1,1304	0,67	0,33	5		0,002	0,023	0,5	1	0,16	0,040		459,45	461,54	461,58	
rectangular pipe	**pipes fullfilled**	763	ks = 1,0mm	DN 2600	5,32	0,14	0,04	85		0,003	0,001	0	1,2	1	0,006			461,58	461,59	
DN 400 pipe out of the filter chamber	**pipes fullfilled**	84,78	ks = 0,5 mm	DN 400	0,1256	0,67	1,3	1		0,001	0,023	0,5	1	0,77	0,054			461,59	461,64	< 465,00
			Anzahl Filterkammern: 18 Stk		Q/Kam	84,78	l/s													
					=> case: 18 out of 20 chambers are active															
Water level filter chamber			nozzle bottom																461,50	
			filter material															+	1,50	
			water overflow															+	2,00	
			water level filter chamber																**465,00**	

Q max = 3965 l/s (14274 m³/h)

Influent ultrafiltration to influent sand filtration chamber

Station	comments	Qt Qm	pipe- roughness	pipe size	A	v	Js	L	h	hr = Jp * L	v²/2g	inlet losses	outlet losses	other losses	ΣHz	sole station X	sole station X-1	WL station X	WL station X-1	comments
Rückstaufaktor:	1	[l/s]		[m²]	[m²]	[m/s]	[‰]	[m]	[m]	[m]	[m]	[m]	[m]	[m]	[m]	[m]	[m ü. NN]	[m ü. NN]	[m ü. NN]	[m ü. NN]
Pit Influent ultrafiltration		3965																	460,75	
pipe DN 1200	**pipes fullfilled**	3965	ks = 1,0mm	3,3912	3,2	1,24	1,1	5		0,006	0,078	1	1	1	0,240	457,80	457,70	460,75	460,99	
rectangular 3 m² pipe	**pipes fullfilled**	3965	ks = 1,0mm	3 m² pipe	3	1,32	0,71	50		0,036	0,089	1	1	1	0,303	457,70	457,70	460,99	461,29	
DN 2000 pipe	**pipes fullfilled**	3965	ks = 1,0mm	DN 2000	3,14	1,26	0,69	246		0,170	0,081	0,5	0,3	1,5	0,357	457,70	458,60	461,29	461,65	
spillway of filtrate tank in continuous flow mode		3965	bride of water fall [m] =		3	c =	0,55	µ =	0,64								461,00	incompletely water fall		
									hü =	1,174									462,17	
DN 1200 pipe of 1 train	**pipes fullfilled**	1983	ks = 0,5 mm	DN 1200	1,1304	1,75	2,12	5		0,011	0,157	0,5	1	0,16	0,271		459,45	462,17	462,44	
rectangular pipe	**pipes fullfilled**	1983	ks = 1,0mm	DN 2600	5,32	0,37	0,1	85		0,009	0,007	0	1,2	1	0,024			462,44	462,47	
DN 400 pipe out of the filter chamber	**pipes fullfilled**	220,3	ks = 0,5 mm	DN 400	0,1256	1,75	8	1		0,008	0,157	0,5	1	0,77	0,364			462,47	462,83	< 465,00
			Anzahl Filterkammern: 18 Stk		Q/Kam	220,33	l/s													
					=> case: 18 out of 20 chambers are active															
Water level filter chamber			nozzle bottom																461,50	
			filter material															+	1,50	
			water overflow															+	2,00	
			water level filter chamber																**465,00**	

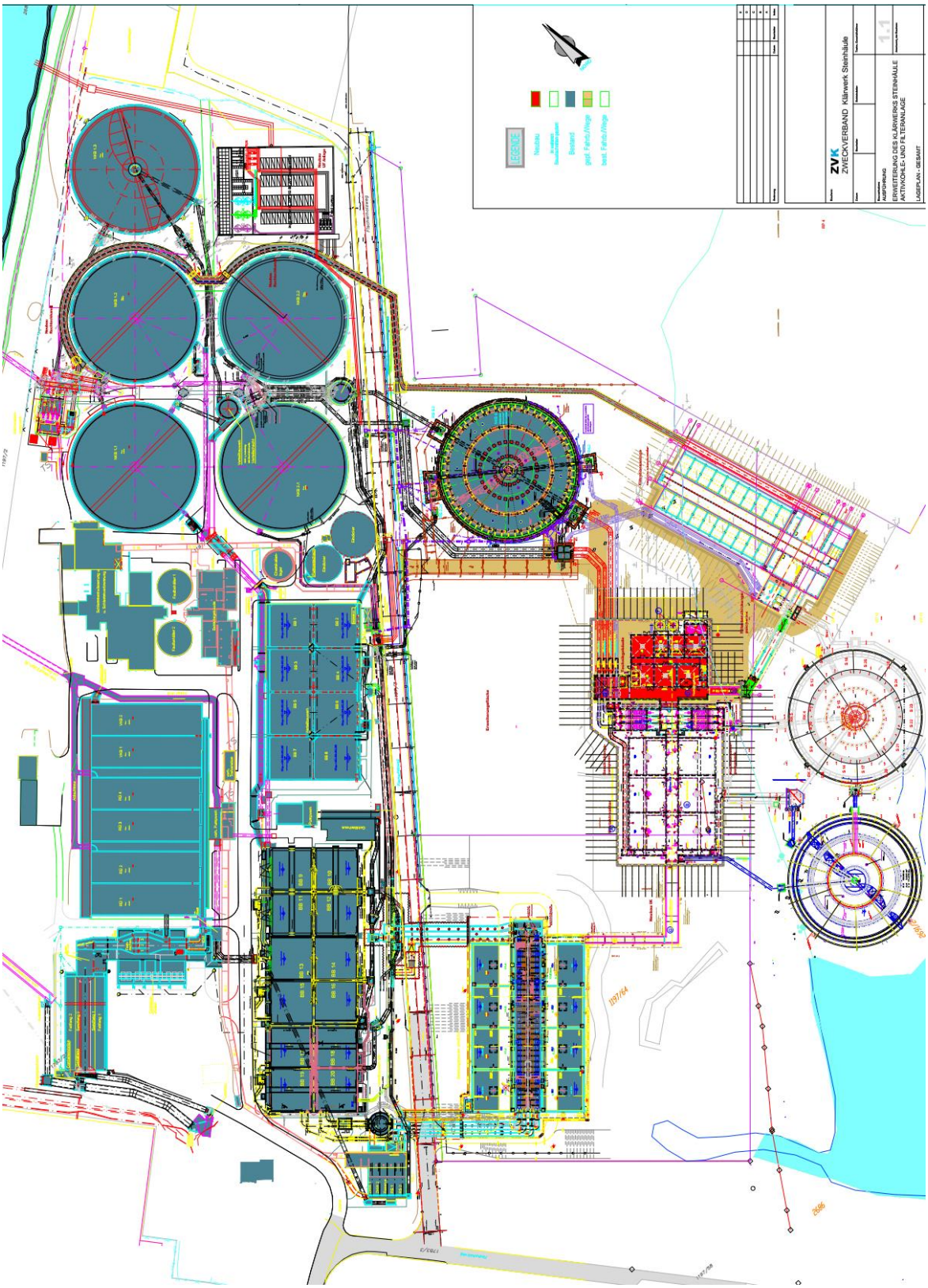


Figure 10-16 Plan of the WWTP Steinhäule with the full-scale UF plant.

10.7.2 Biofilm analyses in feed and UF filtrate samples to show bacterial shifts according to different treatment processes

Biofilm analyses were executed to analyze the phyle in the different treated wastewater (UF SE, UF AWTPAC, UF SE+C). The study results are illustrated in Figure 10-14. Biofilm samples of secondary effluent and activated carbon treated secondary effluent had a high bacterial diversity. The main protagonists of the relative abundance of bacteria species in the feed of the UF pilot plant were predominantly *Actinobacteriota, Chloroflexi, Cyanobacteria, Firmicutes,* and especially *Proteobacteria*. In contrast to the biofilm samples, the wastewater samples of secondary effluent and activated carbon treated secondary effluent exhibited different abundances of *Bacteroidota* and *Acidobacteriota*. The ultrafiltration of the feeds resulted in a significant bacteria shift to *Proteobacteria, Firmicutes* and *Bacteroidota* in filtrate and biofilm samples. Continuous 0.5 mg/L sodium hypochlorite dosage resulted in an additional bacteria shift whereas the previous predominant *Proteobacteria* significant decreased. In contrast, the other phyla like *Firmicutes, Bacteroidota* and *Actinobacteriota* increased. These bacteria species seemed to be more resistant to the chlorination process than *Proteobacteria*. Biofilm analyses in chlorinated filtrate tank revealed no bacteria growth.

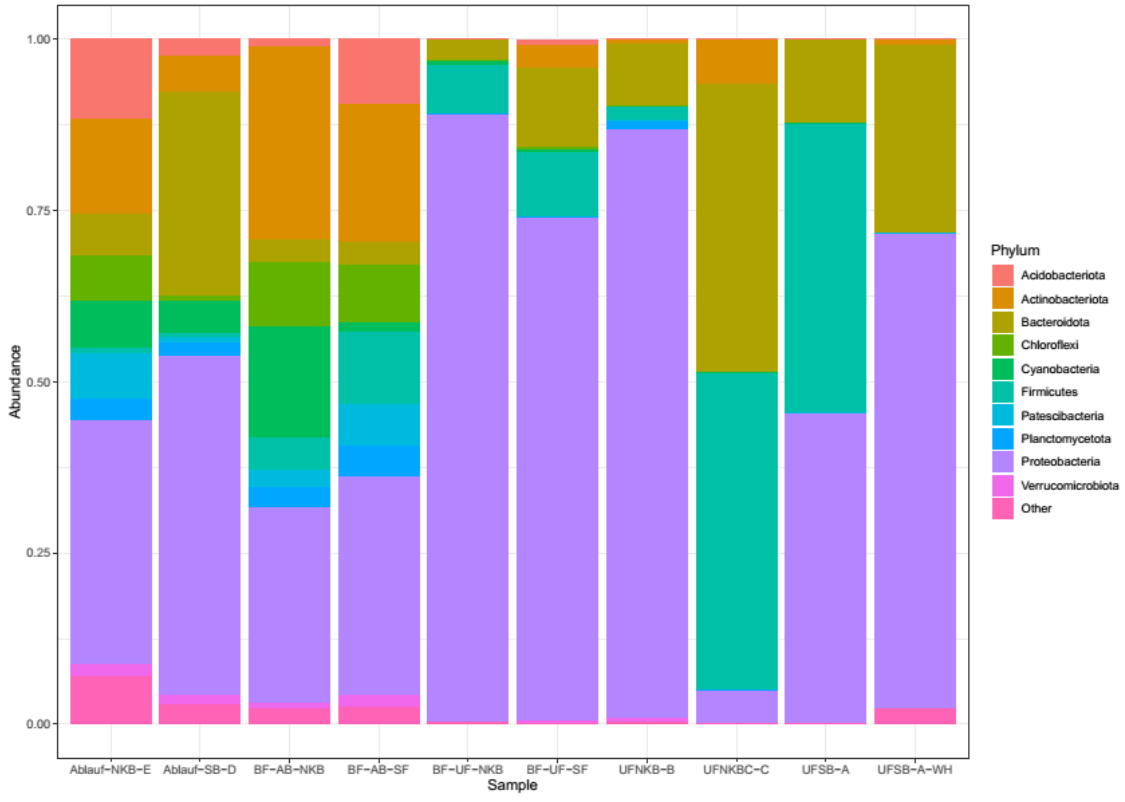


Figure 10-17 Relative abundance of bacteria species in wastewater samples after 31 days of xposure: Ablauf-NKB-E = secondary effluent; Ablauf-SB-D = activated carbon treated secondary effluent; UF-NKB-B = ultrafiltered secondary effluent; UF-NKBC-C = ultrafiltered secondary effluent plus 0.5 mg/L sodium hypochlorite; UF-SB-A = ultrafiltered activated carbon treated secondary effluent sample 1; UF-SB-A-WH = ultrafiltered activated carbon treated secondary effluent sample 2. Determination of bacteria species in following biofilm samples after 31 days of exposition: BF-AB-NKB = secondary effluent; BF-

AB-SF = activated carbon treated secondary effluent; BF-UF-NKB = ultrafiltered secondary effluent; BF-UF-SF = ultrafiltered activated carbon treated secondary effluent.

The predominant phyla of bacteria species analyzed in wastewater was already studied by Numberger et al. (2019). While the inflow of a WWTP had predominantly bacteria species of *Firmicutes, Proteobacteria, Bacteroidetes* and *Acinobacteria*, the secondary effluent was dominated by *Proteobacteria, Bacteroidetes, Firmicutes, Planctomycetes, Actinobacteria, Verrucomicrobia* and *Acidobacteria*. Since bacteria can also be distinguished in their size, especially the ultramicrobacteria (UMB) can be characterized by very small sizes with less than 0.1 µm³ in volume (Duda et al., 2012). Therefore, it seems that small bacteria species in this study like *Proteobacteria, Firmicutes* and *Bacteroidota* analyzed in UF filtrate were selected from the bacteria diversity in the feeds due to size exclusion of the pore sizes of the membrane filtration process. The breakthrough effect of bacteria through the membrane module was already reported by Maejima et al. (2018). Maejima et al. (2018) investigated in membrane filtration and reported that 141 filterable bacteria, predominantly the phyla *Proteobacteria, Bacteriodetes, Firmicutes* and *Actinobacteria* passed through the membrane with 0.22 µm of pore size.

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