Beurteilung von Ausbaumaßnahmen in Kläranlagen zur Verringerung der Ausbreitung von fakultativ pathogenen Antibiotika-resistenten Keimen in Oberflächengewässern

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DISSERTATION

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Kurzfassung

Die Dissemination von Antibiotika-Resistenzen gilt als eine der größten Bedrohungen der menschlichen Gesundheit des 21. Jahrhunderts. Während die Resistenzsituation in der Klinik gut untersucht ist und permanent überwacht wird, ist wenig über die Verbreitung von Antibiotika-resistenten Bakterien und Resistenzdeterminanten im aquatischen Ökosystem bekannt. Über Abschwemmungen von mit Gülle gedüngten Feldern und die Einleitung von gereinigtem Abwasser werden Oberflächengewässer chronisch mit Antibiotika, deren Metaboliten sowie Antibiotika-resistenten Keimen und Resistenzgenen belastet, woraus ein potentielles Gesundheitsrisiko resultiert. Thema der vorliegenden Dissertation ist es, die Resistenzsituation in fäkal belasteten Oberflächengewässern zu erfassen und verschiedene Abwasserreinigungstechnologien bezüglich ihrer Eliminationsleistung von fakultativ pathogenen und Antibiotika-resistenten Keimen zu bewerten.

Der Nachweis von gegen Reserveantibiotika-resistenten Bakterien und klinisch relevanten Resistenzdeterminanten wie *mecA* und $bla_{\text{CTX-M}}$ über den gewählten kulturbasierten Ansatz zeigt, dass diese – wenn auch im Vergleich zur Klinik in einer geringeren relativen Häufigkeit – in der aquatischen Umwelt präsent sind. Im Gegensatz zu klinischen Isolaten zeigten aus der Umwelt isolierte Staphylokokken selten high-level Resistenzen, sondern eine häufig nicht konstitutive, fein regulierte Expression der Resistenzgene. Insgesamt war die Diversität von *erm*-Genen, resultierend in einer Vielzahl von Phänotypen, in der Umwelt deutlich größer als die aus der Klinik berichtete.

Anhand der gefundenen Speziesverteilung der Staphylokokken im Oberflächengewässer war es möglich qualitativ zwischen den einzelnen Eintragsquellen zu differenzieren. Als eine effektive Möglichkeit, die erhöhte Konzentration an fakultativ pathogenen und Antibiotika-resistenten Keimen nach Starkregenereignissen im fluviatilen Ökosystem zu verringern, stellte sich die Leitung des mit Niederschlag verdünnten Abwassers, das aufgrund der Kapazitätsüberschreitung nicht in der Kläranlage behandelt werden kann, über einen Retentionsbodenfilter heraus. Die derzeit als vierte Reinigungsstufe in Kläranlagen getestete Ozonung mit nachgeschalteter Filtration konnte die Konzentration an fakultativ pathogenen und Antibiotika-resistenten *E. coli*, Enterokokken und Staphylokokken im Kläranlagenablauf um etwa eine log-Stufe reduzieren. Anhand von Laborexperimenten konnte gezeigt werden, dass Antibiotika-Resistenz per se nicht direkt mit einer geringeren Empfindlichkeit gegenüber Ozon gekoppelt ist und die teilweise beobachtete Zunahme des Anteils resistenter *E. coli*- und Staphylokokken-Isolate während der Ozonung multikausal erklärt werden muss.

Anhydroerythromycin, ein Metabolit mit einer vernachlässigbar geringen antibiotischen Wirkung, kann eine Kreuzresistenz gegen drei verschiedene Antibiotika-Klassen induzieren. Dafür reichen picomolare Konzentrationen, die praktisch ubiquitär in der aquatischen Umwelt gemessen werden, aus. Das bedeutet, dass sogar Abbauprodukte die Expression von Resistenzen beeinflussen und möglicherweise einen Austausch von Resistenzgenen fördern bzw. zur positiven Selektion resistenter Keime beitragen. Dies verdeutlicht, wie wichtig es ist, Abwasserreinigungsverfahren weiter hinsichtlich ihrer Eliminations-

leistung von Antibiotika, deren Metaboliten sowie von fakultativ pathogenen und Antibiotika-resistenten Keimen zu verbessern, damit auch in Zukunft noch wirksame Antibiotika zur Therapie von Infektionen zur Verfügung stehen.

Abstract

Antibiotic resistance is one of the most serious health threats of the 21th century. Whereas resistance levels are permanently surveyed in hospital, little is known about the fate and the behavior of antibiotic resistant bacteria living in aquatic environments. There is a chronic release of antibiotic resistant bacteria and resistance determinants into receiving water bodies due to erosion of fields fertilized with manure and effluents of sewage treatment plants, which is connected with a risk potential for human and animal health. The present thesis addresses the resistance levels in fecal contaminated surface waters and evaluates advanced sewage treatment technologies concerning their elimination of facultative pathogenic and antibiotic resistant bacteria.

The detection of bacteria with resistance against last resort antibiotics as well as of clinically relevant resistance genes like *mecA* or $bla_{\text{CTX-M}}$ with the chosen culture-based approach shows that they are – albeit in a lower frequency compared to hospital – present in the aquatic environment. In contrast to clinical isolates staphylococci obtained from the aquatic environment revealed only rarely high-level resistances but a frequently not constitutive expression of resistant genes possibly adapted to the subinhibitory antibiotic concentrations in their habitat. The diversity of *erm*-genes resulting in a variety of phenotypes was higher in river water than in hospital.

Based on species distribution of *Staphylococcus*, a qualitative discrimination between the different contamination sources was possible. In case of storm, when the capacity of the sewage treatment plant is exceeded, leading the sewage diluted with storm water through

retention soil filters would be an effective option to reduce numbers of facultative pathogenic and antibiotic resistant bacteria in fluvial ecosystems. Ozonation with downstream filtration as advanced sewage treatment technology led to a further reduction of facultative pathogenic and antibiotic resistant *E. coli*, enterococci and staphylococci in the effluent of the sewage treatment plant in the order of one log-decade. Experiments simulating the ozonation-step in the laboratory showed that antibiotic resistance is not directly connected with a reduced sensitivity against ozone; susceptibility against oxidative stress is determined by several factors.

The detection that picomolar concentrations of anhydroerythromycin, which can be almost ubiquitous measured in aquatic environments, are able to induce cross-resistance against three different antibiotic classes shows that even metabolites in very low concentrations effect the expression of resistances, may trigger the dissemination of resistance genes and may select for antibiotic resistant bacteria. These findings indicate the importance of further improvements in sewage treatment technologies focusing on elimination of antibiotics, their metabolites and facultative pathogenic and antibiotic resistant bacteria in order not to lose antibiotics as an effective option to treat infections.

Inhaltsverzeichnis

1. Einleitung

1.1 Motivation und Fragestellung

Antibiotika zählen zu den wichtigsten Entdeckungen der Medizin (Gullberg et al., 2011) und sind seither ein nicht mehr wegzudenkender Bestandteil in der Therapie von bakteriellen Infektionen. So wurden 2011 in Deutschland mehr als 2.300 Tonnen Antibiotika verabreicht, wovon 600-700 Tonnen in der Humanmedizin und 1.706 Tonnen in der Veterinärmedizin verbraucht wurden (GERMAP 2012). Die Entdeckung neuer und die chemische Modifikation bereits bekannter Antibiotika ist notwendig, da etablierte Antibiotika ihre Wirkung aufgrund der rasanten Verbreitung von Antibiotika-Resistenzen verlieren bzw. bereits verloren haben. Als 1940 das von A. Flemming 1928 entdeckte Penicillin als Medikament zugelassen wurde, waren bereits Penicillinase-produzierende Bakterien bekannt (Davies&Davies, 2010). 2001, ein Jahr nachdem Linezolid als Antibiotikum der neuen Antibiotika-Klasse der Oxazolidinone eingesetzt wurde, wurden erste Berichte über Linezolid-resistente *Staphylococcus aureus* publiziert (Tsiodras et al., 2001). Die Dissemination von Antibiotika-Resistenzen gilt als eine der größten Bedrohungen für die menschliche Gesundheit im 21. Jahrhundert (WHO, 2014). Legt man die Abrechnungsdaten deutscher Krankenhäuser einer Abschätzung der Inzidenz von Infektionen mit multiresistenten Erregern zugrunde, wird die Relevanz dieser Thematik deutlich: 2013 wurden 15.000 Infektionen mit Methicillin-resistenten *Staphylococcus aureus* (MRSA), 13.750 Infektionen mit Vancomycin-resistenten *E. faecium* oder *E. faecalis* (VRE) sowie 9.500 Infektionen

mit Extended-spectrum-ß-Laktamase-produzierenden *Escherichia coli* (ESBL) gemeldet, wobei durch jede auf einer Intensivstation erworbene Infektion mit einem solchen Erreger zusätzliche Kosten zwischen 13.725 und 30.000 Euro anfielen. Nach einer Statistik des Gesundheitsministeriums sterben jährlich fast genauso viele Menschen an Infektionen mit Antibiotika-resistenten Bakterien wie durch Alkohol- und Drogenmissbrauch zusammen (Biermann et al., 2014).

Während das Resistenzniveau und -verhalten in der Klinik gut untersucht ist und permanent überwacht wird, ist relativ wenig darüber bekannt, wie sich diese humanpathogenen, Antibiotika-resistenten Keime, die beispielsweise über Abwasser in die aquatische Umwelt gelangen, im Ökosystem verhalten. Möglicherweise wird durch die chronische Belastung von Gewässern, die häufig auch als Trinkwasserreservoir oder zur Erholung genutzt werden, mit multiresistenten Bakterien ein Reservoir für Antibiotika-Resistenzgene generiert, dessen potentielles Risiko für die menschliche und tierische Gesundheit derzeit nicht abschätzbar ist.

Diese Fragestellung aufgreifend, beschäftigt sich die vorliegende Arbeit mit der Effizienz verschiedener erweiterter Abwasserreinigungs-technologien, wie beispielsweise Ozonung oder Aktivkohle-Filtration, bezüglich der Elimination fakultativ-pathogener und Antibiotika-resistenter Bakterien. Außerdem wird, um den Effekt solcher Maßnahmen abschätzen zu können, die Belastung von Oberflächengewässern, in welche Kläranlagenabläufe eingeleitet werden, hinsichtlich der Konzentration fäkaler Indikatororganismen und Staphylokokken sowie deren Resistenzsituation untersucht.

1.2 Antibiotika-resistente Bakterien in der aquatischen Umwelt

Wichtige ökosystemare Funktionen von Oberflächengewässern sind ihre Nutzung als Trinkwasserreservoir, zur Bewässerung landwirtschaftlicher Nutzflächen und zur (Nah-) Erholung. Um aquatische Ökosysteme zu schützen, eine Akkumulation von Schadstoffen zu verhindern und die Biodiversität zu erhalten, wurden in den letzten Jahren mehrere Richtlinien auf europäischer (Europäische Wasserrahmenrichtlinie) wie auch auf internationaler Ebene (Water Safety Plan der WHO) erlassen.

Das aquatische Ökosystem zählt zu den wichtigsten Lebensräumen von Bakterien (Vaz-Moreira et al., 2014) und gilt als bedeutendes Reservoir für Antibiotika-Resistenzgene (Baquero et al., 2008). Viele heute bekannte Antibiotika werden von Mikroorganismen produziert und fungieren als Boten- und Signalstoffe (Martínez, 2008). Um sich vor den von anderen Mikroorganismen produzierten Substanzen zu schützen, entstanden/entstehen „Abwehrmechanismen“. Antibiotika-Resistenzen sind mutmaßlich in der Umwelt entstanden und existierten bereits in der prä-antibiotischen Ära (Martínez, 2008), wobei bisher sehr wenig über das natürliche Resistenzniveau und die Regulation der Resistenzgene im aquatischen Ökosystem bekannt ist.

Aufgrund des massiven Einsatzes von Antibiotika sowohl in der Human- als auch in der Veterinärmedizin werden Oberflächengewässer chronisch mit Antibiotika, deren Metaboliten sowie Antibiotika-resistenten Keimen und Resistenzdeterminanten belastet. Antibiotika werden in der Tiermast dem Futter der Tiere mit dem Ziel beigemischt, deren Wachstum zu fördern (seit 2006 in der EU verboten) bzw. den Ausbruch/die Ausbreitung von Krankheiten zu verhindern. Durch diesen

Selektionsdruck erwerben zur natürlichen Flora gehörende Bakterien Multiresistenzen, die dann über die Ausscheidungen der Tiere und die Düngung agrarisch genutzter Flächen in den Boden bzw. über Oberflächenabschwemmungen in Gewässer gelangen. Antibiotika werden mit gleichen Zielen in Aquakulturen eingesetzt und finden – wenn auch in geringerem Umfang – Anwendung als Pflanzenschutzmittel (Abb.1.1).

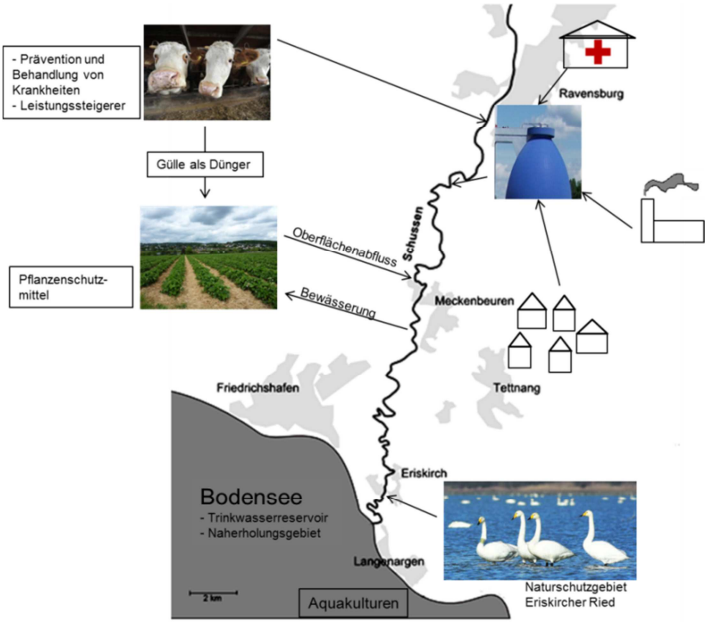


Abb.1.1: Eintragspfade von Antibiotika, deren Metaboliten sowie Antibiotika-resistenten Keimen und Resistenzdeterminanten in die aquatische Umwelt

(Quellen: Karte: Kartharina Peschke, Universität Tübingen; Mapdata: ©OpenStreetMap contributors, license: http://opendatacommons.org/licenses/dbcl/1.0/; Bilder:

Singschwäne:http://www.suedkurier.de/storage/pic/cms2skol/lokales/news/bodensee/friedrichshafen/1939225_1_frx_26_Eriskircher_Ried__Foto_.jpg; Erdbeerfeld: http://geo.hlipp.de/photos/01/07/010770_21a6d29b.jpg; Kühe: http://bilder.t-online.de/b/63/00/84/84/id_63008484/tid_da/kuehe-im-stall.jpg)

Neben der Landwirtschaft ist die Einleitung von Kläranlagenablauf (gereinigtem Abwasser aus Krankenhäusern, Alten- und Pflegeheimen, Siedlungen, Schlachthöfen, Industrie etc.) als eine wichtige Eintragsquelle von Antibiotika und Antibiotika-resistenten Keimen in Oberflächengewässer zu nennen. Zur Behandlung bakterieller Infektionen werden Antibiotika in der Klinik und die Therapie weiterführend dann Zuhause eingenommen und gelangen als Muttersubstanz oder Metabolit zusammen mit Bakterien, die möglicherweise als Folge der Therapie Resistenzen erworben haben, über das Abwasser in die Kläranlage. Letztere, und dort vor allem das Belebtschlammverfahren, werden aufgrund optimaler Bedingungen (hohe Bakteriendichte, hohe Nähr- und Sauerstoffkonzentration) als „hot spots“ für den intra- und interspezifischen Austausch von Resistenzgenen diskutiert (Jury et al., 2013). Weniger als 5% der Antibiotika-Resistenzgene sind chromosomal determiniert (Nwosu, 2001), der Großteil liegt also auf mobilen genetischen Elementen (Plasmiden, Transposons, Genkassetten-Integrons) die über Konjugation (Übertragung durch direkten Zellkontakt) übertragen werden können. Als weitere Verbreitungsmechanismen sind Transformation (Aufnahme freier DNA) sowie Transduktion (Übertragung durch Bakteriophagen) zu nennen. Ob und mit welcher Frequenz horizontaler Gentransfer stattfindet bzw. inwieweit Antibiotika-resistente Bakterien positiv selektiert werden, ist neben den genannten Faktoren auch von abiotischen Parametern wie pH-Wert, Lichtverhältnissen oder Salzkonzentrationen abhängig. Diskutiert wird auch, inwieweit subinhibitorische Antibiotika-Konzentrationen zur Verbreitung von Resistenzdeterminanten und zur Rekombination bzw. Entstehung von Resistenzen durch spontane Mutationen führen (Schwartz et al., 2003),

bzw. es durch Metallkonzentrationen oder bestimmte Substrate, deren Resistenzen bzw. Enzyme für deren Verwertung auf demselben genetischen Element determiniert sind, zu einer Co-Selektion kommt (Baker-Austin et al., 2006).

Darüber wie sich die über die verschiedenen genannten Eintragswege (Abb. 1.1) emittierten Antibiotika-resistenten Bakterien in der aquatischen Umwelt verhalten, ist derzeit wenig bekannt: Möglicherweise kommt es – stimuliert durch die in der aquatischen Umwelt detektierbaren subinhibitorischen Antibiotika-Konzentrationen – zu einer Übertragung von Resistenzgenen auf autochthone Mikroorganismen mit der Konsequenz einer Generierung eines Antibiotika-Resistenzgenpools, verbunden mit einem derzeit nicht einschätzbaren gesundheitlichen Risiko (Baquero et al., 2008; Czekalski et al., 2012).

1.3 Aufbau der Arbeit

Die vorliegende kumulative Dissertation beginnt mit einer thematisch hinführenden Einleitung, die sich mit der Relevanz Antibiotika-resistenter Keime in der Humanmedizin und der sich daraus ergebenden Fragestellung bezüglich deren Dissemination in der aquatischen Umwelt beschäftigt (Kapitel 1). Anschließend folgt eine kurze Vorstellung des Arbeitsgebietes sowie den zur Beurteilung der Effizienz der verschiedenen Klärtechniken bezüglich der Elimination fakultativ pathogener Keime ausgewählten Bakteriengattungen und den zur Bestimmung des Resistenzniveaus ausgesuchten Antibiotika (Kapitel 2). Die nachfolgenden Kapitel (Kapitel 3, 4, 5, 6, 7 und 8) fokussieren

Teilaspekte der Fragestellung, wobei zu Beginn des jeweiligen Kapitels, dessen Inhalt in den Kontext der Fragestellung eingeordnet wird und die wichtigsten Ergebnisse in Stichpunkten zusammengefasst werden, bevor das reproduzierte Manuskript abgedruckt wird. Die in Kapitel 3, 4, 5 und 7 abgedruckten Manuskripte sind bereits veröffentlicht; das in Kapitel 6 abgedruckte Manuskript ist zur Veröffentlichung eingereicht. In Kapitel 8 werden bisher nicht publizierte Ergebnisse zu den Untersuchungen bezüglich der Keimbelastung und Resistenzsituation im Freiland dargestellt und hinsichtlich des Einflusses von Kläranlagen auf fluviatile Ökosysteme diskutiert. In der sich anschließenden Synopse (Kapitel 9) werden die Ergebnisse, die in den vorangegangenen Kapiteln diskutiert wurden, verknüpfend dargestellt und die daraus resultierenden Schlüsse gezogen.

2. Hintergrund

Nachfolgend werden kurz das Arbeitsgebiet sowie die zur Beurteilung der Eliminationsleistung fakultativ pathogener Bakterien gewählten Gattungen und die zur Charakterisierung der Resistenzsituation getesteten Antibiotika vorgestellt.

2.1 Rahmen der Arbeit und Arbeitsgebiet

Die vorliegende Arbeit wurde im Rahmen von „SchussenAktiv*plus*“, eines von 12 Projekten, die im Rahmen der Fördermaßnahme „Risikomanagement von neuen Schadstoffen und Krankheitserregern“ (RiSKWa), die vom Bundesministerium für Bildung und Forschung initiiert und über drei Jahre (2012-2014) finanziert wurde, durchgeführt. Ziel des Projektes war es, verschiedene Abwasserreinigungs-technologien in Mischentwässerungssystemen bezüglich ihres Potentials zur Reduktion von Mikroverunreinigungen und Keimen zu untersuchen, um die Gewässerqualität des Bodenseezuflusses Schussen weiter zu verbessern.

Die Schussen, ein etwa 1,6 km nördlich von Bad Schussenried entspringender und nach einer Fließstrecke von etwa 60 km bei Eriskirch in den Bodensee mündender Fluss, entwässert ein Gebiet von 815 km^2. Das damit größte Einzugsgebiet aller baden-württembergischen Zuflüsse zum Bodensee ist im Gegensatz zum Einzugsgebiet der Argen, die im Rahmen des Projektes als weniger anthropogen beeinflusstes Referenzgewässer beprobt wurde, mit 11%

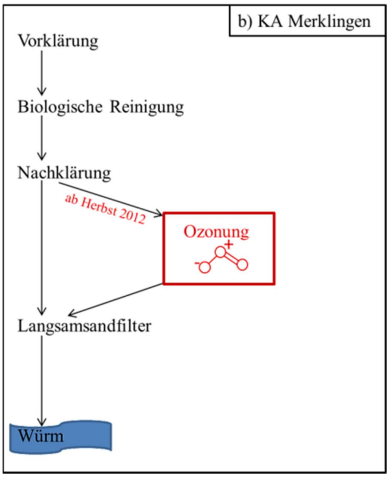
Siedlungsfläche und rund 200.000 Einwohnern relativ dicht besiedelt. Die Entwässerung der Siedlungsbereiche erfolgt hauptsächlich über ein Mischkanalsystem; insgesamt 20 kommunale Kläranlagen unterschiedlicher Ausbaugröße und technischer Ausstattung, an welche etwa 98% der Haushalte angeschlossen sind, sowie mehr als 100 Regenüberlaufbecken schlagen (gereinigtes) Abwasser direkt oder indirekt in die Schussen ab. Mit einem mittleren Abfluss von 11 m^3/s liefert die Schussen circa 5% des Zuflusses in den Bodensee (Auerbach et al., 2009), der als Trinkwasserreservoir für 4 Millionen Menschen in 320 Städten und Gemeinden genutzt wird.

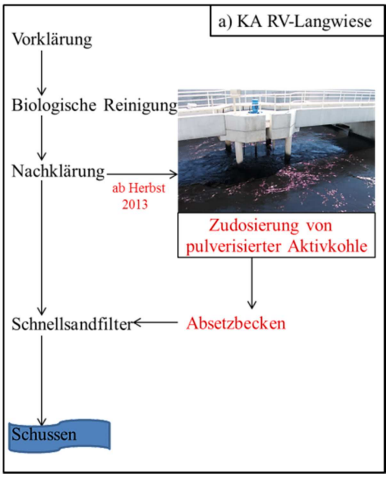
Im Rahmen des Projektes wurden fünf Testsysteme, drei Kläranlagen unterschiedlicher Ausbaugröße sowie ein Regenüberlaufbecken und ein Retentionsbodenfilter, untersucht, die in der nachfolgenden Tabelle (Tab. 2.1) und den Fließschemata (Abb. 2.1) kurz beschrieben werden.

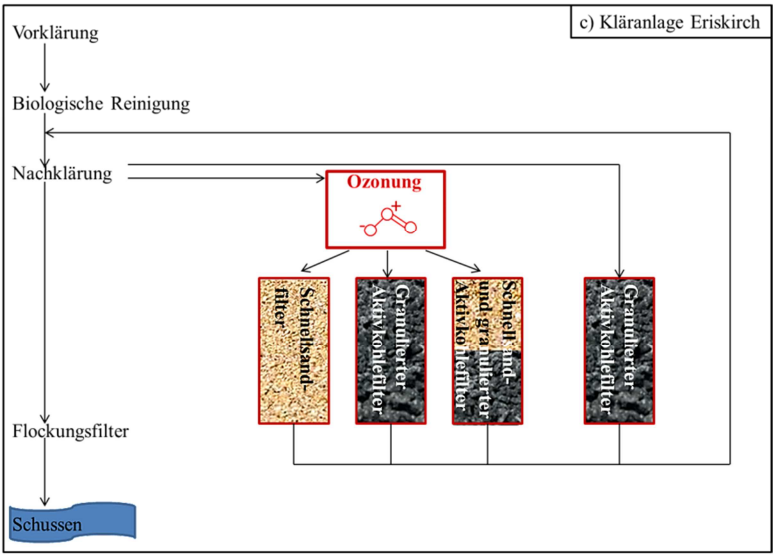
Tab. 2.1: Charakterisierung der Testsysteme

Testsystem	**Größe**	**Getestete Maßnahme(n)**
Kläranlage Ravensburg-Langwiese	170.000 EGW	- Aktivkohlefiltration (pulverisiert; großtechnisch)
Kläranlage Eriskirch	50.000 EGW	- Flockungsfiltration (großtechnisch) - Schnellsandfiltration nach Ozonung (Pilotanlage) - Aktivkohlefiltration (granuliert) im Anschluss an Ozonung (Pilotanlage) - Kombination aus Sand- und Aktivkohlefiltration (granuliert) im Anschluss an Ozonung (Pilotanlage) - Aktivkohlefiltration (granuliert; ohne vorherige Ozonung; Pilotanlage)
Kläranlage Merklingen	2.300 EGW	Ozonung vor Langsamsandfiltration (großtechnisch)
Regenüberlaufbecken Mariatal	10.700 m^3	Schräglamellenklärer (Teilstrombehandlung)
Retentionsbodenfilter Tettnang	2.000 m^2	

(EGW: Einwohnergleichwert)


b) KA Merklingen
Vorklärung
Biologische Reinigung
Nachklärung
ab Herbst 2012
Ozonung
Langsamsandfilter
Würm


a) KA RV-Langwiese
Vorklärung
Biologische Reinigung
Nachklärung
ab Herbst 2013
Zudosierung von pulverisierter Aktivkohle
Absetzbecken
Schnellsandfilter
Schussen


c) Kläranlage Eriskirch
Vorklärung
Biologische Reinigung
Nachklärung
Ozonung
Schnellsand-filter
Granulierter Aktivkohlefilter
Schnellsand- und granulierter Aktivkohlefilter
Granulierter Aktivkohlefilter
Flockungsfilter
Schussen

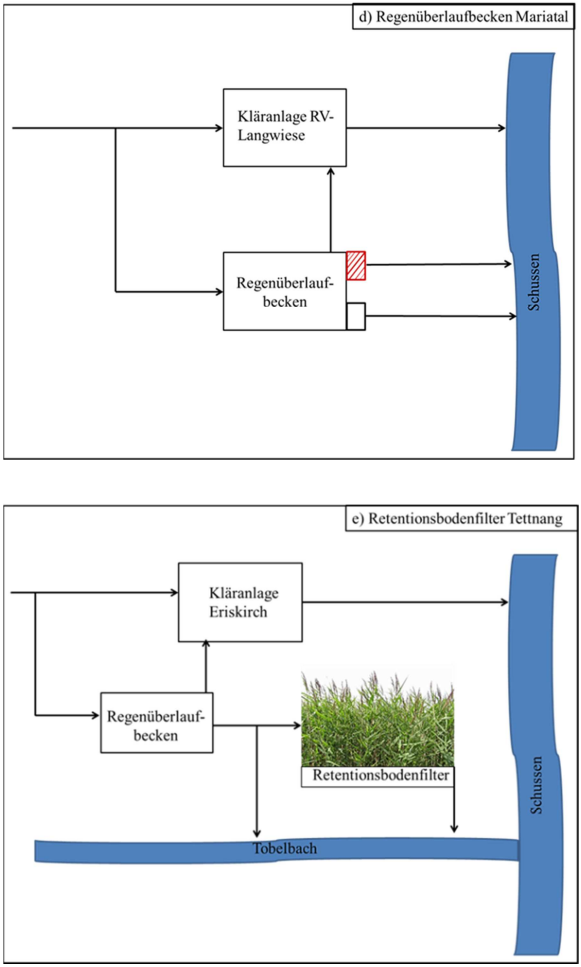


Abb. 2.1: Fließschemata der fünf Testsysteme: a) Kläranlage Ravensburg-Langwiese, b) Kläranlage Merklingen, c) Kläranlage Eriskirch, d) Regenüberlaufbecken Mariatal, e) Retentionsbodenfilter Tettnang. „Neue“ Abwasserreinigungstechnologien sind mit roter Farbe markiert.

(Quellen der Bilder: Schilf: http://www.rieselfelder-windel.de/uploads/pics/Schilfwald_JA.JPG; Sand: http://www.trikkiworld.com/images/bg/bg_sand/25012011/sand006.jpeg; Aktivkohle: http://hajoon.de/wp-content/uploads/2013/12/Aktivkohle.png)

Die Schussen wurde auf ihrer Fließstrecke zwischen Ravensburg und der Mündung in den Bodensee, in der auch die untersuchten Kläranlagen Ravensburg-Langwiese und Eriskirch sowie das Regenüberlaufbecken Mariatal ihren Ablauf in die Schussen einleiten, im Rahmen von 8 Probenahmen an vier Stellen bei unterschiedlichen hydrologischen Verhältnissen beprobt. Parallel dazu wurde jeweils eine Probe aus der Argen, dem als weniger anthropogen belastet geltenden Referenzgewässer, untersucht. In der nachfolgenden Karte (Abb. 2.2) sind die Testsysteme sowie die Freilandprobenahmestellen eingezeichnet.

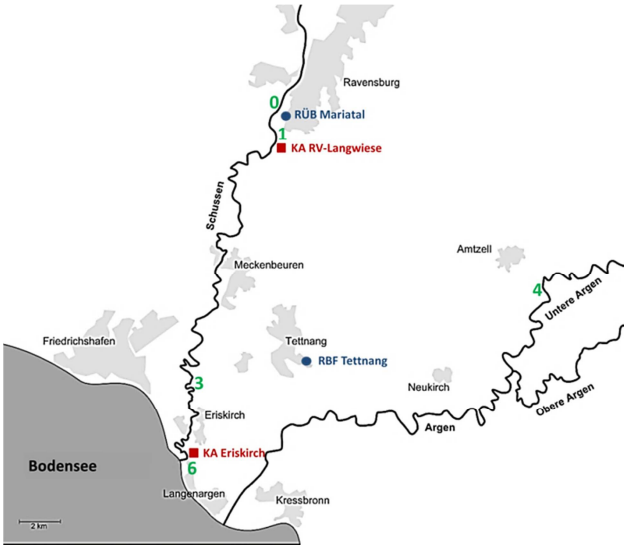


Abb. 2.2: Untersuchungsgebiet mit den beiden untersuchten Kläranlagen (rot), den Regenwasserentlastungssystemen (blau) sowie den 5 Freiland-Probenahmestellen (grün)

(Quelle der Karte: Kartharina Peschke, Universität Tübingen; Mapdata: ©OpenStreetMap contributors, license: http://opendatacommons.org/licenses/dbcl/1.0/)

2.2 Test-Organismen: *Escherichia coli*, Enterokokken und Staphylokokken

Um die Eliminationsleistung sowie die Resistenzsituation in den Testsystemen und im Freiland beurteilen zu können, wurden diese – stellvertretend für eine Vielzahl von (resistenten) Bakterien – für die fakultativ humanpathogene Spezies *Escherichia coli* sowie für die beiden Gattungen *Enterococcus* und *Staphylococcus* untersucht.

2.2.1 Fäkale Indikatororganismen: *Escherichia coli* und Enterokokken

Escherichia coli und intestinale Enterokokken haben ihren Verbreitungsschwerpunkt im Intestinaltrakt von Mensch und Tier (10^6-10^7 KBE/g Stuhl), gehören also zu deren physiologischer Flora. Aufgrund ihrer relativ langen Überlebensdauer in der aquatischen Umwelt im Vergleich zu anderen potentiell pathogenen Bakterien wurden sie im Rahmen des „Konzeptes der fäkalen Indikatororganismen“ als Anhaltspunkt für eine mehr oder weniger lange zurückliegende fäkale Verunreinigung und einem damit verbundenen gesundheitlichen Gefährdungspotential durch über Faeces übertragene Krankheiten ausgewählt. Darauf basierend wird auch die Konzentration von *E. coli* und intestinalen Enterokokken in Gewässern in der EU-Badegewässerrichtlinie als ein Parameter zur Beurteilung für deren Gewässergüte herangezogen.

Tab. 2.2: Grenzwerte der EU-Badegewässerrichtlinie (2006) für fäkale Indikatororganismen (Binnengewässer)

	Enterokokken	*Escherichia coli*
Ausgezeichnete Qualität	200 KBE/100 mL	500 KBE/100 mL
Gute Qualität	400 KBE/100 mL	1.000 KBE/100 mL

Auf der Grundlage der 95-Perzentil-Bewertung; KBE: Koloniebildende Einheiten

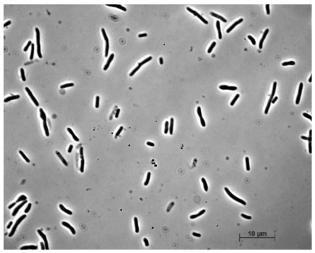


Abb. 2.3: *Escherichia coli* DSMZ 1103

E. coli sind Gram-negative, fakultativ anaerobe und etwa 4 µm lange Stäbchen, die aufgrund ihrer peritrichen Begeißelung beweglich sind (Abb. 2.3). Abhängig von der Ausstattung mit Virulenzmerkmalen können *Escherichia coli* sowohl als Erreger von Magen-Darm-Erkrankungen, von extraintestinalen Infektionen wie Harnwegs-infektionen als auch von Beatmungspneumonien und Sepsen in Erscheinung treten (GERMAP 2012). Die Verbreitung von für Extended-Spectrum-ß-Laktamasen (ESBL) kodierenden Genen (z.B. bla_{TEM}, bla_{SHV} oder $bla_{\text{CTX-M}}$) ist insofern problematisch, da diese Enzym-produzierenden Erreger dann praktisch nur noch mit Carbapenemen behandelt werden können. Carbapeneme haben in klinischen Studien starke Nebenwirkungen gezeigt und gelten als nephrotoxisch.

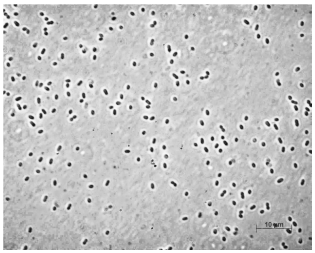


Abb. 2.4: *Enterococcus faecalis* DSMZ 12956

Enterokokken können als Gram-positive, fakultativ anaerobe, unbewegliche und im Durchmesser circa 1 µm messende Kokken beschrieben werden (Abb. 2.4). Die Gattung *Enterococcus* umfasst derzeit 53 Spezies, wozu sowohl Besiedler des menschlichen und tierischen Darms als auch pflanzenassoziierte Spezies zählen. Manche Spezies der Gattung *Enterococcus* wie auch manche Subspezies von *Escherichia coli* sind als humanpathogen klassifiziert und können schwere Infektionen hervorrufen: Enterokokken sind die zweit- bis dritthäufigsten Erreger nosokomialer Infektionen, wobei vor allem *Enterococcus faecium* und *Enterococcus faecalis* als Erreger von Harnwegsinfekten, Wundinfektionen, hauptsächlich im Abdominalbereich, Sepsen und Endokarditiden zu nennen sind (GERMAP 2012). Infektionen mit diesen Keimen sind insbesondere dann problematisch, wenn auch das Reserveantibiotikum Vancomycin aufgrund der Multiresistenz des Erregers nicht mehr greift (VRE: Vancomycin-resistente *E. faecium* und *E. faecalis*).

2.2.2 Staphylokokken

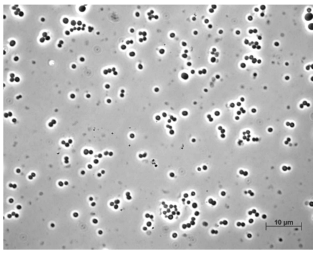


Abb. 2.5: *Staphylococcus aureus* ssp. *aureus* DSMZ 346

Im Gegensatz zu *E. coli* und den Enterokokken sind Staphylokokken keine fäkalen Indikatororganismen, sondern besiedeln Haut und Schleimhäute von Menschen und Tieren (Mensch: 10^3-10^6 KBE/cm^2), können aber auch aus der Luft sowie aus Bodenproben isoliert werden. Die Gattung *Staphylococcus*, Gram-positive, im Durchmesser 1 µm große Kokken, die fakultativ auch anaerobe Bedingungen tolerieren, umfasst derzeit 76 Spezies und Subspezies (Abb. 2.5). Diese wurden basierend auf Multilocus-Sequenzierungen in 15 Cluster-Gruppen und basierend auf phänotypischen Charakteristika in 6 Spezies-Gruppen eingeteilt (Lamers et al., 2012). In der Klinik wird vor allem zwischen *Staphylococcus aureus*, einer Spezies, die das aktive Protein Koagulase synthetisieren und damit Blutplasma koagulieren kann, und Koagulase-negativen Staphylokokken differenziert. *S. aureus* gilt als einer der wichtigsten Erreger im humanmedizinischen Bereich; vor allem Methicillin-resistente *Staphylococcus aureus* (MRSA), die häufig noch gegen weitere Antibiotikaklassen eine Resistenz erworben haben, stellen eine große Herausforderung in der Therapie von Infektionen mit solchen Erregern dar (GERMAP 2012). Aber auch in der Tiermedizin breiten sich diese multiresistenten *S. aureus* (livestock-associated MRSA), beispielsweise als Erreger von Mastitiden bei Milchkühen, aus und

können auf den Menschen übertragen werden. Eiternde Abszesse, Besiedlung von klinischen Plastikteilen (z.B. Kathetern, Magensonden) und das sogenannten Toxische Schocksyndrom sind häufig auf eine Infektion mit Staphylokokken zurückzuführen (GERMAP 2012).

2.3 Getestete Antibiotika

Um das Resistenzniveau von *E. coli*, Enterokokken und Staphylokokken in den jeweiligen Testsystemen sowie im fluviatilen Ökosystem zu bestimmen, wurden Antibiotika verschiedener Klassen gewählt, die in der Humanmedizin zur Behandlung von Infektionen mit diesen Erregern ihren Einsatz finden. Zielstrukturen, Wirktyp und -spektrum der getesteten Antibiotika sind in Tabelle 2.3 zusammengestellt.

Tab. 2.3: Zielstrukturen und Wirkspektrum der ausgewählten Antibiotika

Antibiotika-Klasse		**Getestetes Antibiotikum**	**Wirktyp, -spektrum**	**Zielstruktur**	**Getestet gegen**
ß-Laktame	Penicilline	Ampicillin	Bakterizid; Breitband-Antibiotikum	Zellwand (Inhibition der D-Alanin-Transpeptidase)	*E. coli*, Enterokokken
		Oxacillin	Bakterizid; gegen Staphylokokken	Zellwand (Inhibition der Transpeptidierung; PBP2a)	Staphylokokken
	Cephalosporine	Cefotaxim	Bakterizid; Gram-positive und Gram-negative Bakterien	Zellwand (Inhibition der Transpeptidase; PBP3)	*E. coli*
	Carbapeneme	Ertapenem	Bakterizid; vor allem gegen Gram-negative *Enterobacteriaceae*, Reserve-Antibiotikum	Zellwandsynthese (Inhibition der D-Transpeptidase)	*E. coli*
Fluorochinolone		Ciprofloxacin	Bakterizid; Breitband-Antibiotikum	DNA-Replikation (Inhibition der Gyrase bzw. der Topoisomerase IV)	*E. coli*, Enterokokken, Staphylokokken
Sulfonamide / Trimethoprim		Cotrimoxazol	In Kombination bakterizid; Gram-positive und Gram-negative Bakterien	Folsäuresynthese (Blockade der Dihydropteroat-Synthase und Dihydrofolat-Reduktase)	*E. coli*
Glykopeptide		Vancomycin	Bakterizid; Gram-positive Bakterien	Zellwandsynthese (Inhibition der Transglykosilierung)	Enterokokken, Staphylokokken
Chloramphenicole		Chloramphenicol	Bakteriostatisch; Breitband-, Reserve-Antibiotikum	Proteinbiosynthese (Inhibition der Translation durch Bindung an 50S ribosomale Untereinheit)	Enterokokken
Makrolide		Erythromycin	Bakteriostatisch; Gram-positive Bakterien	Proteinbiosynthese (Bindung an 50S ribosomale Untereinheit)	Enterokokken, Staphylokokken
Lincosamide		Clindamycin	Bakteriostatisch; Gram-positive Bakterien (außer Enterokokken)	Proteinbiosynthese (Bindung an 50S ribosomale Untereinheit)	Staphylokokken

PBP: Penicillin-bindendes Protein

Die Bedeutung der einzelnen gewählten Substanzklassen, sowohl in der Human- als auch in der Veterinärmedizin, spiegelt sich in den Verbrauchsmengen, die in Tabelle 2.4 aufgelistet sind, wider.

Tab. 2.4: Gegenüberstellung der Antibiotika-Verbrauchsmengen in Human- und Veterinärmedizin mit dem Resistenzniveau in der Klinik und im aquatischen Ökosystem

Antibiotika-Klasse		**Getestetes Antibiotikum**	**Anteil resistenter Isolate**		**Verbrauchsmengen**	
			Aquatische Umwelt (Eigene Ergebnisse)	**Klinik (stationärer Bereich)[1]**	**Humanmedizin (Mio DDD 2011)[2, 3]**	**Veterinär-medizin (abgegebene t 2011)[2]**
ß-Laktame	Penicilline	Ampicillin	*E. coli*: 15%; Enterokokken: 4,4% (*E. faecium*: 9,7%; *E. faecalis*: 0,4%)	*E. coli*: 51,0%; *E. faecalis*: 0,4%; *E. faecium*: 93,0%	90,6 (DDD Ampicillin und Oxacillin: 2g →181,2 t)	498
		Oxacillin	Staphylokokken: 1,1%	*S. aureus*: 17,1%; KNS: 57,4%		
	Cephalo-sporine	Cefotaxim	*E. coli*: 0,9%	*E. coli*: 12,2%	77,5 (DDD Cefotaxim: 4g →310 t)	9
	Carbapeneme	Ertapenem	*E. coli*: 0%	*E. coli*: 0,1%		
Fluorochinolone		Ciprofloxacin	*E. coli:* 3,1%; Enterokokken: 6,3% (*E. faecium*: 13,4%; *E. faecalis*: 1,3%); Staphylokokken: 0,1%	*E. coli*: 21,4%; keine Angabe zu Resistenzniveau von Enterokokken; *S. aureus*: 26,8%; KNS: 48,0%	37,5 (DDD Ciprofloxacin: 0,5-1g → 18,75-37,5 t)	10
Sulfonamide / Trimethoprim		Cotrimoxazol	*E. coli:* 7,6%	*E. coli*: 28,0%	15,5 (DDD SXT: 1,92g → 53,8 t)	188
Glykopeptide		Vancomycin	*E. faecium*: 0%; *E. faecalis*: 0%;	*E. faecalis*: 0,2%; *E. faecium*: 13,2%; *S. aureus*		

		Staphylokokken: 0%	und KNS: 0%		
Chloramphenicole	Chlor-amphenicol	Enterokokken: 1,4% (*E. faecium*: 0,9%; *E. faecalis*: 2,5%)	keine Angabe zu Resistenzniveau von Enterokokken		
Makrolide	Erythromycin	Enterokokken: 20,2% (*E. faecium*: 41%; *E. faecalis*: 8,7%); Staphylokokken: 18,7%	keine Angabe zu Resistenzniveau von Enterokokken; *S. aureus*: 24,3%; KNS: 61,7%	52,5 (DDD Erythromycin: 2g → 105 t)	145
Lincosamide	Clindamycin	Staphylokokken: konstitutiv: 3,3%; induzierbar: 3,5%	*S. aureus*: 23,3%; KNS: 46,8%	6,6 (zusammen mit Strepto-graminen und Fusidinsäure) (DDD Clindamycin: 1,2-1,8g → 7,9 – 11,9 t)	15

Bestimmung des Resistenzniveaus in der aquatischen Umwelt: Staphylokokken (n=2259; davon wurden 10 als *S. aureus* identifiziert, die aber gegen alle 5 getesteten Antibiotika sensibel waren); *E. coli* (n=1236); Enterokokken (n=1166, darunter 434 *E. faecium* und 237 *E. faecalis*; intrinsische low-level Vancomycin-Resistenz von *E. gallinarum* und *E. casseliflavus* nicht berücksichtigt). [1]:ARS 2013 Gesamtdeutschland (n: zwischen 13.000 und 40.000 Isolaten; KNS = Koagulase-negative Staphylokokken); [2]: GERMAP 2012; [3]: GVZ, 2007 (DDD = defined daily doses)

Aufgrund der unterschiedlichen Eigenschaften hinsichtlich der Stabilität und Adsorption an organische Partikel (z.B. an Belebtschlamm oder Bodenpartikel) korrelieren die Verbrauchsmengen nur bedingt mit den in der Umwelt detektierten Antibiotika-Konzentrationen (Tab. 2.5).

Tab. 2.5: Antibiotika-Konzentrationen in der aquatischen Umwelt

Antibiotika-Klasse	**Antibiotikum/ Metabolit**	**Konzentration (ng/L)**	**Wasserprobe**	**Quelle**
Makrolide	Anhydroerythromycin	400 (Mittelwert)	Kläranlagenablauf (Kenzingen, Baden-Württemberg)	Umweltbundesamt Deutschland, 2005
		50-94	Oberflächenwasser (Wannsee, Berlin)	Heberer et al., 2008
		49 (Maximum)	Grundwasser (Baden-Württemberg)	Sacher et al., 2001
Linkosamide	Clindamycin	5 (Median)	Kläranlagenablauf (Australien	Watkinson et al., 2009
		1 (Median)	Oberflächenwasser (Australien)	
		15-48	Oberflächenwasser (Wannsee, Berlin)	Heberer et al., 2008
Sulfonamide	Sulfamethoxazol	50 (Median)	Kläranlagenablauf (Australien)	Watkinson et al., 2009
		400 (Median)	Kläranlagenablauf	Hirsch et al., 1999
		480 (Maximum)	Oberflächenwasser (Deutschland)	
		100-326	Oberflächenwasser (Wannsee, Berlin)	Heberer et al., 2008
		8 (Median)	Oberflächenwasser (Australien)	Watkinson et al., 2009
Trimethoprim	Trimethoprim	10 (Median)	Kläranlagenablauf (Australien)	Watkinson et al., 2009
		320 (Median)	Kläranlagenablauf (Deutschland)	Hirsch et al., 1999

		200 (Maximum	Oberflächenwasser (Deutschland)	
		7-49	Oberflächenwasser (Wannsee, Berlin)	Heberer et al., 2008
		3 (Median)	Oberflächenwasser (Australien)	Watkinson et al., 2009
Chloramphenicole	Chloramphenicol	60 (Maximum)	Oberflächenwasser	Hirsch et al., 1999

Der ß-Laktamring der Penicilline beispielsweise ist relativ instabil, was dazu führt, dass Muttersubstanzen dieser Antibiotika-Klasse in der Umwelt praktisch nicht detektiert werden (Kemper, 2008). Trotzdem war die Resistenz gegenüber Ampicillin bei den 1236 getesteten *E. coli*-Isolaten die am häufigsten detektierte Resistenz. Bezüglich der in Oberflächengewässern gemessenen Antibiotika-Konzentrationen und der in der aquatischen Umwelt gefundenen Abundanz verschiedener Resistenzen war keine direkte Korrelation erkennbar. In der Klinik ist der Anteil resistenter Isolate, der – im Gegensatz zum aquatischen Ökosystem – relativ gut mit dem Verbrauch der einzelnen antibiotischen Substanzen in der Humanmedizin korreliert, deutlich höher als in Abwässern und Oberflächengewässern (Tab. 2.4). Inwieweit eine Antibiotika-Resistenz für das Überleben in der aquatischen Umwelt einen Vorteil (oder zumindest keinen Nachteil) darstellt und die in Oberflächengewässern detektierten subinhibitorischen Antibiotika-Konzentrationen (auf Bakterienzellen bakteriostatisch bzw. bakterizid wirkende Antibiotika-Konzentrationen liegen im mg/L-Bereich) zu einer positiven Selektion bestimmter Resistenzen und deren Dissemination beitragen, wird in der Wissenschaft derzeit diskutiert (z.B. Ohlsen et al., 2003; Zhang et al., 2009).

Einen Teilaspekt dieser Fragestellung thematisierend, beschäftigt sich Kapitel 3 mit der Fähigkeit von Anhydroerythromycin, einem Makrolid-Metaboliten, eine Kreuzresistenz gegen MLS_B-Antibiotika zu induzieren. Darauf hinführend wird nachfolgend der Wirkmechanismus von Makroliden und die Resistenzmechanismen gegen diese kurz – ohne zu sehr ins Detail zu gehen – beschrieben.

2.3.1 Wirk- und Resistenzmechanismus von Makroliden, Linkosamiden und Streptograminen der Klasse B bei Staphylokokken

Makrolide, Linkosamide und Streptogramine der Klasse B (MLS_B) stellen zwar aufgrund ihrer unterschiedlichen chemischen Struktur unterschiedliche Antibiotikaklassen dar, haben aber die gleiche Zielstruktur: Durch die Bindung an die 23S rRNA inhibieren sie sterisch die Proteinbiosynthese.

Mehrere Resistenzmechanismen gegen MLS_B-Antibiotika sind bekannt: Effluxpumpen, welche das antimikrobiell wirksame Molekül aus der Zelle transportieren, Enzyme (z.B. Esterasen), welche das Antibiotikum chemisch verändern und Methylasen, welche die Zielstrukturen entsprechend modifizieren, sodass das Antibiotikum nicht mehr an der Zielstruktur binden und die Translation nicht behindern kann.

Den letztgenannten Mechanismus genauer betrachtend, bindet ein zur Induktion geeignetes Molekül (z.B. Makrolide mit einem 14- oder 15-gliedrigen Laktonring) in der Regulatorregion des für die Methylase kodierenden *erm*-Gens an die mRNA. Dies führt dazu, dass sich die Sekundärstruktur der mRNA so verändert, dass die Shine-Dalgarno-Sequenz (blaues Rechteck in Abb. 2.6), die 4-14 Basen vor dem Startcodon der Methylase liegt, für das Ribosom zugänglich wird und die Methylase translatiert werden kann. Dadurch, dass die Bindestellen der MLS_B-Antibiotika an der rRNA relativ nahe beieinander liegen, führt die Mono- bzw. Dimethylierung der rRNA durch die Methylase zur Kreuzresistenz gegen alle MLS_B-Antibiotika.

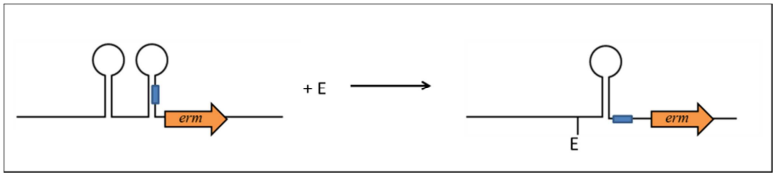


Abb. 2.6: Schematische Darstellung der Sekundärstruktur der mRNA im Bereichder Regulatorregion.

Blaues Rechteck: Shine-Dalgarno-Sequenz; E: Erythromycin als Beispiel für eine MLS_B-Resistenz induzierende Substanz.

Ist die Resistenz – wie gerade beschrieben – induzierbar, ist die Zelle nur dann gegen MLS_B-Antibiotika unempfindlich, wenn ein geeignetes induzierendes Molekül an die mRNA bindet. Mutationen (Duplikationen, Deletionen oder Basenaustausche) in der Regulatorregion des *erm*-Gens können zu dessen konstitutiven Expression führen, und der Zelle – unabhängig davon, ob ein geeignetes induzierendes Molekül präsent ist – Resistenz gegen alle MLS_B verleihen (Leclercq, 2002).

3. Demonstration of staphylococci with inducible macrolide-lincosamide-streptogramin B resistance MLS_B in sewage and river water and of the capacity of anhydroerythromycin to induce MLS_B

Wie bereits angekündigt beschäftigt sich das nachfolgend abgedruckte Manuskript mit der Speziesdiversität von Staphylokokken und deren Resistenzmustern in Abwässern und Oberflächengewässern, wobei insbesondere auf die Fähigkeit von Antibiotika-Metaboliten Kreuzresistenzen zu induzieren, eingegangen wird. Nachfolgend sind die wichtigsten Ergebnisse zusammengestellt:

- Die Konzentration an Staphylokokken war im Fluss größer als im Kläranlagenablauf: Entweder es existiert eine andere Eintragsquelle oder aber Staphylokokken kommen natürlicherweise im aquatischen Ökosystem vor und können sich dort auch vermehren.
- Die Speziesdiversität der aus der aquatischen Umwelt isolierten Staphylokokken war deutlich größer als in der Klinik. Während in der Klinik vor allem *Staphylococcus aureus* aus Infektionsherden isoliert wird, dominierten in der Umwelt Spezies, die als Mitglieder der Saprophyticus- und der Sciuri-Gruppe identifiziert wurden. *Staphylococcus aureus* scheint in der aquatischen Umwelt keine Rolle zu spielen; nur 0,4% der Umweltisolate waren *S. aureus*.
- Der relative Anteil resistenter Staphylokokken nahm während der Passage durch die Kläranlage ab und war im Freiland am geringsten. Insgesamt betrachtet war aber der Anteil resistenter Isolate auch im Rohabwasser deutlich geringer als in der Klinik,

wobei man in diesem Zusammenhang berücksichtigen muss, dass manche Spezies deutlich häufiger Antibiotika-Resistenzgene besitzen als andere und in der Konsequenz die detektierte Speziesdiversität und –abundanz das Resistenzniveau determinierte.

- Auch wenn in der aquatischen Umwelt über den gewählten kulturbasierten Ansatz keine MRSA isoliert wurden, zeigt der genotypische Nachweis von *mecA* in Koagulase-negativen Staphylokokken, dass das für die Resistenz kodierende Gen im aquatischen Ökosystem präsent ist.
- Der Anteil induzierbar gegen MLS_B-Antibiotika resistenter Staphylokokken an Erythromycin-resistenten Isolaten war im Freiland höher als im Kläranlagenzu- und -ablauf.
- Anhydroerythromycin, ein Metabolit des Makrolid-Antibiotikums Erythromycin, das in einem Konzentrationsbereich von ng/L praktisch ubiquitär in Kläranlagenabläufen, Oberflächengewässern und sogar im Grundwasser detektiert werden kann und selbst eine vernachlässigbar geringe antibiotische Wirkung besitzt, induzierte in picomolaren Konzentrationen eine Kreuzresistenz gegen MLS_B. Die Tatsache, dass die in der Umwelt vorhandenen Konzentrationen ausreichen und eine Induktion bereits nach 10-minütiger Kontaktzeit ausgeprägt war, stellt ein potentielles Risiko für die menschliche und tierische Gesundheit dar.
- Es konnte zum ersten Mal gezeigt werden, dass Anhydroerythromycin als Inducer für die Kreuzresistenz gegenüber MLS_B-Antibiotika wirkt.

Reproduziert von: Heß, S., Gallert, C. (2014): Demonstration of Staphylococci with Inducible Macrolide-Lincosamide-Streptogramin B Resistance MLS_B in Sewage and River Water and of the Capacity of Anhydroerythromycin to Induce MLS_B. FEMS Microbiololgy Ecology 88, 48-59

Abstract

Staphylococci causing diseases in humans and animals are well described, whereas not very much is known about the staphylococci in natural ecosystems. Due to increased consumption of antibiotics, multiresistant species are released with excrements. Therefore, 1048 staphylococci from raw and treated sewage and from receiving water bodies were isolated, identified and tested for resistance against erythromycin, clindamycin, oxacillin and ciprofloxacin. More resistant staphylococci were present in raw sewage (33.8 %) than in treated sewage (24.9 %) or river water (10.9 %). Of all isolates, 20.2 % were resistant against the macrolide erythromycin which can induce cross resistance against lincosamides and streptogramin B antibiotics ($iMLS_B$). Erythromycin is metabolized to anhydroerythromycin and excreted which urine into sewage. The question arise wheather anhydroerythromycin can also induce resistance against MLS_B antibiotics in staphylococci. This was investigated with antibiotic susceptibility tests (D-tests) and macrodilution assays. Staphylococci with $iMLS_B$ phenotype in river water were more numerous (27.8 %) than in treated sewage (18.9 %). The most common MLS_B-resistance gene was *erm*C. Traces of erythromycin and anhydroerythromycin

(1 ng L^{-1}) induced already resistance against clindamycin after only 10 min. exposure. This is reported for the first time and is relevant for risk assessment.

3.1 Introduction

Pollution of the environment with antibiotics and antibiotic-resistant bacteria of different sources is an increasing problem world-wide (Kümmerer, 2004). Even with a state-of-the-art sewage treatment antibiotic-resistant bacteria leave sewage treatment plants with the purified sewage and may cause a spread of antibiotic resistance genes by horizontal gene transfer and establishment of multiresistant bacteria in the environment.

The annual consumption of erythromycin and of other macrolides in human and veterinary medicine was about 70 t in Germany in the last decade (Witte *et al*., 2011; Küster *et al*., 2013). In humans 50 – 70 % of erythromycin is inactivated. The parent compound and metabolites are excreted via bile and feces. The common inhibition target of macrolides, lincosamides or streptogramin B (MLS_B) is protein biosynthesis (Leclercq & Courvalin, 2002). Several resistance genes against the above mentioned antibiotics have been reported. Expression of only one of the 40 known genes of the *erm*-gene family, that encode for a N^6-dimethylase, leads to resistance against MLS_B-antibiotics. If a cell possesses an *erm*-gene, erythromycin can attach to mRNA and the start codon of the methylase gene is accessible (Mayford & Weisblum, 1990; Ackermann & Rodloff, 2003). As a result of methylation the target site

of erythromycin is changed and the antibiotic can no longer bind to mRNA and thus inhibit protein biosynthesis. Overlapping binding sites of the above mentioned three classes of antibiotics account for cross-resistance (Leclercq, 2002). Expression of *erm*-genes can either be constitutive or inducible. In the last case the methylase is only synthesized if an inducer is present ($iMLS_B$).

Erythromycin is a lipophilic antibiotic with an alkaline pH (pK_a 8.8). By dehydratation erythromycin-enolether is formed which reacts irreversibly to anhydroerythromycin (Cachet *et al*., 1989; Butler & Weber, 2005). After oral application erythromycin is converted to anhydroerythromycin at low pH in the stomach (Marvola *et al*., 1991, Hassanzadeh *et al*., 2006), which is then excreted with urine. Due to widespread human and veterinary use of erythromycin, anhydroerythromycin is present in sewage (90 – 6000 ng L^{-1}; Umweltbundesamt Germany 2005; Yang *et al*., 2006; BLAC 2003; Sachverständigenrat für Umweltfragen 2007), in surface water (up to 486 ng L^{-1}; Santos *et al*., 2010) and groundwater (from 0.61 up to 49 ng L^{-1}; López-Serna *et al.*, 2013, Sacher *et al*., 2001).

Anhydroerythromycin as such has little antimicrobial activity (Lakritz & Wilson, 1997; Hansen *et al*., 1999). However, the structure of the C3-cladinose sugar, which is responsible to induce resistance against MLS_B-antibiotics by erythromycin is unchanged. Furthermore anhydroerythromycin is able to induce resistance to the parent drug and to carbomycin (Majer, 1981) and therefore the question arises whether anhydroerythromycin might also act as an inducer of resistance against MLS_B-antibiotics such as clindamycin.

For a detailed investigation of resistance of staphylococci in sewage treatment plants and in the receiving water bodies against MLS_B antibiotics more than 1000 *Staphylococcus* strains were isolated from sewage and surface water and were tested against 4 erythromycin, ciprofloxacin, oxacillin and clindamycin. A special focus was layed on inducible resistance against clindamycin by erythromycin and anhydroerythromycin. Another aspect was whether very low concentrations of anhydroerythromycin in aqueous ecosystems of 1 ng L^{-1} could induce resistance against clindamycin and finally the contact time of staphylococci with anhydroerythromycin to obtain clindamycin-resistance was investigated.

3.2 Materials & Methods

3.2.1 Sampling sites and sampling

The Schussen and the Argen are two tributaries of Lake Constance in Southern Germany. Lake Constance serves as a reservoir of drinking water for many cities in Baden Wuerttemberg as well as for recreation. Treated sewage of the sewage treatment plant (STP) Ravensburg-Langwiese with a capacity of 170,000 population equivalents (PE; including sewage from one hospital with 520 beds and 8 senior residences) and Eriskirch with a capacity of 40,000 PE (including sewage from one hospital with 185 beds and 2 senior residences) is disposed into the river Schussen, whereas treated sewage of the STP Merklingen with a capacity of only 2400 PE (including only sewage from one 1 senior residence) flows into the Würm. No wastewater from

slaughter houses is treated in the three STPs. At 7 sampling campaigns twenty-four-hour composite samples of raw sewage and effluent of the STP's before and after ozonation, sand or charcoal filtration for inactivation or further removal of bacteria, respectively, were taken. During 4 sampling campaigns surface water downstream of sewage inlet pipes into the river (random samples) was additionally collected. In addition samples were also taken from influents and effluents of the storm water overflow basin Mariatal and from the soil filter Tettnang (Triebskorn *et al.*, 2013; www.schussenaktivplus.de). The water samples were filled into 1 L autoclaved glass bottles and stored refrigerated at 4 ^{o}C after sampling. Preparation and filtration of samples, if necessary, was finished latest 48 h after sampling.

3.2.2 Strain isolation and identification

Chapman-Stone agar containing 0.05 g L^{-1} sodium azide was used for isolation of staphylococci. Undiluted and 10-fold diluted raw sewage samples were plated on agar dishes. To obtain staphylococci from river water 10 to 40 mL and from effluents of STP's 25 to 400 mL of respective samples had to be filtered through 0.45 µm sterile membrane filters with a diameter of 4.7 cm. The filters were then placed on Chapman-Stone agar plates. After incubation for 48 h at 37 °C, 20 colonies of each sample were streaked on Mannitol-Salt agar and re-streaked once after growth. Single colonies were then re-grown on DEV-nutrient agar and then suspended in 0.9 % NaCl solution to an optical density of McFarland = 0.5. Tests on Micronaut-Staph®-microtiter plates (MERLIN, Gesellschaft für mikrobiologische

Diagnostika mbH) were inoculated with 100 µL of this suspension for identification and for testing antibiotic susceptibility. Staphylococci which could not be identified at species-level with the Staph®-microtiter plates were identified at the genus-level with *Staphylococcus*-specific PCR, targeting the *tuf*-gene (Martineau *et al*., 2001).

3.2.3 Antibiotic susceptibility testing and D-Test

Susceptibility to oxacillin (5 µg), ciprofloxacin (5 µg) and erythromycin (15 µg) was tested by the disc-diffusion test with Mueller-Hinton agar according to German industrial standard (Deutsche Industrie Norm, DIN) 58940 (2011) and to clindamycin (2 µg) according to Clinical and Laboratory Standards Institute (CLSI, 2011). For inoculation a hundred-fold diluted bacterial suspension (McFarland of 0.5) from an overnight culture was prepared. Six-mm discs, impregnated with respective antibiotics (Becton Dickenson, Franklin Lakes, New Jersey, USA) were placed on the agar plate. After incubation for 18 h at 37 °C zones of inhibition around each antibiotic-containing disc were measured and interpreted according to the breakpoints listed in the guidelines for the DIN and CLSI test.

The D-Test was performed according to CLSI (2011) with all strains which were resistant to erythromycin and susceptible to clindamycin in the agar-diffusion test. A 1.0 McFarland suspension with biomass of an overnight culture, grown on DEV-nutrient agar, of respective strains was used for inoculation of Mueller-Hinton plates. The clindamycin discs (2 µg) were placed 17 mm apart (centre to centre) from erythromycin

discs, that were soaked with different amounts of erythromycin (0.2 ng - 15 µg) or acidified erythromycin (= anhydroerythromycin; 4 ng – 15 µg). After incubation at 37 °C for 18 h bacterial lawn showing flattening of the inhibition zone ("D"-formation) around clindamycin discs adjacent to erythromycin discs indicated the $iMLS_B$-phenotype, in contrast to the round inhibition zone of a D-test-negative isolate. Susceptibility of staphylococci against erythromycin and anhydroerythromycin was tested with antibiotic discs on Mueller-Hinton agar plates that contained 15 µg erythromycin and 15 µg anhydroerythromycin. As a control commercially distributed erythromycin discs containing 15 µg erythromycin (Becton Dickenson) were used.

3.2.4 Macrodilution

To test for minimal inducer concentrations 3 ml Mueller-Hinton broth supplemented with 1 mg L^{-1} clindamycin were inoculated with 30 µL (1 %) of a 0.5 McFarland suspension of a *Staphylococcus* colony from a DEV-nutrient agar plate (overnight culture) in the presence or absence of 1 ng L^{-1} of the inducer erythromycin or anhydroerythromycin and incubated at 37 °C. After 24 h of incubation at 37 °C any turbidity indicating growth was interpreted as induction of resistance against clindamycin. To determine the time span for establishment of resistance (mediated by e.g. *erm*-gene expression and tested phenotypically by growth), colonies from DEV-nutrient agar grown overnight were suspended in 3 mL Mueller-Hinton broth that contained 1 µg L^{-1} of the inducer erythromycin or anhydroerythromycin. After 10 minutes of

incubation at 37 °C one mL of the suspension was centrifuged and washed in 1 mL 0.9 % NaCl solution twice to eliminate the respective inducer. Thirty µL of a 0.5 McFarland suspension of these cells were inoculated in 3 mL Mueller-Hinton broth containing 1 mg L^{-1} clindamycin in the absence of the inducer. The respective growth control without any antibiotics added was used to check the influence of centrifugation and washing on cell survival. After 24 h of incubation at 37 °C any growth in the broth with clindamycin was recorded as induced resistance. To test the resistance of all D-test-positive staphylococci against clindamycin the macrodilution assay was used. Thirty µL of respective D-test-positive staphylococci strains in a 0.5 McFarland suspension were inoculated in 4 test tubes: the first containing only 3 ml Mueller-Hinton broth (growth control), the second filled with 3 ml Mueller-Hinton broth that was supplemented with 1 mg L^{-1} clindamycin and the third and fourth filled with 3 ml Mueller-Hinton broth that was supplemented with 1 mg L^{-1} clindamycin and 0.4 mg L^{-1} erythromycin or 0.4 mg L^{-1} anhydroerythromycin, respectively. After incubation for 24 h at 37 °C any turbidity was interpreted as induced resistance to the lincosamide. Macrodilution tests were repeated at least once.

3.2.5 Detection of iMLS$_B$ resistance genes

All isolates that phenotypically revealed inducible clindamycin-resistance were tested for the presence of the iMLS$_B$-resistance genes *erm*A, *erm*B, *erm*C, *erm*F, *erm*G, *erm*Q, *erm*T and *erm*43. Amplification reactions were performed with a total volume of 25 µL containing:

0.625 units True Start HS Taq DNA Polymerase, 2.5 mM $MgCl_2$ (Thermo Fisher Scientific, Waltham, Massachusetts, USA), 0.25 mM of each dNTP, 10 µM of both primers and 0.5 µL of template DNA extracted with phenol-chloroform. Used primer-sequences and PCR-protocols for *erm*A, *erm*B, *erm*C, *erm*F, *erm*G and *erm*Q were published by Koike *et al*. (2010), for *erm*T by Feßler *et al*. (2010) and for *erm*43 by Schwendener & Perreten (2012). Successful DNA-extraction of all strains that were tested negative for the 8 *erm*-genes was checked for PCR amplificability of a *tuf*-gene fragment characteristic of the genus *Staphylococcus* (Martineau *et al*., 2001). The PCR-products were analysed on a 2 % agarose-gel in order to identify the respective amplicons.

3.2.6 Solutions

Ten mg clindamycin (Sigma) were dissolved in 2 mL deionized water and made sterile by filtration through a 0.2 µm membrane filter. This stock solution was stored at 4 °C. Solutions of erythromycin (Roth, Karlsruhe) with different concentrations were prepared in 40 mM phosphate buffer, pH 7.0, directly before use. Commercially available anhydroerythromycin (Sigma) was also tested but according to the manufacturer product sheet, the purity was only in the order of 95 % and the pH was not mentioned at all. Therefore, for preparing anhydroerythromycin, erythromycin was dissolved in deionized water and the pH lowered below 3 by addition of 50 µL 0.1 M HCl. Anhydroerythromycin solutions were prepared at least 2 h before use and stored at room temperature (21 °C). Dehydration of erythromycin to

anhydroerythromycin and the stability of anhydroerythromycin under weak alkaline conditions (no back reaction after dehydratation) was tested by improvement of the "reduced" antimicrobial activity in an agar-diffusion test with Mueller-Hinton agar plates and respective erythromycin-susceptible strains.

3.3 Results

3.3.1 *Staphylococcus* isolates and species diversity

During sewage treatment cell numbers of staphylococci (median values) decreased up to 4 $\log_{10}$ orders from initially 2.7 x 10^4 to 2.3 x 10^0 cfu per 100 mL (Fig. 3.1). In surface waters the cell numbers were higher with median values of 2.6 x 10^1 cfu per 100 mL (Fig. 3.1).

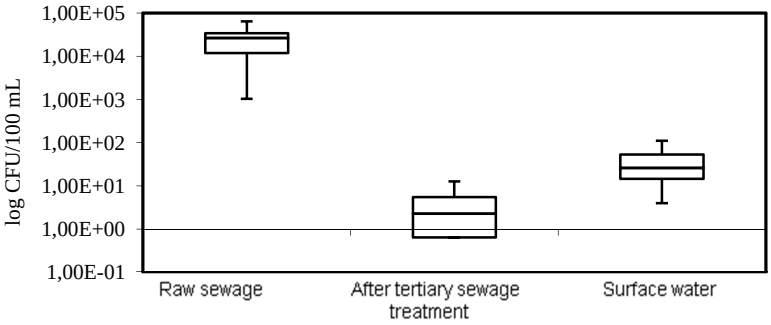


Fig. 3.1: Colony forming units (cfu) of *Staphylococcus spec.* per 100 mL of raw sewage (12 samples), sewage after tertiary treatment (23 samples) and surface water (20 samples). Boxes represent 50 % of all measured values and lines median values. Wiskers represent minimal and maximal values.

From three sampling-campaigns 1048 staphylococci were isolated and identified at species level. Most *Staphylococcus* isolates (Table 3.1) belonged to the Saprophyticus- (57.3 %) and the Sciuri-group (21.5 %). More staphylococci belonging to the Sciuri-group were obtained from river water (24.4 %) than from tertiary treated sewage (12.7 %). *S. saprophyticus* ssp. *saprophyticus* and *S. xylosus* were the most numerous isolated staphylococci. In the receiving river waters the percentage of *S. saprophyticus* ssp. *saprophyticus* decreased in favor of *S. xylosus*. About 20 % of the *Staphylococcus* isolates could not be classified within the two groups mentioned above (Table 3.1), but only 4 *Staphylococcus aureus* strains were isolated from all samples.

Table 3.1: Diversity and antibiotic susceptibility of isolated *Staphylococcus* species from sewage and receiving river water

Source	Raw sewage						After sewage treatment						After tertiary sewage treatment						Schussen/Argen rivers					
Species	No	No-r	E	CC		Ox	No	No-r	E	CC		Ox	No	No-r	E	CC		Ox	No	No-r	E	CC		Ox
				c	i					c	i					c	i					c	i	
S. aureus	1	0					0	0					2	0					1	0				
S. capitis ssp. *capitis*	1	1	1				1	1	1				1	0					0	0				
S. capitis ssp. *ureolyticus*	0	0					1	0					0	0					0	0				
S. cohnii ssp. *cohnii*	16	14	14	1	1	4	13	8	8		1	1	14	11	11		5	1	1	0				
S. cohnii ssp. *urealyticum*	10	6	6	1	4		8	2	2				4	1	1				3	1	1	1		
S. chromogenes	0	0					0	0					1	0					3	0				
S. epidermidis	4	3	2			1	3	0					8	1	1				16	7	7		3	
S. gallinarum	2	0					0	0					2	0					6	1	1			
S. haemolyticus	1	0					0	0					2	0					2	0				
S. hominis	5	2	2			1	5	3	3			1	7	5	5				3	0				
S. kloosii	7	2	2		1		8	2	2				5	1	1			1	7	1	1			
S. lentus	9	0					19	0					13	3	2	3			9	1				1
S. saprophyticus ssp. *saprophyticus*	40	12	12	5			82	24	24	1	2		86	18	18		4	1	47	9	9		4	1
S. sciuri	37	3	3				47	3	1	1		2	15	1		1			76	8	7	6		1
S. simulans	0	0					2	0					4	0					3	0				
S. warneri	0	0					0	0					5	0					20	3	3	1		
S. xylosus	35	13	13	4	1		47	4	4	1			30	5	5	1			128	4	4		2	
S. spec.	45	16	16	3	3	1	29	7	7	1	2		22	9	9		1		24	3	3		1	
Sum	213	72	71	14	10	7	265	54	52	4	5	4	221	55	53	5	10	3	349	38	36	8	10	3
Percentage of resistance		33.8						20.4						24.9						10.9				

Numbers are number of isolates; No = number of total isolates, No-r = number of resistant isolates; other numbers = isolates resistant against the respective antibiotic. E = erythromycin, CC = clindamycin (c: constitutive, i: inducible), Ox = oxacillin. Ciprofloxacin was also tested but no *Staphylococcus* species resistant against ciprofloxacin was obtained within the 1048 isolates; after sewage treatment: effluents of secondary sedimentation and ozonation as well as of the storm water overflow basin and the soil filter; after tertiary sewage treatment: effluent of sand and charcoal filtration or of flocculation filtration

3.3.2 Antibiotic-resistance

None of the 4 *S. aureus* isolates was resistant against one of the 4 tested antibiotics (Table 3.1). Regarding antibiotic resistance, *Staphylococcus cohnii* ssp. *cohnii* was the most resistant species: 75 % of the isolates of this species were resistant at least to one of the tested antibiotics (Table 3.1). The percentage of antibiotic resistant staphylococci decreased during wastewater treatment from 33.8 % in raw sewage to 24.9 % in the tertiary treated sewage and was even lower in surface water with only 10.9 % (Table 3.1). Among 1048 *Staphylococcus* isolates 20.2 % were resistant against erythromycin, whereas only 3 % were resistant against clindamycin and 1.6 % against oxacillin. No ciprofloxacin resistant *Staphylococcus* isolates were obtained (Table 3.1). Twenty-nine of 212 erythromycin resistant *Staphylococcus* isolates (13.7 %) were constitutively resistant against clindamycin and 35 erythromycin resistant *Staphylococcus*-isolates (16.5 %) revealed an inducible clindamycin resistance (Table 3.1). The percentage of isolates that were resistant against erythromycin and clindamycin in river water was higher (22.2 %) than in tertiary treated sewage (5.6 %).

3.3.3 D-test and inducible resistance against MLS_B

All *Staphylococcus*-isolates that were resistant against erythromycin and susceptible to clindamycin (Table 3.1) were either D-test positive (Fig. 3.2a) or D-test negative (Fig. 3.2b).

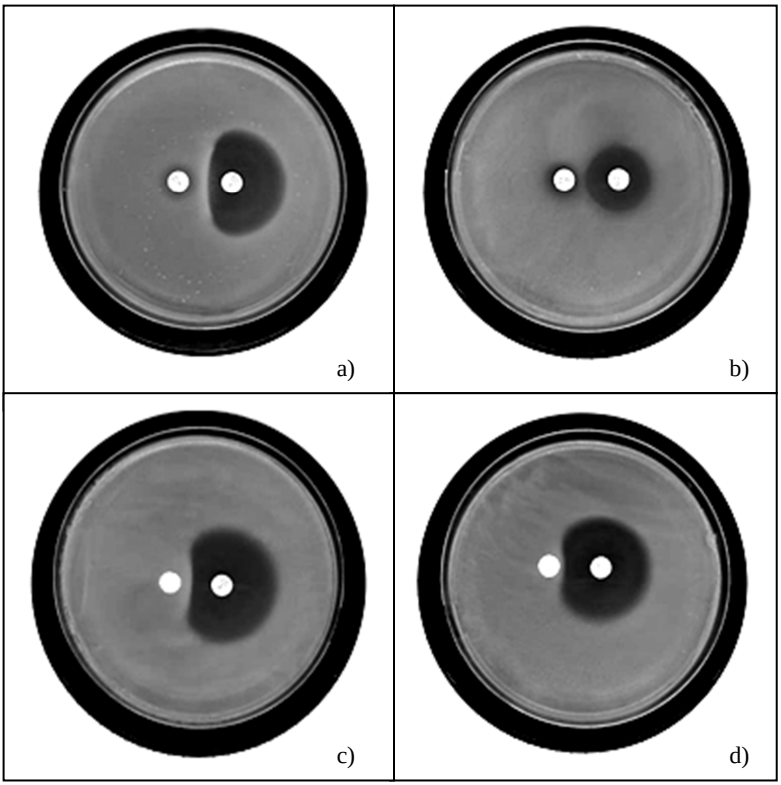


Fig. 3.2 a-d: D-tests with *Staphylococcus* isolates. 3.2a) D-test-positive *S. cohnii* ssp. *cohnii* with 15 µg erythromycin E (left disc) and 2 µg clindamycin CC (right disc). 3.2b: D-test-negative *S. cohnii* ssp. *cohnii* with 15 µg erythromycin E (left disc) and 2 µg clindamycin CC (right disc). 3.2c, d: D-test-positive *S. cohnii* ssp. *cohnii* with only 25 ng erythromycin E (3.2c) or 25 ng anhydroerythromycin (3.2d, left discs) and 2 µg clindamycin (right discs).

D-test-negative *Staphylococcus* isolates were detected in high numbers in raw sewage (66.2 %) as well as in the effluent (tertiary treated sewage) of the 3 STP's (75.5 %), but a lower number (50.0 %) was detected in the river water of Schussen and Argen (Fig. 3.3, dark grey sector). The percentage of erythromycin resistant, inducible clindamycin resistant (D-test-positive) isolates was higher in river water (27.8 %) than in raw and in treated sewage (14 - 19 %). In conclusion the percentage of staphylococci with constitutive or inducible resistance to clindamycin was highest in river water (50.0 %; Fig. 3.3, both light grey sectors).

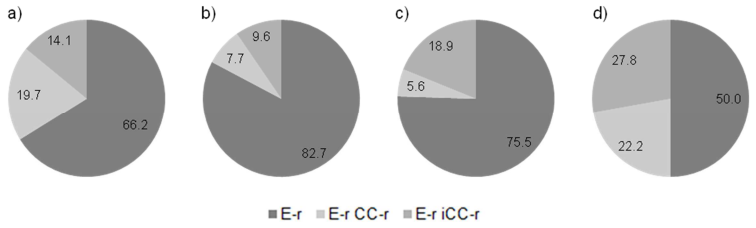


Fig. 3.3: Percentage of *Staphylococcus* isolates that were erythromycin resistant (E-r), erythromycin and constitutively clindamycin resistant (E-r CC-r) and erythromycin and inducible clindamycin resistant (E-r iCC-r) in a) raw sewage (n=71), b) during sewage treatment (n=52), c) treated sewage (n= 53) and d) in river water of the Schussen and Argen (n=36).

Out of the 17 identified *Staphylococcus* species/subspecies of Table 3.1 $iMLS_B$ isolates of 6 species/subspecies were found: *S. cohnii* ssp. *cohnii*, *S. cohnii* ssp. *urealyticus*, *S. epidermidis*, *S. kloosii*, *S. saprophyticus* ssp. *saprophyticus*, *S. xylosus* (Table 3.2).

Table 3.2: Macrodilution and D-test to determine the minimum erythromycin and anhydroerythromycin concentrations for induction of clindamycin resistance in different iMLS$_B$ *Staphylococcus* isolates

Isolated and identified species	Macrodilution (ng L^{-1})		D-test (ng)	
	Erythromycin	Anhydro-erythromycin	Erythromycin	Anhydro-erythromycin
S. cohnii ssp. *cohnii*	1	1	0.3	19.5
S. cohnii ssp. *urealyticum*	1	1	1.35	6
S. epidermidis	1	1	0.2	4
S. kloosii	1	1	0.35	20
S. saprophyticus ssp. *saprophyticus*	1	1	0.4	7.5
S. xylosus			1.85	25

Aside of phenotypical tests for inducible clindamycin resistance, all isolates were checked for the presence of the prominent iMLS$_B$ resistance genes (*erm*A, *erm*B, *erm*C, *erm*F, *erm*G, *erm*Q, *erm*T and *erm*43, respectively), that were hitherto found in staphylococci (Table 3.3). The most common MLS$_B$ resistance gene *erm*C was detected in 60 % of the clindamycin resistant isolates, whereas *erm*A was only detected in 2 isolates (5.7 %). Twelve of 35 isolates revealed inducible resistance to clindamycin, but none of the 8 *erm*-genes mentioned above could be detected.

Table 3.3: Detected *erm*-genes in our $iMLS_B$ resistant isolates of *Staphylococcus spec.*

	*erm*A	*erm*C	no *erm*B, *erm*F, *erm*G, *erm*Q, *erm*T, *erm*43
Raw sewage		4 *S. cohnii* ssp. *urealyticum* 1 *S. cohnii* ssp. *cohnii* 1 *S. xylosus* 1 *S. kloosii* 1 *Staphylococcus spec.*	2 *Staphylococcus spec.* 1 *S. saprophyticus* ssp. *saprophyticus*
Treated sewage	1 *S. cohnii* ssp. *cohnii* 1 *Staphylococcus spec.*	1 *S. saprophyticus* ssp. *saprophyticus* 1 *Staphylococcus spec.*	1 *S. saprophyticus* ssp. *saprophyticus*
Tertiary treated sewage		2 *S. saprophyticus* ssp. *saprophyticus* 1 *S. cohnii* ssp. *cohnii* 1 *Staphylococcus spec.*	4 *S. cohnii* ssp. *cohnii* 1 *S. saprophyticus* ssp. *saprophyticus*
Schussen/Argen		4 *S. epidermidis* 4 *S. saprophyticus* ssp. *saprophyticus*	2 *S. xylosus* 1 *Staphylococcus spec.*

3.3.4 Dehydration of erythromycin to anhydroerythromycin

Anhydroerythromycin formation after acidification of erythromycin was tested by determining the reduced antimicrobial activity with respective erythromycin susceptible strains (Fig. 3.3). The inhibition zone of anhydroerythromycin was about 60 % lower as for erythromycin (11 mm compared to 26 mm, exclusive disc diameter; Fig. 3.4a-c) indicating reduced antimicrobial activity of the metabolite.

Anhydroerythromycin does not react back to erythromycin at a neutral or alkaline pH. This was proven by testing the antimicrobial activity of neutralized anhydroerythromycin in the agar diffusion test (Fig. 3.4d). The inhibition zone of 17 mm (inclusive disc diameter) was as low as it was for anhydroerythromycin (Fig. 3.4c).

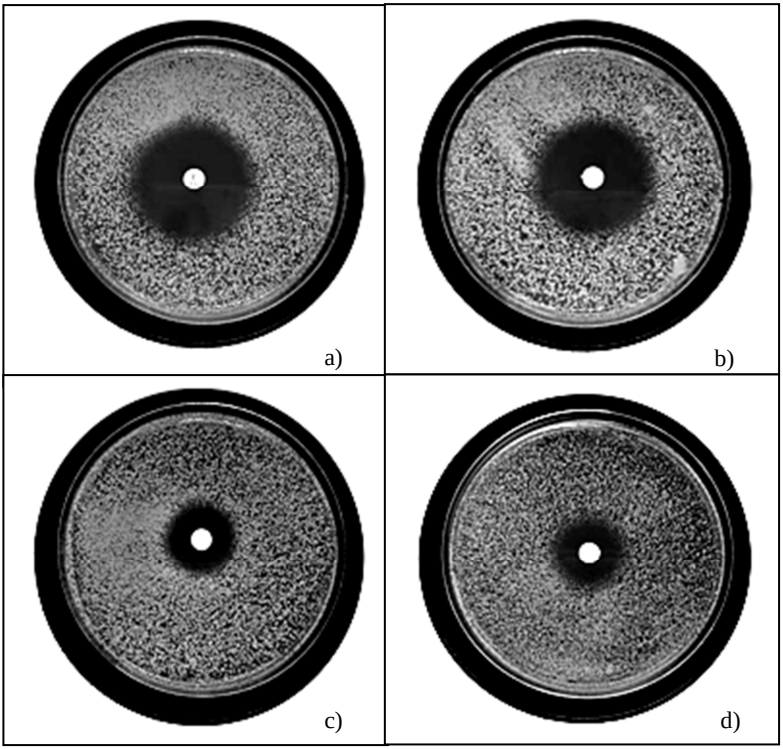


Fig. 3.4: Agar diffusion test of *S. xylosus* on Mueller-Hinton agar plates after incubation at 37 °C for 18 h. a) commercial and b) freshly spotted disc containing 15 µg erythromycin, c) freshly spotted disc containing 15 µg anhydroerythromycin and d) freshly spotted disc containing 15 µg neutralized anhydroerythromycin pH 7.

3.3.5 Anhydroerythromycin, an inducer for clindamycin resistance

In the D-test the typical flattening of the inhibition zone was already seen, when erythromycin and anhydroerythromycin were only dropped in extremely low amounts of 25 ng on the adjacent blank disc (Fig. 3.2c, d). The minimal erythromycin and anhydroerythromycin loads of discs for a positive D-test of $iMLS_B$ isolates were not identical: they varied between 0.2 and 1.85 ng erythromycin and 4 and 25 ng anhydroerythromycin (Table 3.2). With the exception of one *S. xylosus* isolate, all D-test-positive *Staphylococcus* strains in agar diffusion tests did also grow in the macro dilution test (Table 3.2). One ng L^{-1} of erythromycin or anhydroerythromycin was sufficient to induce clindamycin. Induction of clindamycin-resistance was confirmed by growth in test tubes containing erythromycin (Fig. 3.5b) or anhydroerythromycin as inducers (Fig. 3.5c), whereas no growth was obtained in the test tubes containing only clindamycin (Fig. 3.5d).

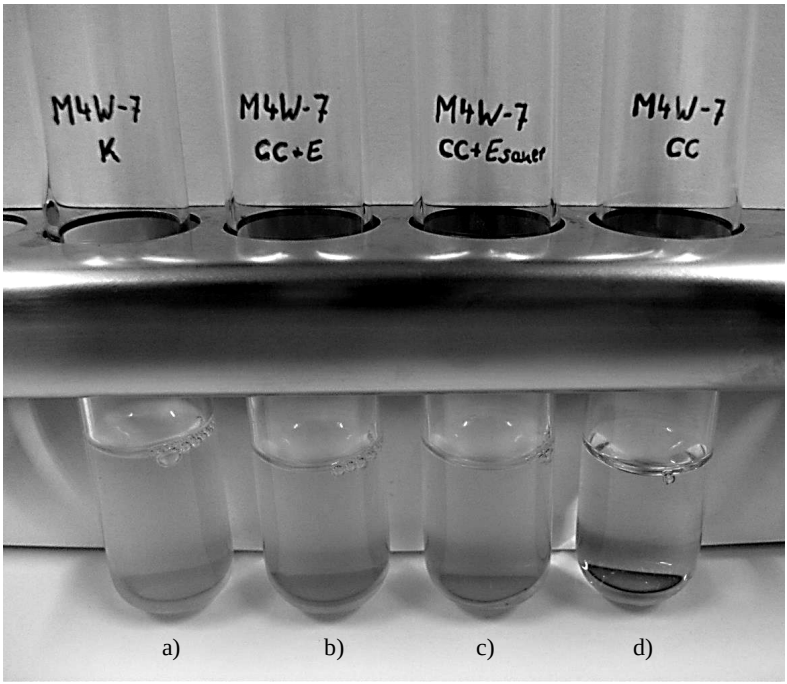


Fig. 3.5: Growth of an $iMLS_B$ phenotype *S. epidermidis* isolate in a) Mueller-Hinton broth (control), b) Mueller-Hinton broth + 1 mg L^{-1} clindamycin + 1 ng L^{-1} erythromycin, c) Mueller-Hinton broth + 1 mg L^{-1} clindamycin + 1 ng L^{-1} anhydroerythromycin and d) Mueller-Hinton broth + 1 mg L^{-1} clindamycin (non-induced control).

Ten minutes of incubation with 1 µg L^{-1} of erythromycin or anhydroerythromycin were enough to induce resistance of staphylococci against clindamycin: After 24 h of incubation all tested strains grew to a similar optical density (Fig. 3.6a-d).

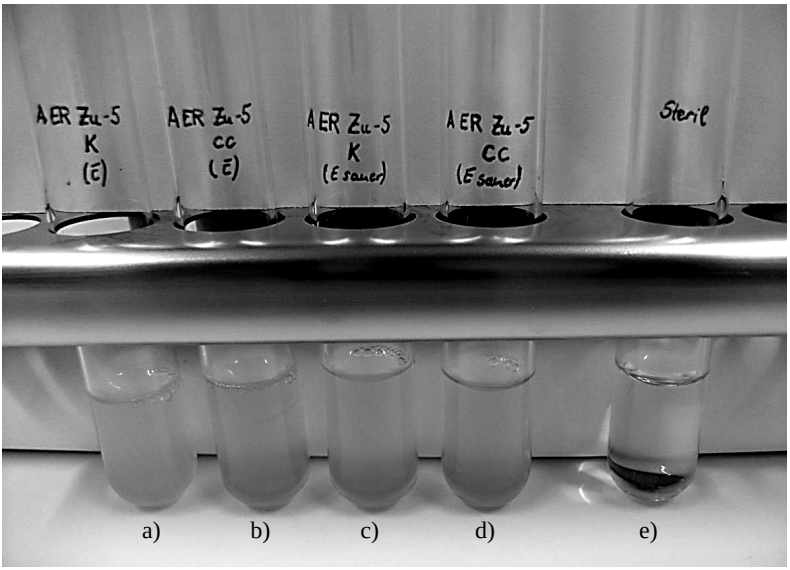


Fig. 3.6: Growth of erythromycin induced, $iMLS_B$ phenotype *S. cohnii* ssp. *urealyticum* in Mueller-Hinton broth (a) or Mueller-Hinton broth + 1 mg L^{-1} clindamycin (b) and of anhydroerythromycin-induced cells in Mueller-Hinton broth (c) or Mueller-Hinton broth + 1 mg L^{-1} clindamycin (d). Vial (e) was a sterile control.

3.4 Discussion

Although in clinical specimen *Staphylococcus spec.* are well investigated, systematic investigations of their role in sewage and environmental aquatic samples were rarely reported. Faria *et al.* (2009) found 10^3 – 10^5 cfu of staphylococci per 100 mL in treated sewage, whereas after discharge of STP effluent into the Czarna Hańcza river in Poland staphylococci varied between "undetectable" and 1.7 x 10^4 cfu per 100 mL (Niewolak & Opieka, 2000). Thus, the population density of staphylococci in river water seemed to be highly variable, which was

consistent with results of the present study. In water samples of the Schussen and Argen river from four sampling campaigns cell numbers of staphylococci varied from 6.5 x 10^0 to 2.5 x 10^2 cfu per 100 mL. One reason for this variability could be that *Staphylococcus* species enter a viable but non-culturable state, which was described for *S. aureus* in a biofilm (Pasquaroli *et al*., 2013).

In most literature sources staphylococci from environmental samples were only identified to the genus level (Niewolak & Opieka, 2000). The prominent human pathogen *S. aureus* and coagulase negative staphylococci (CNS) were just differentiated by testing coagulase activity. Our *Staphylococcus* isolates from different sources (Table 3.1) were identified frequently as members of the Saprophyticus-group (57.3 %) with the predominant species *S. saprophyticus* in sewage samples and *S. xylosus* in river samples (Table 3.1). Similar results were obtained by Faria *et al*. (2009), who reported the dominance of representatives of the Saprophyticus-group in wastewater samples (78.7 %). In their study only one 48 isolates was identified as *S. aureus* by a positive coagulase test. In our study only four strains out of 1048 *Staphylococcus* strains were identified as *S. aureus* (Table 3.1), indicating a weak survival of *S. aureus* in wastewater. Since species such as *S. aureus* or *S. hyicus, S. xylosus, S. cohnii, S. sciuri, S. lentus, S. haemolyticus, S. hominis, S. capitis, S. lugdunensis* and *S. epidermidis* could originate from animals (Aarestrup *et al.*, 2000; Werckenthin *et al*., 2001; Zhang *et al*., 2009) as well as from human excrements, it is not possible to assign isolates from river water to a certain origin by species identification.

Contrary to extensive investigations in clinics, where the consumption of antibiotics corresponded well with the evolution of antibiotic resistance mechanisms (e.g. Witte *et al*., 2011) the resistance situation in aquatic environments was rarely investigated. Due to the use of antibiotics in hospitals, for home medication or for treating animal diseases, non-metabolized or partially metabolized drugs are introduced with excrements via sewage or manure in varying, but measureable amounts into the environment and may cause acquisition of resistances by the indigenous microflora. Whereas clinical isolates of *S. haemolyticus* and *S. epidermidis* were highly resistant against clindamycin, oxacillin, ciprofloxacin and erythromycin, the percentage of resistant isolates from sewage effluents and river water against these antibiotics, with the exception of erythromycin, was much lower, presumably due to the much lower concentrations of exposition. For erythromycin a significantly higher percentage of resistant isolates was found (Table 3.4). A similar high percentage of erythromycin resistant CNS strains in different wastewater samples (21.3 %) as in our study (24 %, Table 3.4) was reported by Faria *et al*. (2009). In the last decade the relevance of erythromycin in medicine was, however, decreasing. Erythromycin was substituted by ketolides, azalids and new macrolides because of better pharmacokinetics and less side effects (Witte *et al*., 2011).The higher percentage of $iMLS_B$ phenotypes of erythromycin resistant isolates from surface water as compared to raw sewage and treated sewage (Fig. 3.3) cannot be explained by selective pressure of clindamycin. The clindamycin concentration in sewage (17 ng L^{-1} average effluent values of STP's in Germany) is higher than in surface water. Aquatic samples on average contain 9 ng L^{-1} clindamycin (BLAC,

2003), which is sub-inhibitory in terms of antimicrobial activity. According to DIN 58940 (2011) more than 0.5 mg L^{-1} clindamycin are required to suppress bacterial growth.

Table 3.4: Comparison of antibiotic resistant *Staphylococcus* isolates with environmental concentrations of antibiotics found in sewage or surface water and annual antibiotics consumption

	Clindamycin	Oxacillin	Ciprofloxacin	Erythromycin
Percentage of resistant clinical isolates of *S. haemolyticus/S. epidermidis* (Witte *et al*., 2011)	47.6/47.5	89/73.8	85.4/66.7	92.7/67.8
Percentage of resistant isolates before and after sewage treatment (this study, Table 3.1)	2.3 (6.8 if inducible resistance is included)	1.4	0	24.0
Percentage of resistant isolates of river water (this study, Table 3.1)	2.3 (inclusive inducible resistance: 5.2)	0.9	0	10.3
Average antibiotic concentration of STP effluents in Germany (ng L^{-1}, in brackets maximum value; BLAC, 2003)	17 (130)	<BG (30)	<BG (144)	137** (6000)
Average antibiotic concentration in surface waters of Germany (ng L^{-1}, in brackets maximum value; BLAC, 2003)	9 (30)	<BG (<BG)	<BG (28)	1** (460)
Antibiotics consumption in human medicine (kg total in Germany 2001; BLAC, 2003)	16,080	118.1	17,973	19,199
Antibiotics requirement for veterinary medicine (t total in Germany 2005*. In brackets main application; Küster *et al*., 2013)	12.1 (infections of the respiratory tract; mostly in poultry farming)	199.2 (infections of the respiratory and the intestinal tracts, sanious infections)	3.7 (infections of the respiratory and the intestinal tracts)	52.6 (infections of the respiratory tract and of the udder)

<BG: below detection limit; *numbers represent total amounts of lincosamides, penicillins, chinolones, macrolides. **detected as anhydroerythromycin

Regarding the distribution of the different resistance phenotypes of clinical samples the dominance of $iMLS_B$ and $cMLS_B$ isolates over MS_B types (no resistance against clindamycin) is conspicuous. Delialioglu *et al*. (2005) for instance found that 54.9 % of CNS isolates from clinical samples were constitutively ($cMLS_B$) or inducibly ($iMLS_B$) resistant to MLS_B antibiotics and only 18.2 % of the CNS isolates with MS_B phenotype were detected. $cMLS_B$ and $iMLS_B$ phenotypes are normally more numerous than the MS_B phenotype, regardless if only *S. aureus* or the typical nosocomial CNS (e.g. *S. epidermidis, S. haemolyticus* ; Witte *et al*., 2011) were examined (Lina *et al*., 1999; Gul *et al*., 2008; Gherardi *et al*., 2009). Possibly, the distribution of our erythromycin resistant *Staphylococcus* isolates can be explained by the species diversity. 74.6 % of all *S. saprophyticus* ssp. *saprophyticus*-isolates, that were resistant to erythromycin, were neither inducibly nor constitutively resistant to clindamycin (Table 3.1). In river water this species was less dominant than in sewage, and other species like *S. epidermidis*, *S. sciuri* or *S. warneri* were isolated more often (Table 3.1). Unfortunately studies on the distribution of resistance genes against antibiotics of the MLS_B group are very rare. Inducible clindamycin resistance of clinical isolates often is based on the expression of *erm*A, *erm*B or *erm*C. Animal isolates mostly express *erm*B (Lina *et al*., 1999). This finding is supported by several investigations with clinical isolates: *erm*A and *erm*C are the predominant *erm*-genes found in *S. aureus* and CNS (Fiebelkorn *et al*., 2003; Gherardi *et al*., 2009). Among our isolates that according to the D-test revealed an inducible clindamycin resistance, *erm*C was the most frequently detected resistance gene. At present more than 40 members of the *erm*-gene family are known and still more *erm*-genes are found (e.g. Schwendener & Perreten, 2012). Twelve of our 35

$iMLS_B$ isolates had none of the 8 resistance genes mentioned earlier. It might be that the diversity of this gene family is higher in the aquatic environment as in a clinical environment due to the occurrence of more different *Staphylococcus* species and horizontal gene transfer within different species.

It is known for long that erythromycin could induce resistance to macrolides, lincosamides and streptogramin B antibiotics in staphylococci (Weisblum *et al*., 1971; Sandler & Weisblum, 1989; Mayford & Weisblum, 1990). In this paper, the induction of resistance to clindamycin by erythromycin and by anhydroerythromycin in the pico molar range (1 ng L^{-1}) is reported for the first time. Measured concentrations of erythromycin and of anhydroerythromycin in surface waters, which very often serve for recreation or as reservoirs for drinking water are thus high enough for induction of resistance to clindamycin that belong to the MLS_B classes. Only 10 minutes contact time of *Staphylococcus* isolates with either erythromycin or anhydroerythromycin are sufficient to induce and to establish resistance to clindamycin (Fig. 3.6), which remains as long as clindamycin is present. Induction of resistance against clindamycin by erythromycin/anhydroerythromycin might even require less time. In consequence infections with induced clindamycin resistant staphylococci while swimming or bare foot walking on the shore side cannot be treated with clindamycin, an often used antibiotic to treat infections with staphylococci in human medicine. Subinhibitory concentrations of clindamycin and the presence of anhydroerythromycin in river water might possibly support the spread of *erm*-genes. The $iMLS_B$ phenotype is detected in pathogenic and non-pathogenic

Staphylococcus species from humans and animals. Such *Staphylococcus* strains could play a role as a reservoir for resistance genes and are possibly the origin for their spread. The observation that anhydroerythromycin is a potent inducer for resistance against lincosamides even at pico molar concentrations must be considered as a wide-spread potential risk for human health. The quantitative retention of antibiotics during sewage treatment by adsorption onto powdered charcoal or oxidation by ozonation or H_2O_2-/UV treatment should be intended since very low residual concentrations of an antibiotic in the sub inhibitory range or even of a metabolite may induce resistance against different classes of antibiotics.

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4. Resistance behavior of inducible clindamycin-resistant staphylococci from clinical samples and from aquatic environments

Um herauszufinden, ob sich das Resistenzverhalten von Umweltisolaten, die permanent subinhibitorischen Antibiotika-Konzentrationen ausgesetzt sind, und von klinischen Isolaten, welche mit inhibitorischen Antibiotika-Konzentrationen behandelt werden, unterscheidet, wurde das Verhalten der in der vorangegangenen Studie beschriebenen iMLS$_B$-Staphylokokken mit klinischen Staphylokokken, die ebenfalls eine induzierbare Clindamycin-Resistenz zeigten, verglichen:

- Im Vergleich zur Klinik, in der eine induzierbare MLS$_B$-Resistenz praktisch ausschließlich auf die Expression von *erm*(A) oder *erm*(C) zurückzuführen ist, war die *erm*-Gendiversität in der aquatischen Umwelt deutlich größer.
- Anhand des phänotypischen Verhaltens gegen Makrolide, wobei sich Oleandomycin als bester Marker herausstellte, ist es möglich auf das für die Kreuzresistenz kodierende *erm*-Gen zurückzuschließen: Das kodierende *erm*-Gen bestimmt den Phänotyp und nicht die Herkunft des Staphylokokken-Isolates oder die Spezies. Dies ist insbesondere dann von Bedeutung, wenn man berücksichtigt, dass *erm*(C)-positive Isolate deutlich häufiger mutierten (und dadurch konstitutiv gegen alle MLS$_B$-Antibiotika resistent wurden) als beispielsweise *erm*(A)-, *erm*(43)-exprimierende Staphylokokken oder Isolate mit bisher unbekanntem *erm*-Gen. Dies gilt es bei der Entscheidung, welches

Antibiotikum zur Behandlung einer Infektion mit einem iMLS$_B$-*Staphylococcus* herangezogen werden soll, zu berücksichtigen.

- *erm*(43)-exprimierende Staphylokokken und Isolate mit bisher nicht bekanntem *erm*-Gen zeigten – im Gegensatz zur high-level Erythromycin-Resistenz von *erm*(A)- und/oder *erm*(C)-positiven Staphylokokken – eine low-level-Erythromycin-Resistenz. Isolate mit minimalen Hemmkonzentrationen in diesem niedrigen Bereich (3-16 mg/L) werden in der Klinik bisher – basierend auf den derzeit gültigen klinischen Grenzwerten – zum Teil nicht als resistent erkannt und in der Folge Infektionen mit solchen Erregern möglicherweise falsch therapiert.
- Der Bereich der minimalen Hemmkonzentrationen gegen Clindamycin, den die Umweltisolate zeigten, war deutlich größer als bei klinischen iMLS$_B$-Staphylokokken.

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Abstract

In this study species diversity of staphylococci with inducible resistance to macrolides, lincosamides and streptogramin B (MLS$_B$) isolated from clinical samples, sewage and river water was investigated. Inducible clindamycin-resistance was tested using D-test and macrodilution-assays. Inducible cross-resistance (iMLS$_B$-phenotype) was examined by

PCR of *erm* gene classes *A, B, C, F, G, Q, T* and *43.* Although *ermC* was the most frequently detected resistance gene in iMLS$_B$-phenotypes of environmental staphylococci (61.2 %) resistance genes encoding for iMLS$_B$ were more diverse than in staphylococci from hospital samples. In 22.4 % of iMLS$_B$-staphylococci from aquatic environments none of the eight tested *erm* genes was found. Those isolates and *erm43* expressing *Staphylococcus lentus* displayed low erythromycin-MICs (3-16 µg ml^{-1}) compared to *ermC*-positive environmental staphylococci (≥ 256 µg ml^{-1}). Contrary to clinical isolates with clearly defined resistance behavior, resistance patterns against MLS$_B$ and MICs for clindamycin of environmental isolates were more divers. Although the abundance of iMLS$_B$-staphylococci in the aquatic environment was lower than in staphylococci from hospital samples, the diversity of resistance genes encoding for this phenotype seemed to be higher. Oleandomycin is the best marker to correlate iMLS$_B$-phenotype and the respective *erm* gene. The phenotypical behavior of environmental isolates may differ from the resistance pattern of clinical iMLS$_B$-staphylococci expressing *ermA* or *ermC*, and this should be considered for successful treatment of infections.

4.1 Introduction

Macrolides and lincosamides are frequently medicated human and veterinary antibiotics to treat infections caused by staphylococci (Lina *et al.*, 1999; Lüthje & Schwarz, 2006). Mechanisms of resistance against these drugs include drug inactivation, export of the antibiotic and ribosomal modification and result in a variety of phenotypes (Leclercq,

2002). The last mentioned mechanism, leading to combined resistance against macrolides, lincosamides and streptogramin B (MLS_B) in staphylococci, is based on the expression of one or more *erm* genes of the classes *A, B, C, F, G, Q, T, Y, 33* and *43* (Wendland *et al.*, 2013). These genes code for methylases that modify the target site(s) in 23S rRNA and by this inhibit the binding of MLS_B (Wendland *et al.*, 2013). Expression of *erm* genes can either be inducible or constitutive. Cells with inducible resistance against MLS_B are sensitive to these drugs as long as no adequate inducer is present. It depends on the sequence in front of the respective *erm* gene which drug is able to act as inducer and this may serve to distinguish *erm* genes (Leclercq, 2002). Cells that constitutively express one *erm* gene are resistant against all MLS_B. Due to deletions, insertions and point mutations, mainly in the region of the translational attenuator, the tertiary structure of the rRNA can be changed leading to accessibility of the start codon. Due to these changes the presence of an rRNA-binding inducer is no longer necessary and the expression of the *erm* gene becomes constitutive. The frequency of occurrence and the type of mutation differ depending on the structure of the regulatory leader region as well as on the copy number (Daurel *et al.*, 2008). The localization of these genes varies: whereas *ermA* is usually borne by transposon Tn554, *ermC* is often found on small plasmids (Daurel *et al.*, 2008; Leclercq, 2002). The transferability of these genetic elements may explain the detection of *erm* genes in Gram-positive as well as in Gram-negative genera (Leclercq, 2002). There are many clinical studies examining the abundance of inducible and constitutively expressed MLS_B-resistant staphylococci, especially of *S. aureus* with comparable results: 70-80 % of clinical staphylococci are resistant against macrolides–and about 75 % of them reveal ribosomal

modifications (Delialioglu *et al.*, 2005; Gul *et al.*, 2008; Witte *et al.*, 2011). However, the phenomenon of inducible resistance to MLS$_B$ is not restricted to staphylococci that were isolated from human or animal samples. Although the percentage of macrolide-resistant staphylococci in the aquatic environment is lower (20 % versus 70-80 % in clinical samples) (Faria *et al.*, 2009; Heß & Gallert, 2014), 31.1 % of them reveal inducible or constitutive resistance to clindamycin (Heß & Gallert, 2014). Whereas clinical isolates with inducible MLS$_B$-resistance, which are genetically almost exclusively encoded by *ermA* or *ermC*, are phenotypically well characterized (Daurel *et al*., 2008; Di Modugno *et al.*, 2002; Hamilton-Miller & Shah, 2000), very little is known about "environmental"-iMLS$_B$-staphylococci. For successful medication after infection, identification and information about their phenotypic behavior is necessary. To assess this potential risk for human and animal health, based on the genetic reservoir of staphylococci from aquatic environment, 49 iMLS$_B$-staphylococci were isolated from sewage or from river water and were phenotypically characterized according to their resistance patterns against macrolides. Their reaction to inhibitory concentrations of the lincosamide clindamycin was investigated and compared with a reference group of 19 clinical *Staphylococcus* isolates.

4.2 Materials and methods

4.2.1 Sampling and strain isolation

During eight sampling campaigns in 2012 and 2013, 24-h composite samples of raw sewage as well as effluents of different treatment steps in the sewage treatment plants (STPs) in Eriskirch, RV-Langwiese and Merklingen were collected together with river water of Schussen and Argen, two tributaries of Lake Constance in Southern Germany. The river water samples were taken downstream of sewage inlet pipes (random samples, 5 sampling campaigns). Furthermore, water samples were also taken from influents and effluents of the storm water overflow basin in Mariatal and the soil filter in Tettnang (for details concerning sampling locations and the *Staphylococcus* species diversity in the respective compartments see Heß & Gallert, 2014; Triebskorn *et al.*, 2013; www.schussenaktivplus.de). The water samples were taken in 1 l autoclaved glass bottles and cooled to 4 °C after sampling. For isolation of staphylococci 100 µl-samples of undiluted and 10-fold diluted raw sewage were plated on Chapman-Stone agar dishes containing 0.05 g l^{-1} sodium azide. To obtain isolates, 10 to 40 ml river water and 25 to 400 ml of STP effluents were filtered through 0.45-µm sterile membrane filters with a diameter of 4.7 cm for concentration. The filters were placed on Chapman-Stone agar and incubated at 37 °C. After 48 h, colonies were streaked on mannitol-salt agar and re-streaked once after growth. The clinical staphylococci were obtained from Städtisches Klinikum Karlsruhe and were isolated by the staff of the municipal hospital from samples of different patients, mainly from smears of skin and mucous membranes but also smears of wounds and blood cultures.

4.2.2 Identification, antibiotic susceptibility testing and D-test

The isolates were identified by their physiological reactions on Micronaut-Staph®-microtiter plates (MERLIN, Gesellschaft für mikrobiologische Diagnostika, Bornheim, Germany). For this purpose single colonies that grew overnight on DEV-nutrient agar were suspended in 0.9 % NaCl solution to an optical density of McFarland = 0.5. Every well of the microtiter plate was inoculated with 100 µl of this suspension. After 24 h of incubation at 37 °C, physiological reactions in the wells of the plates were recorded by photometer analysis. Isolates that could not be identified at species level with the Staph®-microtiter plates were identified at the genus level with *Staphylococcus*-specific PCR, targeting the *tuf*-gene (Martineau *et al.*, 2001).

Susceptibility to erythromycin (15 µg; Becton Dickenson, Franklin Lakes, NJ), oleandomycin (15 µg; Oxoid Deutschland GmbH, Wesel, Germany), clarithromycin (15 µg; Oxoid), azithromycin (15 µg; Oxoid), spiramycin (100 µg; Oxoid), tylosin (30 µg; MAST Diagnostica GmbH, Reinfeld, Germany) and telithromycin (15 µg; Oxoid) was tested by the disc diffusion test according to DIN 58940 (Deutsche Industrie Norm, 2011) and to clindamycin (2 µg) according to the Clinical and Laboratory Standards Institute (CLSI, 2011). For inoculation 100 µl of a hundred-fold diluted bacterial suspension (McFarland = 0.5), prepared from an overnight culture on DEV-nutrient agar, were plated on a Mueller-Hinton agar. Six-mm discs, impregnated with the respective antibiotic, were placed on the agar plate and after 18 h of incubation at 37 °C zones of inhibition around each antibiotic-containing disc were measured.

Inducible clindamycin-resistance was tested by the D-test according to CLSI (2011) with all erythromycin-resistant and clindamycin-susceptible strains. For this purpose 100 µl of a 1.0 McFarland suspension prepared with biomass of an overnight culture on a DEV-nutrient agar were streaked on a Mueller-Hinton agar plate. The 2 µg clindamycin-containing discs were placed 17 mm apart (center to center) from the discs impregnated with 15 µg erythromycin. After incubation at 37 °C for 18 h, the bacterial lawn showing flattening of the inhibition zone around the clindamycin discs adjacent to the erythromycin discs indicated the iMLS$_B$-phenotype (Fig. 4.1(a)). To verify the result of the D-test the erythromycin induced clindamycin-resistance was tested with the macrodilution assay as previously described (Heß & Gallert, 2014).

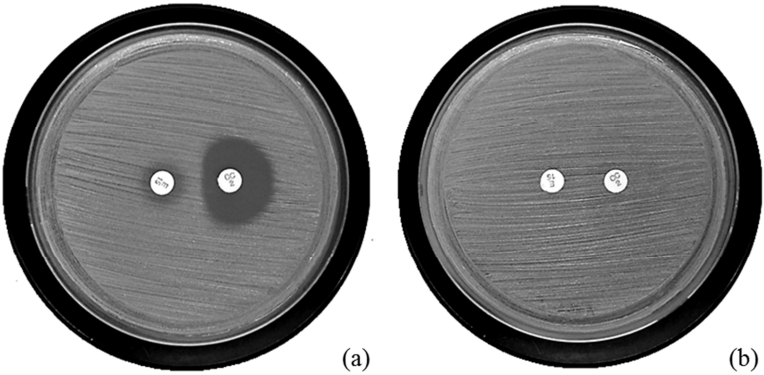

Fig. 4.1: D-test of an iMLS$_B$ *S. cohnii* ssp. *urealyticum* strain isolated from sewage before (a) and after mutation (b). "D-formation" around a 2 µg clindamycin containing disc adjacent to a disc spotted with 15 µg erythromycin by the "wild strain" (a) and acquired constitutive resistance against clindamycin by a culture that was pregrown in the presence of 5 µg clindamycin ml^{-1} (b)

4.2.3 Determination of MIC

For determination of the MIC of erythromycin and clindamycin, colonies from an overnight culture on DEV-nutrient agar were suspended in 0.9 % NaCl to an optical density of McFarland = 1.0. Hundred µl of the bacterial suspension were streaked on a Mueller-Hinton agar and an Etest-strip (bioMérieux, Marcy-L'Etoile, France), soaked with increasing concentrations of the respective antibiotic (0.016 to 256 µg ml^{-1} from one end to the other), was layed on it. After incubation at 37 °C for 18 h, the interception of bacterial lawn/inhibition zone with the concentration marked on the strip was read.

4.2.4 Detection of *erm* genes

Inducible clindamycin-resistance was examined by testing for the resistance genes *ermA, ermB, ermC, ermF, ermG, ermQ, ermT* and *erm43*. DNA-amplification was performed in a total volume of 25 µl containing 0.625 units True Start HS Taq DNA Polymerase, 2.5 mM $MgCl_2$ (Thermo Fisher Scientific, Waltham, MA), 0.25 mM of each dNTP, 10 µM of both primers (double concentration for the detection of *erm43*) and 0.5 µl of template DNA that was extracted with phenol/chloroform. Used primer sequences and PCR protocols were described previously (Feßler *et al.*, 2010; Koike *et al.*, 2010; Schwendener & Perreten, 2012). Successful DNA extraction of all strains that were tested negative for the eight *erm* genes, was checked by PCR amplificability of a *tuf* gene fragment specific for the genus *Staphylococcus* (Martineau *et al.*, 2001). The PCR products were

separated on a 2 % agarose gel strained with ethidiumbromide to identify the respective amplicons.

4.2.5 Mutation experiments

To test the reaction of the 68 D-test positive (iMLS$_B$) *Staphylococcus* isolates against inhibitory concentrations of clindamycin in the absence of an inducer, growth on clindamycin screening-plates was checked. For this purpose 10 µl of bacterial suspension, prepared with biomass of an overnight culture on DEV-nutrient agar (10 µl of McFarland = 0.5 → ~ 10^6 cells) of the respective strain, was dropped on a Mueller-Hinton agar without antibiotic (non-inhibited growth control; Fig. 4.2(a)) and on plates with 1, 2 (see Fig. 4.2(b)), 5 and 10 µg clindamycin ml^{-1}.

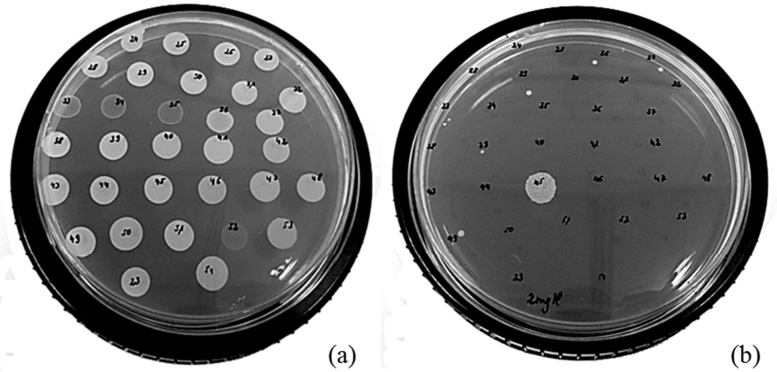

Fig. 4.2: Screening-plates of 32 environmental iMLS$_B$ staphylococci expressing *erm*C or *erm*A. (a) Mueller-Hinton agar plate without clindamycin (growth control; the 4 grey spot represent *S. epidermidis*); (b) Mueller-Hinton agar plate containing 2 µg clindamycin ml^{-1}: One strain had a very high mutation frequency with confluent growth after 48 h incubation. Single colonies were seen earlier. In another "drop-inoculation" 2 mutations can be seen whereas in each of 5 inoculation spots 1 mutation occurs

All plates were incubated at 37 °C up to 48 h. Colonies grown on the clindamycin screening-plates (one up to many, depending on the mutation rate) were streaked on a Mueller-Hinton agar containing 5 µg clindamycin ml^{-1} in order to get biomass for MIC-determination with Etest-strips. If their MIC in the clindamycin-Etest was now ≥ 256 µg ml^{-1}, these strains were considered as mutated and were used as an inoculum for another D-test (e.g. Fig. 4.1(b). No inhibition zone was visible around the disc containing 2 µg clindamycin).

Strains that were originally "resistant" against < 0.75 µg clindamycin ml^{-1} (classified as non-resistant) but did grow in the presence of 1, 2 µg clindamycin ml^{-1} on the screening-plate were transferred on a Mueller-Hinton agar that contained 5 µg clindamycin ml^{-1}. If they did not grow they were considered as adapted (more resistant to clindamycin than the original "wild strains"). Their MIC against clindamycin was checked with clindamycin-Etest strips. In order to determine the frequency of mutation the number of grown colonies was correlated to the number of cells dropped on the plate.

4.3 Results

4.3.1 Species diversity and abundance of *erm* genes

Thirteen of the 19 clinical *Staphylococcus* isolates with iMLS$_B$-phenotype belonged to *Staphylococcus aureus*, the most prominent species causing human infections. All environmental isolates were coagulase negative *Staphylococcus* spec. (CNS) belonging to 7 identified and 5 non-identified isolates (Table 4.1). Whereas *S.*

epidermidis was obtained from human samples as well as from water samples, *S. hominis* with inducible MLS_B-resistance was not isolated from aquatic samples. In general the species diversity showing cross-resistance to MLS_B compounds was higher in sewage and in river water than in hospital samples. This may be due to the fact that only "special" coagulase negative staphylococci, isolated from human specimen, were obtained from the hospital. Remarkably no *S. aureus* was isolated from aquatic samples (Table 4.1). In our study 69.2 % of the clinical *S. aureus* isolates with inducible cross-resistance carried the *ermA* and 30.8 % carried the *ermC* gene. The predominantly detected *erm* gene in clinical CNS (100 %) and environmental CNS (61.2 %) was *ermC* (Table 4.1). *ermA* could only be detected in two of the 49 (4.1 %) environmental CNS. The inducible resistance to MLS_B of all *S. lentus* strains, isolated from sewage and river water, was genetically determined by *erm43*. Eleven of 49 environmental stains (22.4 %) with $iMLS_B$-phenotype were negative for the *erm* gene classes *A, B, C, F, G, Q, T, 43* (Table 4.1).

Table 4.1: Abundance of *erm* genes on species level of staphylococci from clinical and environmental sources and reaction to inhibitory clindamycin-concentrations

Species	*ermA*			*ermC*			*erm43*			*erm?*		
	n	n-m	n-a	n	n-m	n-a	n	n-m	n-a	n	n-m	n-a
Isolated from humans												
S. aureus	9	1	0	4	2		0			0		
S. epidermidis	0			2	0		0			0		
S. hominis	0			4	2	1	0			0		
Total number	9			10			0			0		
Isolated from aquatic environment												
S. cohnii ssp. *cohnii*	1	0	0	2	0	0	0			1	0	0
S. cohnii ssp. *urealyticum*	0			5	3	1	0			0		
S. epidermidis	0			4	2	0	0			0		
S. kloosii	0			1	1	0	0			0		
S. lentus	0			0			6	0	3	0		
S. saprophyticus ssp. *saprophyticus*	0			13	5	0	0			4	0	1
S. spec.	1	0	0	3	1	0	0			3	0	1
S. xylosus	0			2	0	0	0			3	0	2
Total number	2			30			6			11		

n: number of isolates; n-m: number of mutated isolates (inducible resistant strains became constitutively resistant after incubation with inhibitory concentrations of clindamycin); n-a: number of adapted isolates (inducible resistant strains stayed inducible resistant but became less susceptible against clindamycin); erm? means that none of the respective erm gene classes (A, B, C, F, G, Q, T and 43) gave a positive PCR product

4.3.2 Phenotypical characterization of clinical and environmental iMLS$_B$-staphylococci with respective *erm* genes

Expression of different *erm* genes results in distinguishable patterns of phenotypical resistance to macrolides (Table 4.2). In contrast to *ermA*-encoded inducible MLS$_B$-resistance in staphylococci, *ermC*-encoded cross-resistance leads to high-level resistances against oleandomycin and clarithromycin. This is clearly visible in the agar-diffusion test displaying no inhibition zones at all (Table 4.2). In comparison to clarithromycin, oleandomycin seems to be a better marker for distinguishing between *ermA* and *ermC* because of higher differences in the inhibition zones, especially for clinical isolates (Table 4.2).

Table 4.2: Phenotypic characterization of clinical and environmental iMLS$_B$-staphylococci carrying different *erm* genes by determination of inhibition zones against different macrolides or MICs against clindamycin

Antibiotic	*ermA*		*ermC*		*erm43*
	Clinical isolates (n=9)	Environmental isolates (n=2)	Clinical isolates (n=10)	Environmental isolates (n=30)	Environmental isolates (n=6)
Erythromycin	- no inhibition zones or < 10 mm - MICs ≥ 256 µg ml^{-1} - heterogeneous expression		- no inhibition zones or < 10 mm - MICs ≥ 256 µg ml^{-1}		- inhibition zones about 10 mm - MICs between 3 and 16 µg ml^{-1}
Oleandomycin	- inhibition zones > 20 mm		- no inhibition zones	- no inhibition zones or < 15 mm	- inhibition zones between 25 and 30 mm
Clarithromycin	- inhibition zones between 11 and 15 mm		- no inhibition zones or < 10 mm		- inhibition zones between 12 and 15 mm
Azithromycin	- inhibition zones between 10 and 14 mm		- no inhibition zones or < 11 mm		- inhibition zones between 9 and 12 mm
Tylosin	- inhibition zones between 24 and 29 mm		- inhibition zones between 20 and 36 mm		- inhibition zones between 23 and 27 mm
Spiramycin	- inhibition zones between 26 and 29 mm		- inhibition zones between 26 and 36 mm		- inhibition zones between 26 and 30 mm
Telithromycin	- inhibition zones between 28 and 44 mm		- inhibition zones between 28 and 41 mm		- inhibition zones between 35 and 40 mm
Clindamycin	- MICs about 0.1 µg ml^{-1}	- MICs between 0.2 and 0.4 µg ml^{-1}	- MICs between 0.06 and 0.2 µg ml^{-1}	- MICs between 0.02 and 0.625 µg ml^{-1}	- MICs between 0.06 and 0.5 µg ml^{-1}

n: number of isolates; MIC: minimal inhibitory concentration of the respective antibiotic

Using oleandomycin as a marker for expression of inducible resistance against MLS$_B$-antibiotics is also possible for environmental

staphylococci with $iMLS_B$-phenotype. Nevertheless it is important to consider that their behavior against this antibiotic is not always as clear as described for clinical isolates. 63.3% of environmental *ermC*-positive staphylococci displayed small inhibition zones around the disc (data not shown).

A possibility to distinguish between *ermA* and *ermC* encoded inducible MLS_B cross-resistance, significant for clinical as well as for environmental isolates, is the determination of MIC against erythromycin: In the Etest *ermC*-positive staphylococci displayed no inhibition zone along the strip (MIC $\geq$ 256 µg ml^{-1}). Isolates with *ermA*-encoded resistance also had such a high MIC for erythromycin (Table 4.2), but contrary to *ermC*, *ermA* was expressed heterologously, which was observable by single colonies growing in the inhibition ellipse (Fig. 4.3).

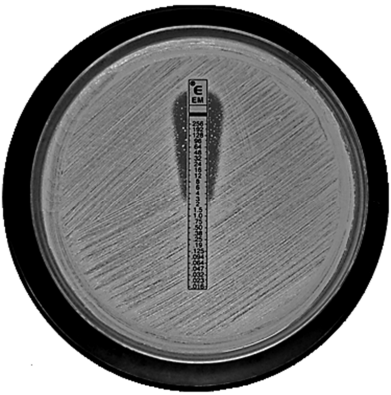

Fig. 4.3: Expression of the *ermA* gene for erythromycin resistance of *S. cohnii* ssp. *cohnii* from sewage: Growth of single colonies in the inhibition ellipse at increasing erythromycin concentrations indicate heterologous expression.

The MIC of erythromycin of *erm43*-possessing *S. lentus* varied between 3 and 16 µg ml^{-1} (Table 4.2) and no heterologous expression could be detected. Except of two staphylococci stains with iMLS$_B$-phenotype not encoded by *ermA, B, C, F, G, Q, T, 43*, all displayed similar low erythromycin-MICs in the mentioned range (data not shown).

The MICs against clindamycin of clinical *ermA*-positive staphylococci were about 0.1 µg ml^{-1}; those of the two environmental staphylococci with the mentioned resistance gene were 0.2 and 0.4 µg ml^{-1}. The same phenomenon could be detected for isolates with iMLS$_B$-phenotype encoded by *ermC*: The MICs of clinical staphylococci were in the range of 0.06 - 0.2 µg ml^{-1} except for one strain of *S. hominis* with a MIC for clindamycin of 0.38 µg ml^{-1}. MICs of the environmental isolates revealed a higher variability in the range between 0.02 and 0.625 µg ml^{-1}. Six of 30 environmental *ermC*-positive CNS had a MIC below 0.06 µg ml^{-1} (20 %) and five of 30 environmental *ermC*-positive CNS had a MIC higher than 0.2 µg ml^{-1} (16.7 %). The differences in the MIC of clindamycin could not be associated with a certain species of staphylococci. Our *erm43*-positive *S. lentus* isolates showed MICs for clindamycin in the range of 0.06 and 0.5 µg ml^{-1} and those of the environmental iMLS$_B$-CNS without *ermA, B, C, F, G, T, Q, 43* displayed MICs between 0.02 and 0.75 µg ml^{-1}. Therefore it seemed that the described phenomenon of a wide range of MICs of clindamycin was characteristic for environmental strains since those of clinical isolates were in a much smaller concentration range. Drawing conclusions about the encoding class of *erm* genes on the basis of the diameters of the inhibition zones around discs that contained tylosin and spiramycin was not possible for clinical as well as for environmental isolates. The diameter of the inhibition zone around the oleandomycin

containing disc was the best marker to assign an iMLS$_B$-*Staphylococcus* isolate to the expression of the respective *erm* gene based on phenotypical observations, independent of its origin.

4.3.3 Reaction of staphylococci to inhibitory clindamycin-concentrations

In order to test if differences concerning the reaction to inhibitory concentrations of clindamycin between clinical and environmental iMLS$_B$-staphylococci exist, growth of the respective strains on Mueller-Hinton agar in the presence of different concentrations of the antibiotic was checked. Forty percent of the clinical and environmental strains of staphylococci, 50 % of the *S. aureus* isolates and 33.3 % of the CNS that expressed the *ermC* gene, mutated (changed their behavior from inducible to constitutive resistance against clindamycin) on screening-plates in the presence of different inhibitory concentrations of clindamycin (Table 4.1 and Fig. 4.2). The frequencies of mutation of clinical and environmental isolates were in the same order (about 1 x 10^{-6}), independent of the species and the origin of the strain (Table 4.3). None of our two environmental *ermA*-positive CNS and of the 6 *S. lentus* strains carrying the *erm43* gene mutated and only one of the 9 clinical *ermA*-positive *S. aureus* strains (11.1 %) changed its susceptibility to clindamycin during the experiment. There was no correlation between mutation frequency and the clindamycin concentration on the screening-plate with respect to the species and to clinical and environmental isolates (Table 4.3). Two isolates, one *ermA*-expressing *S. aureus* and one *ermC*-expressing *S. cohnii* ssp.

urealyticum, showed clearly higher mutation rates (> 2.5 x 10^{-5}, Table 4.3).

Table 4.3: Frequency of mutation of *Staphylococcus* isolates for different clindamycin concentrations in the agar of the screening-plates

Species	*erm* gene	Clindamycin concentration of the screening-plate			
		1 µg ml^{-1}	2 µg ml^{-1}	5 µg ml^{-1}	10 µg ml^{-1}
Isolated from humans					
S. aureus	*ermA*	> 2.5 x 10^{-5}	> 2.5 x 10^{-5}	> 2.5 x 10^{-5}	> 2.5 x 10^{-5}
S. aureus	*ermC*			1 x 10^{-6}	1 x 10^{-6}
S. aureus	*ermC*	1 x 10^{-6}			
S. hominis	*ermC*		1 x 10^{-6}		1 x 10^{-6}
S. hominis	*ermC*	2 x 10^{-6}	6 x 10^{-6}	1 x 10^{-6}	
Isolated from aquatic environment					
S. saprophyticus ssp. *saprophyticus*	*ermC*	1 x 10^{-6}			
S. saprophyticus ssp. *saprophyticus*	*ermC*		1 x 10^{-6}		
S. saprophyticus ssp. *saprophyticus*	*ermC*			1 x 10^{-6}	
S. saprophyticus ssp. *saprophyticus*	*ermC*		1 x 10^{-6}		
S. saprophyticus ssp. *saprophyticus*	*ermC*				1 x 10^{-6}
S. cohnii ssp. *urealyticum*	*ermC*			1 x 10^{-6}	
S. cohnii ssp. *urealyticum*	*ermC*				1 x 10^{-6}
S. cohnii ssp. *urealyticum*	*ermC*	> 2.5 x 10^{-5}	> 2.5 x 10^{-5}	> 2.5 x 10^{-5}	> 2.5 x 10^{-5}
S. epidermidis	*ermC*		1 x 10^{-6}		
S. epidermidis	*ermC*		1 x 10^{-6}	1 x 10^{-6}	
S. spec.	*ermC*		1 x 10^{-6}		
S. spec.	*ermC*		1 x 10^{-6}	1 x 10^{-6}	

Three of the 6 *erm43*-positive *S. lentus* strains (50 %) adapted and grew on the 1 µg clindamycin ml^{-1} screening-plate. Growing on Mueller-Hinton agar plates with this antibiotic concentration was also possible for 4 environmental *Staphylococcus* isolates (36.4 %) whose $iMLS_B$-phenotype was not encoded by *ermA, B, C, F, G, Q, T, 43* (Table 4.1).

4.4 Discussion

In clinical CNS, *ermC* is the predominantly detected *erm* gene (Gherardi *et al.*, 2009; Gul *et al.*, 2008; Lina *et al.*, 1999). Coinciding with this finding, inducible MLS_B-resistance of our environmental CNS was also abundantly encoded by *ermC* (61.2 %). Possibly the wide-spread distribution of this gene in different species of staphylococci in the aquatic environment can be explained by its insertion into small plasmids, usually in the range between 2.3 and 4.4 kb (Wendland *et al.*, 2013). On the contrary, the *ermA* gene, which was integrated into transposons Tn554 and Tn917/Tn551 and present in a single copy or only a few copies in the chromosome, was less frequently detected in the aquatic environment (4.1 %) (Daurel *et al.*, 2008; Wendland *et al.*, 2013). The different abundance of these mobile determinants in the aquatic environment could be explained by different transfer rates. Unfortunately, to our knowledge, there are no data describing terms for horizontal *erm* gene transfer with focus on the different classes and the respective transfer rates in the aquatic ecosystem. Up to date the *erm43* gene was only reported for *Staphylococcus lentus* and detected in strains isolated from humans, dogs and chickens (Schwendener & Perreten, 2012). In this study the *erm43* gene, for the first time, was detected in *S. lentus* strains isolated from the aquatic environment. Whereas inducible

clindamycin-resistance of clinical staphylococci by expression of the *ermA* or *ermC* gene was detected almost universally (Gherardi *et al.*, 2009; Gul *et al.*, 2008; Lina *et al.*, 1999) in staphylococci that were isolated from aquatic environment, the diversity of *erm* genes was higher than in strains isolated in hospitals. Unfortunately investigations about the distribution of *erm* genes in *Staphylococcus* isolates from environmental sources are very rare. Koike *et al*. (2010) examined waste lagoons and subsurface waters from swine farms on the basis of MLS_B methylases for molecular ecology. They reported a high diversity of *erm* genes, but due to their molecular approach and the wide distribution of the recently known about 40 resistance genes of the *erm* gene family (Leclercq, 2002) among Gram-positive as well as among Gram-negative bacteria, it is not possible to assign them to species of the genus *Staphylococcus*.

Phenotypic resistance patterns of *ermA* or *ermC* carrying staphylococci from hospital samples have already been described (Daurel *et al.*, 2008; Di Modugno *et al.*, 2002). The heterologously expressed erythromycin-resistance of our *ermA*-positive environmental staphylococci was also observed by Di Modugno *et al.* (2002) during MIC determinations. Di Modugno *et al.* (2002) distinguished between the two endpoints 'changing from confluent to light growth' and 'changing from light growth to no growth'. As a possible explanation for this phenomenon, they mentioned that, whereas *ermC* leads to dimethylation of a specific residue in 23S rRNA, other genetic determinants may cause mono-methylation at the same position. Up to date, such low erythromycin-MICs as in environmental *erm43*-expressing *S. lentus* strains and in 9 *Staphylococcus* strains with an $iMLS_B$-phenotype, that were not encoded by *ermA, B, C, F, G, Q, T, 43* (3-16 μg ml^{-1}), were associated with other

resistance mechanisms like efflux pumps, which are encoded for instance by *msr*A (Hauschild & Schwarz, 2010). Our phenotypic investigations showed that on the basis of low-level erythromycin resistance, it was not possible to exclude inducible resistance to MLS$_B$-antibiotics and, as a consequence, rather low MICs to erythromycin were not a sufficient criterion to decide if clindamycin could be used to treat an infection caused by such *Staphylococcus* strains. In accordance with our observations, concerning the clindamycin-MICs of clinical isolates, Le Bouter *et al.* (2011) reported MICs for clindamycin of their clinical *ermC*-expressing *S. saprophyticus* strains between 0.03 and 0.12 μg ml^{-1}. To our knowledge such a high fluctuation of the MIC of clindamycin as displayed by our environmental isolates (variation from 0.02 to 0.75 μg ml^{-1}) has not yet been reported for iMLS$_B$-staphylococci, neither for strains of clinical nor of environmental origin. This has implications for medical prescription. In contrary to the finding of Di Modugno *et al.* (2002), who reported that *ermA*-positive iMLS$_B$-staphylococci were significantly more susceptible to tylosin (mean MIC 1.7 μg ml^{-1}) in contrast to their *ermC*-caused resistance (mean MIC 3 μg ml^{-1}), our *ermA*-expressing isolates did not reveal significantly larger inhibition zones compared to those that express the *ermC* gene (Table 4.2).

The mutation frequencies of our environmental *ermC*-expressing CNS (Table 4.3) were higher than those reported from *ermC*-expressing *S. aureus*, which ranged between 1.7 x 10^{-6} and 4.4 x 10^{-8}. The finding of Daurel *et al.* (2008), which indicated that their *ermA*-expressing *S. aureus* mutated 14 times less than *ermC*-possessing *S. aureus*, supports our observation that no correlation exists between species and their mutation frequency. The frequency seems to be dependent on the

encoding *erm* gene, especially on the respective structures of the attenuator region. Whereas the regulatory region of *ermC* comprises only one encoded leader peptide and four inverted repeats, the respective region of *ermA* is longer and more complex. It includes sequences encoding two leader peptides and six inverted repeats (Daurel *et al.*, 2008). The similarity to the attenuator of the *erm43* gene, which is also constructed by sequences encoding two leader peptides (Schwendener & Perreten, 2012), might be a possible explanation for our result that none of the 6 *S. lentus* isolates that expressed the *erm43* gene mutated during incubation with inhibitory clindamycin concentrations. Possibly the clearly higher mutation rates of the two isolates of this study, as well as the fluctuations determined for strains belonging to the same species and possessing the same *erm* gene (as e.g. reported by Daurel *et al.*, 2008), could be explained on the basis of sequence analysis.

To conclude, the abundance of staphylococci with inducible cross-resistance to MLS_B antibiotics that were isolated from aquatic environment was lower than that isolated from hospital samples. The diversity of resistance genes encoding for the mechanism of ribosomal modification and their phenotypic expressions seem to be much more variable. Whereas environmental *Staphylococcus* strains with inducible MLS_B-resistance, encoded by *ermA* or *ermC*, showed phenotypic resistance patterns and reactions to inhibitory concentrations of clindamycin assignable to the respective resistance gene, isolates from sewage and river water that harbored the *erm43* gene or so far not detected resistance genes, did not behave as expected for this resistance mechanism. Collectively, environmental $iMLS_B$-CNS displayed a broader range of reactions against the different MLS_B compounds, possibly as a result of an adaption to the permanently changing

conditions in their ecosystem. For successful treatment of infections caused by such strains it is important to know their phenotypic characteristics for identification and medication with an adequate antibiotic.

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5. Removal of total and antibiotic resistant bacteria in advanced wastewater treatment by ozonation in combination with different filtering techniques

Kläranlagen stellen eine bedeutende Eintragsquelle von Antibiotika, deren Metaboliten sowie Antibiotika-resistenten Bakterien und Resistenzdeterminanten in das aquatische Ökosystem dar. Gerade weil das daraus resultierende potentielle Risiko für die Gesundheit von Mensch und Tier derzeit nicht abgeschätzt werden kann, ist es wichtig, die Belastung von als Vorfluter dienenden Oberflächengewässern weiter zu minimieren. Um die diesbezügliche Effizienz erweiterter Abwasserreinigungstechnologien zu testen, wurde ein Abwasserteilstrom in der Kläranlage Eriskirch mit Ozon begast und anschließend über verschiedene Filter geleitet. Als Bezugspunkt zur Beurteilung dieser um eine vierte Stufe erweiterten Abwasserreinigungsverfahren wurde der Ablauf des derzeit großtechnisch etablierten Flockungsfilters herangezogen.

- Die Ozonung war „der“ keimeliminierende Schritt, der allerdings zu einem erhöhten relativen Anteil Antibiotika-resistenter *E. coli* und Staphylokokken führte.
- Im Gegensatz zu *E. coli* und den Staphylokokken nahm der Anteil resistenter Enterokokken während der Ozonung um 25,4% ab, was mit einer Veränderung in der Speziesverteilung (insbesondere einer Abnahme des Anteils an *E. faecium*-Isolaten) gekoppelt war.
- Teilweise kam es während der Passage durch die der Ozonung nachgeschalteten Filter wie auch des Flockungsfilters zu einem

Anstieg der Konzentration an fakultativ pathogenen Bakterien wie auch des Anteils resistenter *E. coli*, Enterokokken und Staphylokokken. Ein entsprechend angepasster Betrieb der Filter wie regelmäßige Wartung, Intensivierung von Spülintervallen etc. würde aber die beobachtete Zunahme von Keimen minimieren können.

- Die keimeliminierende Wirkung des Ozons überlagerte die teilweise gesehene Zunahme des Anteils resistenter Isolate, sodass die Konzentration fakultativ pathogener und Antibiotika-resistenter Keime in den Abläufen der vierten Reinigungsstufe im Vergleich zum Ablauf des derzeit etablierten Flockungsfilters (tertiäre Reinigungstufe) um etwa eine log-Stufe geringer war.

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Abstract

Elimination of bacteria by ozonation in combination with charcoal or slow sand filtration for advanced sewage treatment to improve the quality of treated sewage and to reduce the potential risk for human health of receiving surface waters was investigated in pilot scale at the sewage treatment plant Eriskirch, Baden-Wuerttemberg/Germany. To

determine the elimination of sewage bacteria, inflowing and leaving wastewater of different treatment processes was analysed in a culture-based approach for its content of *E. coli*, enterococci and staphylococci and their resistance against selected antibiotics over a period of 17 month. For enterococci, single species and their antibiotic resistances were identified. In comparison to the established flocculation filtration at Eriskirch, ozonation plus charcoal or sand filtration (pilot-scale) reduced the concentrations of total and antibiotic resistant *E. coli*, enterococci and staphylococci. However, antibiotic resistant *E. coli* and staphylococci apparently survived ozone treatment better than antibiotic sensitive strains. Neither vancomycin resistant enterococci nor methicillin resistant *Staphylococcus aureus* (MRSA) were detected. The decreased percentage of antibiotic resistant enterococci after ozonation may be explained by a different ozone sensitivity of species: *Enterococcus faecium* and *E. faecalis*, which determined the resistance-level, seemed to be more sensitive for ozone than other *Enterococcus*-species. Overall, ozonation followed by charcoal or sand filtration led to 0.8 - 1.1 log-units less total and antibiotic resistant *E. coli*, enterococci and staphylococci, as compared to the respective concentrations in treated sewage by only flocculation filtration. Thus, advanced wastewater treatment by ozonation plus charcoal or sand filtration after common sewage treatment is an effective tool for further elimination of microorganisms from sewage before discharge in surface waters.

5.1 Introduction

In Germany so far, no breakpoints exist for the concentration of bacteria or even for antibiotic resistant microorganisms in the effluent of wastewater treatment plants (WWTP), since the focus lies on the elimination of carbon, nitrate and phosphate. Currently, sewage treatment of most plants can be divided into three steps: primary and secondary treatment (mostly activated sludge) combined with nitrification/denitrification, phosphate precipitation and/ or filtration as tertiary treatment (Statistisches Bundesamt, 2013). In the last years, the quality of receiving water bodies, which very often serve as drinking water reservoir or for recreation, moved more and more into public interest. This is manifested in different guidelines, for instance in the EU Bathing Water Directive (European Parliament & Council, 2006), which defines quality thresholds for bathing water. Among other parameters, maximal numbers of two fecal indicator groups, *Escherichia coli* and enterococci are fixed. These two bacterial groups were chosen as markers for contamination with human feces (Luczkiewicz et al., 2011). In contrary, staphylococci, typical colonizers of skin and mucous membranes of humans and animals, are not classified as fecal indicators. However, some species and subspecies of enterococci and *E. coli* are also relevant human pathogens.

To achieve the goals manifested e.g. in WHO or EU guidelines, improvements of wastewater purification by fourth treatment techniques, such as UV-irradiation, photo Fenton, chlorination or photocatalysis may be useful (e.g. Diao et al., 2004; Rincón and Pulgarin, 2005; Michael et al., 2012). A positive effect of the mentioned techniques is the reduction of living facultative pathogenic bacteria but

it is unclear if their establishment will lead to a higher percentage of antibiotic resistant bacteria (e.g. Dodd, 2012).

Ozonation is another of the recently tested advanced oxidation processes: molecular ozone decays in three phases if it is dissolved in water (Gehr et al., 2003). Its decay rates depend on various parameters.

If, for instance, alkalinity is low and/or the concentration of organics high, ozone will decay rapidly, forming hydroxyl radicals for non-selective oxidation (Gehr et al., 2003). Hunt and Marinas (1997) found out that molecular ozone primarily inactivated *E. coli*. Up to date, the mode of action and the specific target structures of ozone and its decomposition products in microbial cells are not completely understood: amino acids or proteins, peptidoglycan, lipids in the cell wall and cell membrane, enzymes as well as DNA-molecules may be affected (Dodd, 2012). Furthermore it is still an open question whether antibiotic resistant microorganisms might be less sensitive against oxidative stress that is caused by ozone in aquatic environment.
The effect of different filter materials such as quartz sand or charcoal on the distribution of antibiotic resistance genes is controversially discussed: Grabow et al. (1976) assumed that stony surfaces of biofilters or sand filters are unfavorable for conjugation and could damage sex pili resulting in a stable or even decreasing percentage of antibiotic resistant bacteria. On the other hand, the prolonged hydraulic retention times in the filters may promote horizontal gene transfer. The percentage of antibiotic resistant bacteria may also increase, if temporarily retained microorganisms in a filter would incorporate free DNA fragments of lysed cells after ozonation (Dodd, 2012).

Within the project “SchussenAktiv*plus*” the effect of ozonation followed by sand and/or activated-charcoal filtration per se or in combination was investigated with respect to the removal of total and antibiotic resistant bacteria, *E. coli*, enterococci and staphylococci. The filters were used for post-ozonation treatment to remove e.g. emerging contaminants, estrogenicity or mutagenicity possibly induced by ozone (Magdeburg et al., 2014). For this purpose, part of the sewage entering the wastewater treatment plant of Eriskirch was treated with the mentioned advanced techniques. The concentrations of bacteria as well as the percentage of antibiotic resistant isolates were determined in a culture-based approach and results were compared with those of the effluent of routine tertiary treatment by flocculation filtration.

5.2 Material and methods

5.2.1 WWTP characteristics

The study site is a medium sized WWTP in Eriskirch (Germany) with 40,000 inhabitant equivalents located 0.5 km upstream to the Schussen estuary into Lake Constance. The catchment area has a population of 28,000 inhabitants. Standard wastewater treatment processes comprise mechanical retention of large solids, separation of sand and grit in aerated chambers, settling of suspended solids in primary settling tanks, biological removal of nitrogen and phosphorus by activated sludge units (including chemical phosphorus precipitation) and secondary clarifiers. In an additional flocculation filtration step, again phosphorus precipitation takes place, before the treated water is directed to a

filtration unit consisting of seven cells with a surface area of 22.1 m^2 each filled with 15 cm quartz gravel support layer (4-8 mm), 65 cm quartz sand layer (0.71-1.25 mm) and a 85 cm anthracite layer (1.4-2.5 mm). Back flushing was performed at 6 h intervals from cell to cell, requiring 42 h. Depending on weather conditions this time span may have been shorter. The maximum hydraulic load was 350 l/s. The mean daily run-out was about 10,296 m^3/ d (dry weather minimum 6,468 m^3/ d, rainy weather maximum 20,318 m^3/ d, triennial means 2011-2013) (for details see Triebskorn et al., 2013; www.schussenaktivplus.de).

For the pilot scale study, a partial flow of the effluent (1.5 m^3/h) was piped through an ozonation reactor (contact time 20 min with 0.73 mg O_3/ mg DOC), followed by either sand filtration or granulated activated charcoal (GAC) adsorption or a combination of both techniques. The three filters were identically sized (4 m height, 0.3 m diameter, filter area 0.0707 m^2) and were top-down fed and bottom-up back-flushed. The sand filter (back-flush interval 43 h) contained 0.3 m gravel support layer, a 0. 6 m sand layer (volume 40 l, grain size 0.71 mm – 1.25 mm) and a 0.8 m hydroanthracite layer (volume 55 l, grain size 1.4 mm – 2.5 mm). The GAC filter (back-flush interval 5 h) contained 0.3 m gravel support layer and a 2.2 m charcoal layer (volume 155 l, grain size 0.425 mm – 2.36 mm, manufacturer Chemviron). The combined sand/GAC filter contained 0.6 m sand and 2.2 m GAC. This filter was emptied in August 2013 and filled only with GAC material to check its removal efficiency for bacteria from wastewater that was not treated with ozone before. Fig. 5.1 shows a scheme of the WWTP Eriskirch and the sampling points.

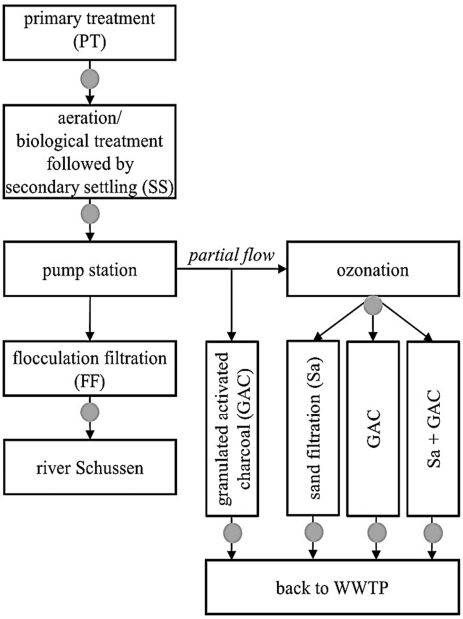

Fig. 5.1: Schematic overview of WWTP Eriskirch and the pilot station with ozonation followed by sand filtration (Sa) and/ or granulated activated charcoal (GAC). The combination filter Sa + GAC was substituted in August 2013 against a GAC filter examining the effect of GAC solely without prior ozonation. Grey dots indicate sampling stations.

5.2.2 Sample collection from the WWTP Eriskirch

In sampling campaigns from May 2012 until December 2013 effluents of primary treatment (ef PT), secondary settling (ef SS), flocculation filtration (ef FF), ozonation (ef Oz), ozonation in combination with sand

filtration (ef Oz+Sa), ozonation in combination with GAC (ef Oz+GAC) or ozonation in combination with sand filtration/GAC adsorption (ef Oz+Sa+GAC) were analysed. Effluent of a filter that was filled with exclusively GAC and operated with conventionally purified sewage without ozonation (ef GAC) served as a control sewage samples were collected volume-proportional over a period of 24 hours and refrigerated in an automatic sampler. Purified sewage for microbiological investigations was sampled in autoclaved glass bottles, which were stored at 4 °C during transport. Processing in the laboratory was finished within 24 h.

5.2.3 Enumeration, isolation and identification of cultivable *E. coli*, enterococci, and staphylococci

For *E. coli*, ECD-agar containing 4-methylumbelliferyl-beta-D-glucuronide (MUG) was prepared according to manufacturer´s instructions (Merck, Darmstadt, Germany). To cover the probable contamination level, samples were either concentrated by membrane filtration (cellulose nitrate, pore size 0.45 µm, Ø 50 mm (Sartorius, Göttingen, Germany)) or directly plated on agar plates. After incubation at 37 °C for 20-24 h, blue fluorescent colonies (resulting from the beta-glucuronidase activity that hydrolyzes MUG) of *E. coli* were counted under UV light.

For *Enterococcus spec.*, membrane filters with appropriate dilutions were incubated on azide nutrient pads (Sartorius) for 40-48 h at 37°C, followed by incubation on kanamycin-aesculin-agar (Merck, Darmstadt)

for 1 h at 44 °C. Red, pink and reddish brown colored colonies with positive aesculin reaction were counted.

For *Staphylococcus spec.*, Chapman-Stone agar containing 0.05 g L^{-1} sodium azide was used. Undiluted and 10-fold diluted raw sewage samples were plated on agar dishes. To obtain staphylococci from effluents after the mentioned sewage treatment combinations, 1 to 400 ml of respective samples were filtered through 0.45 µm membrane filters (Ø 47 mm), which were placed on Chapman-Stone agar plates and incubated for 48 h at 37 °C. Twenty colonies of each sample were streaked on Mannitol-Salt agar and re-streaked once after growth.

Per sampling site 15 typical colonies of *E. coli* and of enterococci were randomly selected and isolated. Presumptive *E. coli* - isolates were further tested for tryptophanase activity with Kovac´s reagent. Separated enterococci – isolates were further streaked on kanamycine aesculin azide agar to confirm positive aesculin reaction.

Gram-positive cocci were identified on Micronaut-Staph®-microtiter plates (staphylococci) / Micronaut-Strep2®-microtiter plates (enterococci) according to manufacturer's instructions (MERLIN, Gesellschaft für mikrobiologische Diagnostika, Bornheim, Germany). *E. coli* was identified by targeting a specific *tuf*-gene fragment (primers and PCR-conditions as described by Maheux et al., 2009). Briefly, the DNA-amplification was performed in a total volume of 25 µl containing 0.625 units True Start HS Taq DNA Polymerase, 2.5 mM $MgCl_2$ (Thermo Fisher Scientific, Waltham, MA), 0.25 mM of each dNTP, 10 µM of both primers and 0.5 µl of template DNA (extraction with phenol/chloroform).

5.2.4 Antibiotic susceptibility testing and detection of antibiotic resistance genes (ARG)

The agar diffusion test according to DIN 58940 (DIN, Deutsche Industrie Norm 2011) was performed with 318 enterococci for antibiotic resistance (AR) against ampicillin (10 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg) and erythromycin (15 µg). The susceptibility of *E. faecium* and *E. faecalis* (VRE) isolates against vancomycin was tested according to Clinical and Laboratory Standards (CLSI, 2011). Vancomycin resistance (VR) was confirmed by the presence of VR genes *van*A-E, G (Depardieu et al., 2004); DNA was amplified in a 25 µl assay containing 0.625 units True Start HS Taq DNA Polymerase, 2.5 mM $MgCl_2$, 0.25 mM of each dNTP, 10 µM of both primers and 0.5 µl of template DNA (extraction with phenol/chloroform). Intrinsic low-level vancomycin-resistances of *E. gallinarum* and *E. casseliflavus* (vanC1- and vanC2-type) were not considered.

AR of 349 *E. coli*-isolates against ampicillin (10 µg), ciprofloxacin (5 µg), cotrimoxazol (1.25 µg trimethoprim/ 23.75 µg sulfamethoxazol; SXT) was also tested with agar diffusion tests (DIN, 2011). For testing AR against cefotaxim (5 µg) clinical breakpoints (EUCAST, 2011) were applied. To detect extended-spectrum-ß-lactamase (ESBL) producing *E. coli* (multidrug-resistant strains causing severe infections and therefore displaying an increasing problem in human and veterinary medicine), all ampicillin resistant isolates were additionally tested against ceftazidim (30 µg) and cefpodoxim (10 µg) according to CLSI (2011). The inhibitory effect of clavulanic acid on ß-lactamase was checked as described by Bradford (2001). According to the definition of Robert-

Koch-Institut (2007), ESBL producers were resistant against cefpodoxim as well as ceftazidim and/or cefotaxim.

Susceptibility of the 636 *Staphylococcus*-isolates against oxacillin (5 µg), ciprofloxacin (5 µg) and erythromycin (15 µg) was tested by the disc-diffusion test with Mueller-Hinton agar according to DIN 58940 (DIN, 2011) and against clindamycin (2 µg) according to CLSI (2011). Oxacillin resistance was confirmed by the detection of *mec*A using previously described primers and PCR-conditions (Predari et al., 1991). The DNA-amplification was performed in a 25 µl assay containing 0.625 units True Start HS Taq DNA Polymerase, 2.5 mM $MgCl_2$, 0.25 mM of each dNTP, 10 µM of both primers and 0.5 µl of template DNA (extraction with phenol/chloroform).

5.3 Results and Discussion

5.3.1 Quantification of cultivable *E. coli*, enterococci and staphylococci in effluent of conventional and of pilot scale advanced treatment processes

The established wastewater treatment with flocculation filtration as a tertiary treatment reduced the number of *E. coli* and enterococci about 2.8 log-units and of staphylococci about 3.4 log-units (Tab. 5.1 and Fig. 5.2), whereas numbers of *E. coli*, enterococci and staphylococci after the flocculation filter fluctuated around one log-unit (Fig. 5.2). Secondary treatment showed highest efficiency in total bacterial removal, which was observed before (George et al., 2002; Hijnen et al., 2000). Probably

this high elimination of bacteria was caused mainly by grazing of protozoa, competition with the prevalent microflora, and sedimentation of bacteria adsorbed to sludge flocs.

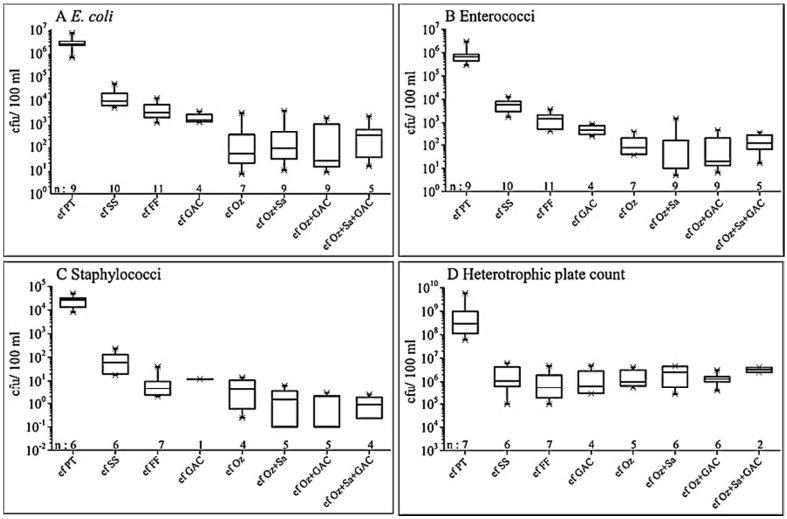

Fig. 5.2: Quantification of cultivable *E. coli* (A), enterococci (B), staphylococci (C) and heterotrophic plate count (D) after their passage through primary treatment (ef PT), secondary settling (ef SS), flocculation filtration (ef FF), filtration with granulated activated charcoal GAC solely (ef GAC), ozonation (ef Oz), ozonation + sand filtration (ef Oz+Sa), ozonation + GAC (ef Oz+GAC), ozonation + sand filtration + GAC (ef Oz+Sa+GAC). n = number of sampling events.

Tab. 5.1: Mean log reduction efficiencies for *E. coli*, enterococci and staphylococci as well as antibiotic resistant isolates achieved by conventional (flocculation filtration (FF)) and advanced wastewater treatment with granulated activated charcoal (GAC) solely, ozonation (Oz), Oz+sand filtration (Sa), Oz+GAC or Oz +Sa+GAC. Calculations were made for each purification step compared to the primary treatment effluent. In brackets are the corresponding cfu/ 100 ml.

elimination efficiency	*E. coli*		enterococci		staphylococci	
calculated to PT	total	antibiotic resistant	total	antibiotic resistant	total	antibiotic resistant
FF	2.8 (4.5×10^3)	2.6 (1.0×10^3)	2.8 (1.4×10^3)	2.6 (5.3×10^2)	3.4 (9.8×10^0)	3.6 (2.5×10^0)
GAC	3.2 (1.9×10^3)	3.1 (3.9×10^2)	3.3 (5.1×10^2)	3.3 (1.1×10^2)	3.3 (1.2×10^1)	3.5 (3.0×10^0)
Oz	3.8 (5.0×10^2)	3.5 (1.3×10^2)	3.9 (1.3×10^2)	4.2 (1.4×10^1)	3.7 (5.6×10^0)	3.7 (1.7×10^0)
Oz+Sa	3.6 (7.5×10^2)	3.8 (6.8×10^1)	3.6 (2.5×10^2)	3.4 (7.9×10^1)	4.1 (2.3×10^0)	4.4 (4.0×10^0)
Oz+GAC	3.8 (4.6×10^2)	3.8 (6.6×10^1)	3.9 (1.3×10^2)	3.6 (5.1×10^1)	4.3 (1.4×10^0)	4.5 (3.0×10^0)
Oz+Sa+GAC	3.7 (6.1×10^2)	3.5 (1.4×10^2)	3.8 (1.6×10^2)	3.6 (5.9×10^1)	4.4 (1.0×10^0)	4.7 (2.0×10^0)

Compared with removal efficiencies of other conventional WWTPs with equivalent treatment techniques, bacterial elimination found in our study was relatively high. Kistemann et al. (2008) reported a reduction of fecal-associated microorganisms of one to maximally three log-units and Faria et al. (2009) measured a reduction of staphylococci in the order of two log-units.

In comparison to the effluent of the established large-scale flocculation filter, numbers of culturable *E. coli* and enterococci after ozonation were about one (*E. coli*) or 1.1 (enterococci) log-units lower. For staphylococci, a possibly higher reduction by using ozone could not be seen due to already low cfu-values in ef SS (mean value: 8.6×10^1 cfu/100 ml; Tab. 5.1 and Fig. 5.2). In accordance with our data, Abegglen et al., (2009) reported that ozonation with 0.4 - 1.1 g O_3/g DOC caused a 1-2 log unit reduction of *E. coli* and enterococci cell concentrations. The duration of ozonation, the concentration of ozone in the sewage and the composition of sewage may influence the efficiency of this advanced treatment technique concerning the elimination of living cells. Xu et al. (2002) measured the effect of the hydraulic retention time in the ozonation unit on the elimination rate. They found no difference of inactivation between 2 and 10 minutes exposure time for a given ozone dose. Reported inactivation rates of different genera and species varied (Gehr et al., 2003; 1989; Xu et al., 2002) and were lower in biofilms (Zimmermann et al., 2011), presumably due to cell protection in deeper layers.

Regarding the mean reduction rates shown in Table 5.1, ozonation is the elimination step for microorganisms during advanced sewage treatment.

The effect of sewage filtration through sand or GAC after ozonation on the number of living cells varied. During some sampling campaigns they were higher in the effluent of the respective filter than in the influent (up to 0.6 log-steps; data not shown). The mean reduction rates of *E. coli* and enterococci after passing the respective filters were in the same order of magnitude as after ozonation (3.6 - 3.9 log-units; Table 5.1). The number of living staphylococci decreased during passage of the filters, whereas the passage though the combined sand- and granular activated charcoal filter was the best option leading to an additional reduction of 0.7 log-units (Table 5.1). The texture of the filter material seemed to be a determining factor for removal of respective particles. A study of Foppen et al. (2010) showed that the attachment on quartz sand filter material was dependent on sphericity, motility, zeta-potential, cell aggregation, lipopolysaccharide composition and the presence/absence of different outer surface proteins. The mentioned characteristics are not genus or species specific as Foppen et al. (2010) described those for a number of *E. coli*-isolates. Therefore, using different filter materials may increase removal efficiency. The mean hydraulic retention times of *E. coli*, enterococci and staphylococci in the respective filters in the STP of Eriskirch are unknown and are probably influenced by back-flushing intervals. Nevertheless, comparison of cell numbers in the respective effluents allows an evaluation of the advanced sewage treatment techniques of the pilot-scale plant. On average, the number of living cells in the effluents of the respective filters after ozonation was about one log-unit lower compared to respective effluent after only tertiary treatment without preceding ozonation (Table 5.1).

5.3.2 Species diversity of enterococci

The most frequently isolated species of the genus *Enterococcus* was *E. faecium* (36.5%), followed by *E. faecalis* (18.2%) and *E. hirae* (14.5%; Table 5.2). Dominance of these species in sewage was observed in different countries in Europe like Sweden, Spain, Portugal and the United Kingdom (e.g. Ferreira da Silva et al., 2006; Luczkiewicz et al., 2011). *E. faecium* and *E. faecalis* are regarded as specific species in human feces, whereas *E. hirae*, *E. gallinarum/E. casseliflavus* and *E. durans* seem to be environmental strains and originate from feces of animals (Luczkiewicz et al., 2011). A shift of the abundance of the different *Enterococcus*-species after ozonation was obvious. In effluent samples of the secondary settling pond (ef SS) and in water samples from aquatic environment, percentages of *E. faecium* and *E. faecalis* were identical (49.1%; Table 5.2). After ozonation, the percentages of *E. faecium* and *E. faecalis* decreased to only 26.3% in favor of *E. hirae*, *E. gallinarum/E. casseliflavus* and *E. durans* (Table 5.2). Possibly those *Enterococcus*-species that were found mainly in aquatic environment had developed mechanisms against oxidative stress and therefore were less “sensitive” against ozone. They survived ozone treatment in larger numbers than *E. faecalis* and *E. faecium*. The pigment produced by *E. casseliflavus* may for instance play a role in the defense against damage by reactive oxygen species similar as already described for the staphyloxanthin that is synthesized by *Staphylococcus aureus* (Clauditz et al., 2006). In the effluents of the respective filters (ef FF, ef Oz+SF, ef Oz+GAC, ef Oz+SF+GAC), *E. faecium* and *E. faecalis* dominated: 57.7% to 78.6% of the isolates of these sampling points were identified as one of the two species (Table 5.2). To exclude that the observed

species shift was an effect of the chosen culture based approach further investigations are needed.

Tab. 5.2: Diversity and antibiotic susceptibility of *Enterococcus* species isolated from the different sampling points (n = absolute numbers; n-r = number of resistant isolates).

	ef PT		ef SS		ef FF		ef GAC		ef Oz		ef Oz+Sa		ef Oz+GAC		ef Oz+Sa+GAC	
	n	n-r	n	n-r	n	n-r	n	n-r	n	n-r	n	n-r	n	n-r	n	n-r
Sum	70		57		74		22		38		26		17		14	
E. avium			1													
E. casseliflavus	16		7		8		5		7		1				1	
E. durans	2				1											
E. faecalis	13	2	8		14	1			4		4		4		7	2
E. faecium	25	12	20	14	34	19	6	4	6	2	11	8	8	6	4	3
E. gallinarum	5	1	5	1	7	1	3		8		4		1			
E. hirae	5	1	16	4	9	3	8	1	6	2	5	2			2	
E. spec.	4				1				7		1		4	1		

5.3.3 Effect of conventional and pilot scale advanced sewage treatment on the percentage of antibiotic resistant *E. coli*, enterococci and staphylococci

To evaluate the effect of the installed advanced sewage treatment techniques in more detail, percentages of antibiotic resistant bacteria in respective samples from conventional treatment units were compared with effluent samples of the established flocculation filter.

The percentage of antibiotic resistant *E. coli* was higher in the effluent of the flocculation filter (FF, 22%) than in effluent of primary treatment (ef PT, 14.5%; see Table 5.3). With few exceptions all *E. coli*-isolates were – if at all – resistant against ampicillin. Some of the ampicillin resistant isolates were additionally resistant against SXT and/or ciprofloxacin. Extended-spectrum-ß-lactamases (ESBL) producing *E. coli* were detected twice (ef PT and ef Oz+GAC). The frequency of distribution of resistance against the four tested antibiotics (Table 5.3) was in accordance with the findings of Luczkiewicz et al. (2010), who also analysed the antibiotic resistance pattern of *E. coli* strains that were isolated from sewage. They isolated one ESBL in the aeration chamber. The fact that ESBL producers can be detected by culture based approaches points out that they are present in respective numbers in sewage.

During ozonation, the percentage of antibiotic resistant isolates increased about 16% (Table 5.3), especially due to an increase of the percentage of ampicillin resistant *E. coli*. The percentage of SXT and ciprofloxacin resistant strains did not change (SXT: 7-8%; ciprofloxacin: about 4%; Table 5.3). Contrary to that, Luczkiewicz et al.

(2010) described an increased percentage of SXT and ciprofloxacin sensitive *E. coli*-isolates during ozonation. However, they also detected differences between the respective antibiotics. So the percentage of cephalosporin resistant isolates, for instance remained stable.

Filtering of sewage after ozonation led to a decrease of the percentage of antibiotic resistant *E. coli* strains, whereas the percentage of resistant isolates was lowest after passing of the sand filter (reduction about 17.7%; according to Table 5.3). Grabow et al. (1976) summarized that, on average, advanced treatment by biofiltration and sand filtration led to a reduction of transferable resistance to one or more drugs in coliforms of about 50%. The authors discussed the structure of the filter material concerning its inhibitory effect on conjugation. However, the question remained why the flocculation filter and the sand-filter after ozonation (comparable construction) had a contrary effect on the percentage of resistant isolates. The percentage of resistant isolates increased about 11.3% during passage of the flocculation filter (Table 5.3). Nevertheless, the percentages of resistant *E. coli* in the effluents of the filters downstream of the ozonation unit were in a comparable order of magnitude or lower than after passing the flocculation filter (according to Table 5.3).

Similar to *E. coli*, the percentage of antibiotic resistant enterococci was about 10.8% higher in effluent (ef FF) of the flocculation filtration unit compared to the percentage in effluent of primary treatment (ef PT; Table 5.3). The resistance-level was determined by resistance against erythromycin. The same trend was seen for ciprofloxacin resistant isolates (Table 5.3). No vancomycin resistant *E. faecium* or *E. faecalis*

(VRE) strains were detected and the resistance-level against the last resort antibiotic "chloramphenicol" was low in the respective samples (at most 2 chloramphenicol resistant enterococci-isolates per sampling point). The percentage of ampicillin resistant isolates of the genus *Enterococcus* was nearly doubled in ef FF (10%) compared to ef PT (5.2%; Table 5.3). Such a resistance pattern was also described by Ferreira da Silva et al. (2006) and by Luczkiewicz et al. (2010). During ozonation, the percentage of antibiotic resistant enterococci decreased about 25.4% (Table 5.3). In accordance to the results of Luczkiewicz et al. (2011), the percentages of erythromycin and ciprofloxacin resistant isolates were lower after ozonation (reduction about 20.7% (erythromycin) and 4.1% (ciprofloxacin) (Table 5.3). A possible explanation for the exactly contrary effect of ozone on enterococci compared to *E. coli* and staphylococci may be connected to the above described shift of the abundance of the respective *Enterococcus*-species: the percentage of isolates identified as *E. faecium* or *E. faecalis* decreased remarkably from 49.1% to 26.3% during ozonation (Table 5.2). The resistance-level was determined by *E. faecium*; 75.9% of the resistant enterococci were *E. faecium* and 59.3% of the isolates belonging to the mentioned species were resistant at least against one of the five tested antibiotics (Table 5.2). Within the enterococci that were found associated with human feces (*E. faecium* and *E. faecalis*) 42.6% were antibiotic resistant in contrast to only 12.9% of the "environment-associated" *E. durans*, *E. hirae*, *E. casseliflavus* and *E. gallinarum* (Table 5.2). Ferreira da Silva et al. (2006) also reported that *E. faecium* was more frequently antibiotic resistant than *E. hirae*. In consequence, the abundance of *E. faecium* determined the resistance-level in the respective sample. Having this in mind, the explosively increased

percentage of antibiotic resistant isolates in the effluents of the respective filters downstream of ozonation (up to 42% in ef Oz+Sa+GAC; Table 5.3) could be better integrated in the general view. The percentages of *E. faecium* and *E. faecalis* in the effluents of the respective filters were in the same order of magnitude as in ef PT (68.6%). The lowest resistance-level was detected in the effluent of the sand-filter downstream of ozonation (28.3%) with 57.7% of the isolates identified as *E. faecium* or *E. faecalis* and the highest in ef Oz+Sa+GAC (42%) in which 78.6% of the enterococci were *E. faecium* or *E. faecalis* (Table 5.2 and 5.3). The percentage of resistant enterococci in the effluent of the large-scale flocculation filter was 33.3% (Table 5.3). In the effluent of the ozone + sand filtration unit the resistance-level was about 5% lower (28.3%). In the effluent of the Oz+GAC unit 39.6% of the enterococci and in the effluent of the Oz+Sa+GAC unit 42% were resistant (Table 5.3).

Contrary to *E. coli* and enterococci, the percentage of antibiotic resistant staphylococci, determined by erythromycin resistance, was about 10% lower in ef FF than in ef PT (Table 5.3). The trend of a decreasing percentage of the resistance-level during sewage treatment by the established treatment process could also be seen for constitutive clindamycin resistance (Table 5.3). No ciprofloxacin resistant *Staphylococcus* was isolated and the percentage of oxacillin resistant isolates fluctuated between 1.5 and 2.5% in the respective samples (data not shown). *mec*A could only be detected in coagulase-negative species and no methicillin resistant *Staphylococcus aureus* (MRSA) was isolated. The detected resistance patterns were in accordance with the results of Faria et al. (2009), who analyzed resistance of coagulase-

negative staphylococci in the influent and the effluent of a sewage treatment plant in Portugal.

The effect of advanced sewage treatment on the percentage of resistant staphylococci was the same as for *E. coli*: the percentage of antibiotic resistant staphylococci increased about 5.5% during ozonation (Table 5.3). After passing the filters downstream of ozonation, the percentage of resistant isolates decreased to a different extent in the effluents of the respective filters. The highest reduction of resistant isolates was detected downstream of ozonation followed by sand filtration (14.5%; according to Table 5.3). In accordance with the results for *E. coli*, the effect of flocculation filtration and sand filtration after ozonation was also contrary. Both filters had a comparable structure; possibly, a closer look on effects of precipitating agents and ozone on cells with special respect to promoting effects on transformation and conjugation rates may help to understand these results.

Tab. 5.3: Percentage of antibiotic resistant *E. coli*, enterococci and staphylococci isolates with respect to the wastewater treatment in standard operating procedures (ef PT, ef SS, ef FF) and advanced wastewater treatment with ozonation (ef Oz) or GAC (ef GAC) solely as well as with ozonation in combination with sand filtration and/ or GAC (ef Oz+Sa, ef Oz+GAC, ef Oz+Sa+GAC). n = number of sampling campaigns (Abbreviations same as Tab. 5.1).

treatment	% resistant *E. coli*				% resistant enterococci					% resistant staphylococci	
	Total (n= no. of samplings)	cipro-floxacin	SXT	ampi-cillin	Total (n= no. of samplings)	cipro-floxacin	ampi-cillin	chloram-phenicol	erythro-mycin	Total (n= no. of samplings)	constitutive clindamycin
ef PT	14.5 (6)	0	8.4	14.5	22.5 (6)	3.6	5.2	3.6	20.1	35.8 (6)	8.9
ef SS	10.7 (5)	4.2	8.3	10.7	36.4 (5)	8.5	7.7	2.9	29.7	25 (6)	3.3
ef FF	22 (7)	0.5	3.9	21	33.3 (7)	7.4	10	2	29.3	25.8 (7)	3.7
ef GAC	19.3 (3)	8.9	14.5	19.2	20.9 (2)	0	0	4.2	20.8	25 (1)	5
ef Oz	26.7 (4)	3.6	7.1	19.6	11 (4)	4.4	9	3.1	9	30.5 (4)	4.9
ef Oz+Sa	9 (5)	0	4	9	28.3 (4)	1.7	9.2	0	24.2	16 (5)	3
ef Oz+GAC	14 (5)	3.2	1.3	12.6	39.6 (4)	8.3	6.3	0	39.6	19.6 (5)	2
ef Oz+Sa+GAC	22.8 (2)	13.6	13.6	18.2	42 (2)	0	4.2	0	41.7	24.7 (4)	0

Compared to the percentage of resistant staphylococci obtained from ef FF, resistance-levels in effluents of the respective filters after ozonation were up to 9.8 % lower (Table 5.3).

Even if the percentage of antibiotic resistant bacteria increased during ozonation or after downstream filtration the concentrations of antibiotic resistant *E. coli*, enterococci and staphylococci in all effluents of the pilot plant were lower compared to the respective concentrations of the effluent of the currently established tertiary treatment step (ef FF; Fig. 5.2 and 5.3). Figure 5.3, which shows the same trend as Figure 5.2, illustrates the overbalancing disinfectant effect of ozone. Establishing ozonation followed by a filter passage would reduce the discharge of total and antibiotic resistant bacteria into the receiving water body by about 0.8 to 1.2 log-units (Table 5.1). Nevertheless, antibiotic resistant bacteria and antibiotic resistance determinants would be released into the aquatic environment. At the moment, the remaining risk and the effect on autochthonous microorganisms cannot be estimated. To improve advanced sewage treatment processes, it would be important to understand the detailed mode of action of ozone on living cells as well as the “interactions” of filter materials with cell envelopes. Investigations concerning terms for conjugation and transformation and the respective rates would help to understand the described observations.

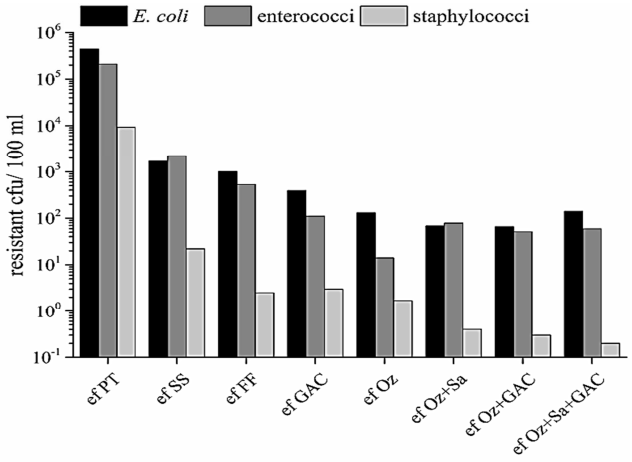

Fig. 5.3 Colony forming units (cfu) of resistant isolates of *E. coli*, enterococci and staphylococci during different treatment steps.

5.5 Conclusion

- Ozonation followed by a filter passage led to an additional reduction of total and antibiotic resistant *E. coli*, enterococci and staphylococci between 0.8 and 1.1 log-units compared to the respective concentrations of the effluent of the currently established tertiary sewage treatment (flocculation filtration). Nevertheless, a potential risk for human and animal health remains, as to date the effect of the discharge of antibiotic resistant bacteria and antibiotic resistance genes on the autochthonous microorganisms in surface water cannot be estimated.

- Treatment with ozone led to an increased percentage of antibiotic resistant *E. coli*- (16%) and staphylococci-isolates (5.5%) and a

decrease of the resistance-level of enterococci (25.4%), whereas differences for the respective antibiotics were observed. A deeper insight into the mode of action of ozone on living cells would help to explain these results and to improve advanced sewage treatment processes.

- Environment-associated *Enterococcus*-species seemed to be less sensitive against ozone than *E. faecium* and *E. faecalis*.

- Filter passages partially caused an increase of the resistance-level. Investigations concerning the effect of the filter material on retention of microorganisms and horizontal gene transfer would help to optimize advanced sewage treatment techniques.

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6. Sensitivity of antibiotic resistant and antibiotic susceptible *Escherichia coli*, *Enterococcus* and *Staphylococcus* strains against ozone

In der Wissenschaft wird momentan diskutiert (z.B. Dodd, 2012), ob der Einsatz von Ozon in der Abwasserreinigung mit dem „negativen“ Effekt einer relativen Anreicherung von Antibiotika-resistenten Keimen verbunden und Antibiotika-Resistenz mit einer geringeren Empfindlichkeit gegen Ozon gekoppelt ist. Auch in der Pilotanlage in Eriskirch wurde nach der Ozonung ein Anstieg des Anteils resistenter *E. coli* und Staphylokokken detektiert. Um diese im vorangegangenen Manuskript beschriebenen Ergebnisse zu verifizieren und das diametral unterschiedliche Verhalten von *E. coli* und Staphylokokken im Vergleich zu den Enterkokken während der Ozonung zu verstehen, wurde diese Reinigungsstufe im Labor simuliert und der Effekt von Ozon auf einzelne Isolate mit unterschiedlichen Antibiotika-Resistenzmustern bestimmt:

- Innerhalb der einzelnen Spezies wurde eine große Varianz hinsichtlich der Empfindlichkeit gegen Ozon detektiert (bis zu 3,8 log-Stufen), die nicht monokausal erklärt werden kann. Antibiotika-Resistenz per se scheint nicht der entscheidende Faktor zu sein.
- Manche Spezies sind aufgrund von artspezifischen Charakteristika, wie beispielsweise die Synthese von Staphyloxanthin bei *Staphylococcus aureus*, weniger empfindlich gegen Ozon als andere. In der Kombination mit dem Ergebnis, dass manche

Spezies häufiger Antibiotika-resistent sind als andere Arten, kann es durch die aus der Ozonung resultierenden Verschiebung in der Abundanz der einzelnen Spezies zu einer indirekten Selektion Antibiotika-resistenter Isolate kommen.

- Die in der Pilotanlage in der Kläranlage in Eriskirch gesehene Reduktion des Anteils resistenter Enterokokken (25,4%) hängt tatsächlich mit der detektierten Verschiebung in der Abundanz der einzelnen *Enterococcus*-Spezies zusammen (der Anteil von *E. faecium* sank während der Ozonung um 15,8%, während der Anteil von *E. casseliflavus* sich von 12,3% auf 18,4% erhöhte): *E. casseliflavus-Isolate* waren unempfindlicher gegen 4 mg/L Ozon als *E. faecium*-Isolate, die aber deutlich häufiger gegen eines der 5 getesteten Antibiotika resistent waren und deren Abundanz in der Konsequenz das detektierte Resistenzniveau bestimmte.

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Abstract

The tolerance of antibiotic susceptible and antibiotic resistant *Escherichia coli*, *Enterococcus* and *Staphylococcus* strains from clinical

and wastewater samples against ozone was tested to investigate if ozone, a strong oxidant applied for advanced wastewater treatment, will affect the release of antibiotic resistant bacteria into the aquatic environment. For this purpose the resistance pattern against antibiotics of the mentioned isolates and their survival after exposure to 4 mg/L ozone was determined. Antibiotic resistance of the tested strains was not correlated with a higher tolerance against ozone. With the exception of ampicillin resistant *E. coli* strains, which seemed to be little more resistant against ozone, *E. coli* strains that were resistant against cotrimoxazol, ciprofloxacin or a combination of the three antibiotics were similarly or less resistant against ozone than antibiotic sensitive strains. Pigment-producing *Enterococcus casseliflavus* and *Staphylococcus aureus* were little more resistant against ozone than non-pigmented species of these genera. The ability of biofilm formation apparently protected bacteria from inactivation by ozone. The relatively large variance of tolerance against ozone may indicate that resistance is most probably dependent on several factors, where antibiotic resistance, if at all, plays not the major role.

6.1 Introduction

Surface water sources very often serve as drinking water reservoir or for recreation and therefore their water quality is of fundamental interest. This is manifested in WHO or EU guidelines, for instance in the European Union Water Framework Directive (EC 2000), which demands to reduce acute and chronic toxicity in water for aquatic organisms and to protect biological diversity and human health. To

achieve these aims, improvements of sewage purification by ultrafiltration or ozonation as additional treatment stages in sewage treatment plants (STPs) are discussed and tested. The main goal of these additional wastewater treatment technologies is a reduction of the concentration of emerging contaminants in the effluent of STPs and respectively in receiving water bodies. A positive side effect of e.g. ozonation is the inactivation of facultative pathogenic bacteria such as *Escherichia coli*, *Enterococcus* or *Staphylococcus* strains in effluents of STPs by several log-decades (Xu et al. 2002; Gehr et al. 2003; Lüddeke et al. 2015). The efficiency of ozone as a disinfectant depends on several parameters. Kinetics of the reactions of ozone are complex due to different reaction rates with the compounds of sewage, speciation of ozone and its decomposition products, which again interact with microorganisms (Gehr et al. 2003). In batch assays Gehr et al. (2003) demonstrated that ozone decayed rapidly and formed hydroxyl radicals for non-selective oxidation, if the alkalinity was low and/or the organic concentration was high. Hunt and Mariñas (1997) reported that molecular ozone was the agent responsible for the inactivation of *E. coli*. Up to date, the exact mode of action and the specific target structures of ozone and of its decay products in microbial cells are not completely understood: Ozone is expected to react rapidly with the unsaturated bonds of phospholipids and lipopolysaccharides in membranes and cell walls, but intracellular amino acids and proteins may also be affected (Dodd 2012). In addition DNA damages may also be caused by ozone (Gehr et al. 2003).

For advanced sewage treatment ozone is applied after secondary treatment either with a diffuser at the bottom of the tank or with a pump-

injector system, injecting small gas bubbles into the influent water of the ozone-reactor. Ozone-concentrations between 2 and 10 mg/L, depending on the dissolved organic carbon (DOC) concentration, are used for final treatment of sewage. The minimally required contact time of ozone with sewage in STPs for a maximal oxidation is difficult to determine. Investigations in the STPs Bad Sassendorf, Duisburg-Vierlinden and Schwerte (Northrhine-Westphalia, Germany) indicated that the reactions triggered by ozone occurred within less than 30 minutes (http://www.lanuv.nrw.de/wasser/abwasser/forschung/pdf/Abschlussbericht_Spurenstoffe_Warburg.pdf).

In his recent review Dodd (2012) raised the question whether antibiotic resistant bacteria have an advantage to cope with oxidative stressors such as ozone in comparison to bacteria that are not resistant against antibiotics. We addressed this problem and investigated if the treatment of conventionally purified sewage with ozone would favor a better survival of antibiotic resistant bacteria than of antibiotic sensitive bacteria. For this purpose the sensitivity of antibiotic resistant and antibiotic susceptible *Escherichia coli*, *Enterococcus* and *Staphylococcus* isolates from clinical samples, sewage and river water against ozone was determined for the first time in detail. Survival of antibiotic susceptible and antibiotic resistant bacterial strains were compared with data obtained from an ozonation unit in a STP.

6.2 Materials and Methods

6.2.1 Laboratory assay simulating the pilot-scale ozonation of the sewage treatment plant (STP) in Eriskirch

In the pilot-scale ozonation unit of the STP of Eriskirch (Baden-Wuerttemberg, Germany) 34 m^3 of mechanical and biological treated sewage with a mean DOC of 5.5 mg/L were separated per day and supplemented with 4 mg/L ozone that was injected by a ventury injector (Xylem Water Solutions GmbH, Großostheim, Germany). After the ozone dosage the sewage was pumped into a closed tank (volume: 0.5 m^3). The contact time of ozone with sewage was 20 minutes, long enough to decompose organic material until ozone exhaustion.

Ozonation experiments in the laboratory were performed with 100 mL aqueous samples in 250 mL glass reactors, equipped with a diffuser and sterile 0.2 µm PTFE filters (Roth, Karlsruhe, Germany) at the in- and outlet. Since the pH in sewage-simulating 20-fold diluted OECD medium (final DOC of 5.5 mg/L as in treated sewage) dropped below 5.5 after ozonation a phosphate-buffered medium (3.87 g/L K_2HPO_4, 2.42 g/L KH_2PO_4, 0.0138 g/L $C_6H_{12}O_6$, 9 g/L NaCl dissolved in deionized water, pH 7.0, autoclaved for 15 minutes) with 5.5 mg DOC per L and a pH of 7 after ozonation was used instead. An ozone-generator (Laborozonisator 301.7, Sander, Uetze-Eltze, Germany) was connected via Norprene tubings with the inlet filter unit of the glass reactor. Ozone was generated from hydrocarbon-free compressed air for three minutes and introduced via the diffuser into the glass reactor. A concentration of 4 mg O_3/L was reached after 3 minutes. Then 0.5 mL of

an overnight culture of the respective strain, grown in DEV-nutrient broth, was inoculated.

After 0.5, 1, 2, 5, 20 and 40 minutes of exposure to ozone, surviving cell numbers were determined. From *E. coli* cultures 10^{-2}, 10^{-3} and 10^{-4}-fold and from *Enterococcus* and *Staphylococcus* cultures 10^{-1}, 10^{-2} and 10^{-3}-fold dilutions were prepared for plating on DEV- nutrient agar (Roth) in duplicate. To determine original cell densities in overnight cultures 100 µL of the dilution steps 10^{-6} and 10^{-7} were plated on DEV-nutrient agar. After 20 h of incubation at 37 °C colonies were counted and inactivation with exposure time to ozone was calculated. The reproducibility of these assays was about +/- 0.3 log-decades.

Ozone decay in the phosphate-buffered medium was determined using potassium indigo trisulfonate according to DIN 38408-3 (DIN, Deutsche Industrie Norm 2011-04). The half-life time of ozone was 6 minutes and 4 seconds (Fig. 6.1). Preliminary experiments revealed, that ozone inactivated bacteria within less than 30 seconds. Between 5 and 20 minutes after inoculation no further inactivation of bacteria was seen, although ozone was not yet depleted (Fig. 6.1).

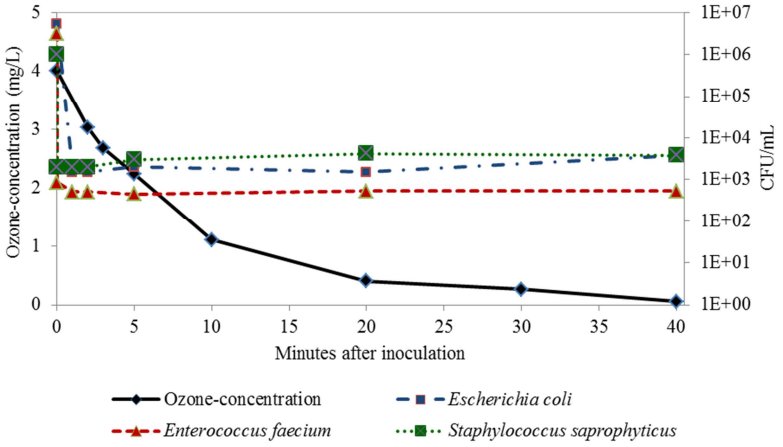

Fig. 6.1: Decrease of the ozone concentration and of colony forming units (cfu) in an *Escherichia coli*, *Enterococcus faecium* and *Staphylococcus saprophyticus* culture in the presence of initially 4 mg/L ozone.

In control assays with an *E. coli*, an *Enterococcus* and a *Staphylococcus* strain in the phosphate buffered medium gassing with air instead of ozone did not change numbers of living cells during incubation (data not shown). For laboratory assays the contact time could thus be restricted to 5 minutes, compared to 20 minutes contact time of sewage with ozone in the pilot-scale ozonation plant in Eriskirch.

6.2.2 Identification and antibiotic susceptibility testing of isolated strains

The tested 52 *E. coli*, 89 *Enterococcus* and 80 *Staphylococcus* strains were isolated within the SchussenAktiv*plus* project during 2012 and

2014 from sewage and river water (for details see Heß&Gallert 2014; Triebskorn *et al*. 2013; www.schussenaktivplus.de). Strains that probably have already survived ozonation (e.g. isolated from the effluent of the STP of Eriskirch) were excluded from this study. Depending on the contamination level of the respective samples, direct plating or the filtration method was used to obtain *E. coli*, *Enterococcus* and *Staphylococcus* isolates.

For cultivation of *E. coli* isolates, ECD-agar (Merck Millipore, Darmstadt, Germany) and ESBL CHROMagar™ (MAST Diagnostica GmbH, Reinfeld, Germany) were used, following the manufacturer's instructions. On ECD-agar, blue fluorescent and indole positive colonies (red-colored after reaction with 10 µL Kovac's reagent) were identified by PCR targeting of a specific *tuf*-gene fragment (Maheux et al. 2009). Briefly, the DNA-amplification was performed in a total volume of 25 µl containing 0.625 units True Start HS Taq DNA Polymerase, 2.5 mM $MgCl_2$ (Thermo Fisher Scientific, Waltham, MA), 0.25 mM of each dNTP, 10 µM of both primers and 0.5 µl of template DNA (extraction with phenol/chloroform).

For cultivation of *Enterococcus* isolates bile-esculin-azide agar (Roth) and VRE CHROMagar™ (MAST Diagnostica GmbH), supplemented with 0.15 g/L sodium azide were used, following the manufacturer's instructions. Black colonies with a halo on bile-esculin-azide agar and pink colonies on VRE CHROMagar™ were identified on species level with Micronaut-Strep2®-microtiter plates according to the manufacturer's instructions (MERLIN, Gesellschaft für mikrobiologische Diagnostika, Bornheim, Germany).

For cultivation of *Staphylococcus* isolates Chapman-Stone agar, containing 0.05 g/L sodium azide, was used. Colonies grown after incubation for 48 h at 37 °C were streaked on Mannitol-Salt agar before they were identified by their physiological reactions on Micronaut-Staph®-microtiter plates (MERLIN, Gesellschaft für mikrobiologische Diagnostika). *Staphylococcus aureus* and *Staphylococcus saprophyticus* isolates from clinical specimen were obtained from Städtisches Klinikum Karlsruhe. Strains were isolated by the staff of the municipal hospital from different patients, mainly from smears of skin and mucous membranes but also from smears of wounds and blood cultures.

Antibiotic resistance (AR) of the *E. coli* isolates against ampicillin (10 µg), ciprofloxacin (5 µg), cotrimoxazol (1.25 µg trimethoprim/ 23.75 µg sulfamethoxazol; SXT) was tested using the agar diffusion test according to Deutsche Industrie Norm (DIN 58940, 2011). For testing AR against cefotaxim (5 µg) clinical breakpoints according to EUCAST (2011) were applied. All ampicillin resistant isolates were additionally tested against ceftazidim (30 µg) and cefpodoxim (10 µg) according to CLSI (2011); the inhibitory effect of clavulanic acid on ß-lactamase was checked as described by Bradford (2001). According to the definition of Robert-Koch-Institut (2007), extended-spectrum-ß-lactamase producers phenotypically were resistant against cefpodoxim as well as against ceftazidim and/or cefotaxim. All isolates that were resistant against the above mentioned antibiotics were classified as ESBL-producers.

AR of the *Enterococcus* isolates was tested against ampicillin (10 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg) and erythromycin (15 µg) according to Deutsche Industrie Norm (DIN 58940, 2011). The

susceptibility of *E. faecium* and *E. faecalis* isolates against vancomycin (VRE) was tested according to Clinical and Laboratory Standards (CLSI, 2011). Vancomycin resistance (VR) was confirmed by the presence of *van*A-E and *van*G genes (Depardieu et al. 2004). Intrinsic low-level vancomycin resistances of *E. gallinarum* and *E. casseliflavus* (*van*C1- and *van*C2-type) were not considered.

Susceptibility of the *Staphylococcus* isolates against oxacillin (5 µg), ciprofloxacin (5 µg) and erythromycin (15 µg) was tested by the disc-diffusion test with Mueller-Hinton agar according to Deutsche Industrie Norm (DIN 58940, 2011) and against clindamycin (2 µg) according to CLSI (2011). Oxacillin resistance was confirmed by the presence of the *mec*A gene using previously described primers and PCR-conditions (Predari et al. 1991). DNA-amplification was performed in a 25 µl assay containing 0.625 units True Start HS Taq DNA Polymerase, 2.5 mM $MgCl_2$, 0.25 mM of each dNTP, 10 µM of both primers and 0.5 µl of template DNA (extraction with phenol/chloroform).

6.2.3 Pigment extraction and quantification

Cells of an overnight culture of the respective *S. aureus* strains, grown in DEV-nutrient broth, were pelleted by centrifugation. The cells were re-suspended in a 0.9 % sodium chloride solution to obtain a turbidity of McFarland 2.0. Two mL of the suspension were used to extract staphyloxanthin and to quantify pigment production as described by Morikawa et al. (2001).

6.2.4 Statistical analysis

For descriptive analysis of the obtained data, box plots were used displaying variation within the respective “clusters”.

6.3 Results and Discussion

6.3.1 Exposure time for inactivation of bacteria by ozone

A Gram-negative *E. coli* strain, a Gram-positive *Enterococcus* isolate and a Gram-positive *Staphylococcus* isolate were exposed to 4 mg/L ozone. Inactivation of cells by more than 3 log-decades occurred within the first 30 seconds contact time from initially $1 – 4 \times 10^6$ to finally $5 \times 10^2 – 5 \times 10^3$ bacteria per mL (Fig. 6.1). No further inactivation occurred later on, although even after 5 minutes more than 2 mg/L ozone was still present, an ozone concentration that was higher than the initial concentration in some pilot-scale ozonation systems. The phenomenon of a rapid initial inactivation of bacteria by ozone seemed to be independent of the applied ozone concentration as Xu et al. (2002) also reported no difference in inactivation of fecal coliforms between 2 and 10 minutes hydraulic retention time for a given ozone dose. Differences in cell wall and membrane architecture between Gram-positive and Gram-negative bacteria seemed not to be the decisive structures that were responsible for survival in the presence of ozone.

6.3.2 Inactivation of *Escherichia coli* by ozone

A relatively large variance of antibiotic susceptible and antibiotic resistant *E. coli* strains was observed for inactivation by ozone (Fig. 6.2).

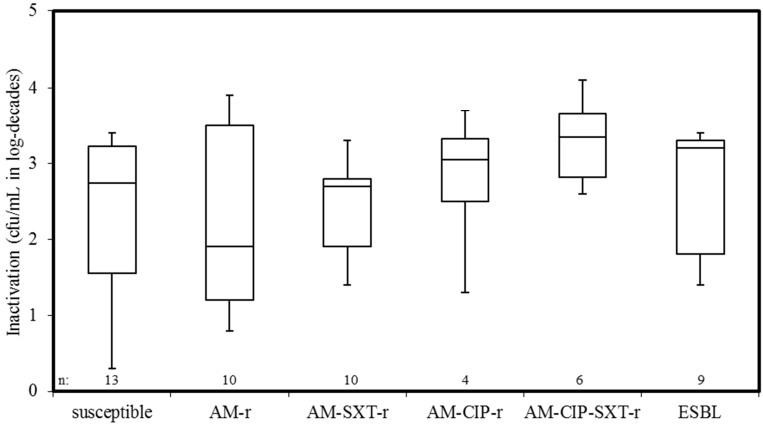

Fig. 6.2: Inactivation of 13 antibiotic susceptible and 39 antibiotic resistant *Escherichia coli* strains that were isolated from sewage and river water samples by 4 mg/L ozone.

Number of tested isolates, n. Resistant strains against ampicillin (AM-r), cotrimoxazol (SXT-r), ciprofloxacin (CIP-r). Extended-spectrum-ß-lactamase-producers, ESBL.

Within the same species some strains were less susceptible against ozone than others (variation of the inactivation of living cells between 0.3 and 4.1 log-decades), independent of whether the strain carried antibiotic resistance genes or not (Fig. 6.2): The median inactivation (2.8 log decades) of the tested antibiotic resistant isolates was in the same order to that of antibiotic sensitive strains. Looking more into

details, the median of the cluster "ampicillin resistant *E. coli*" was the lowest within all antibiotic resistant *E. coli* strains, indicating a lower susceptibility against ozone of ampicillin resistant isolates compared to strains, which were not at all resistant against the tested antibiotics or were resistant against at least one more antibiotic-class (Fig. 6.2). The same trend was also observed in the pilot-scale ozonation plant in Eriskirch: The percentage of ampicillin resistant *Escherichia coli* isolates increased during ozonation about 8.9 % (influent: 10.7 %, effluent: 19.6 %; Lüddeke et al. 2015).

In the STP of Eriskirch, the percentage of ampicillin resistant isolates, which were additionally resistant against at least one more class of antibiotics, decreased from 8.3 % to 3.8 % after ozonation (Lüddeke et al. 2015). This was in line with the laboratory approach, where isolates that were in addition to ampicillin resistant against cotrimoxazol, ciprofloxacin or cefotaxim (ESBL) were more susceptible against ozone, manifested in higher median values for inactivation of living cells (2.7-3.4 log-decades compared to 1.9 log-decades of only ampicillin resistant isolates; Fig. 6.2). Händel et al. (2013) demonstrated that *Escherichia coli* cells apparently compensated "metabolic costs" for antibiotic resistance by physiological adaption. Comparing antibiotic susceptible and antibiotic resistant strains, changes in gene expression levels, mainly of genes for cell wall maintenance, DNA metabolic processes, cellular stress and respiration as well as for the electron transport, were observed. Overall the acquisition of antibiotic resistance primarily seemed not to require much extra energy, but seemed to cause a reduced ecological versatility (Händel et al. 2013). Nevertheless there were apparently differences between mechanisms for antibiotic resistances:

On average, ampicillin resistant *E. coli* strains were less susceptible against ozone, as indicated by the results of the laboratory assays (Fig. 6.2) as well as from pilot plant operation in Eriskirch, but the tested "only" ampicillin resistant *E. coli* isolates showed a very broad range of susceptibility against ozone (the reduction of living cells varied between 0.8 and 3.9 log-decades; Fig. 6.2). The reason could be that several mechanisms for resistance against ß-lactam-antibiotics are expressed, that are more or less energy intensive, e.g. overexpression of the intrinsic *amp*C-gene or the expression of one of the *bla*-genes (e.g. TEM, SHV, CTX-M; Robert Koch Institut 2007).

6.3.3 Inactivation of *Enterococcus* isolates by ozone

Inactivation of *Enterococcus* strains by ozone ranged from 0.2 to 5 log-decades (Fig. 6.3a, b). Although there was a broad range of susceptibility within the respective "clusters" of different species, the median of inactivation of *E. casseliflavus* (1.5 log-decades; Fig. 6.3) by ozone was significantly lower than for *E. gallinarum* (3.8 log-decades), *E. durans* (3.6 log-decades), *E. hirae* (2.5 log-decades), *E. faecalis* (3.0 log-decades) or *E. faecium* (3.2 log-decades; Fig. 6.3).

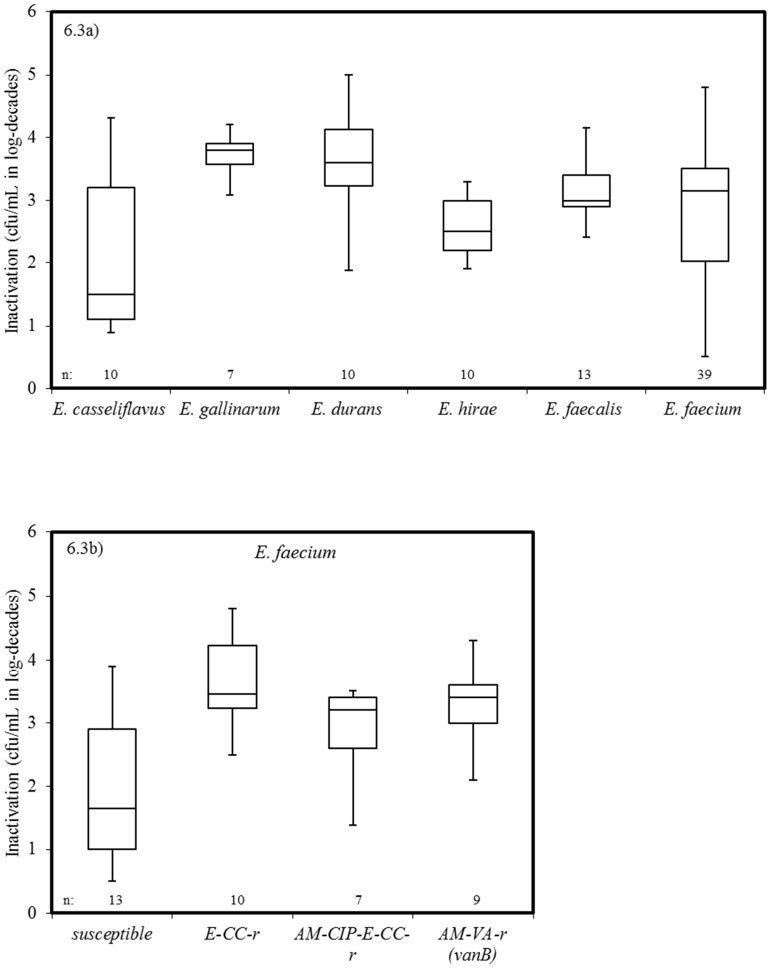

Fig. 6.3a, b: Inactivation of 89 *Enterococcus* strains belonging to 6 *Enterococcus* species (Fig. 6.3a) and of 39 different *E. faecium* strains (splitted in susceptible and resistant against the mentioned antibiotics, Fig. 6.3b) by 4 mg/L ozone.

Number of tested isolates, n; erythromycin (E-r), clindamycin (CC-r), ampicillin (AM-r), ciprofloxacin (CIP-r), vancomycin (VA-r) resistant strains. All *E. casseliflavus*, *E.*

gallinarum, *E. durans*, *E. hirae* and *E. faecalis* strains were susceptible against the tested antibiotics.

A typical feature of the species *Enterococcus casseliflavus* is the production of a yellow pigment leading to intensively yellow colored colonies on agar plates. Pigment formation is a well known strategy for protection against oxidative stress by many Gram-positive bacteria. Investigations of Clauditz et al. (2006) with *Staphylococcus aureus* confirmed that staphyloxanthin, a carotenoid produced by this *Staphylococcus* species, plays a role for fitness and the ability to cope with oxidative stress. Possibly, the pigment itself or intracellular processes coupled with its synthesis are the factors that protect cells against damaging effects of ozone and its decomposition products. Nevertheless, concerning the inactivation of *E. casseliflavus* strains by ozone, which varies over 0.9 - 4.3 log-decades, pigment synthesis itself could not explain the broad range of susceptibility against ozone within the strains of this species. In the STP in Eriskirch, the percentage of isolates that were identified as *E. casseliflavus* was higher after ozonation (18.4 %) than before ozonation (12.3 %; Lüddeke et al. 2015), indicating a higher “resistance” of the respective species against ozone. A notable reduction of *E. faecium* strains in the STP of Eriskirch after ozonation of 15.8 % (Lüddeke et al. 2015) was in accordance with results of the laboratory experiments (Fig. 6.3). Lüddeke et al. (2015) assumed that the recorded shift might explain the drastic decrease of antibiotic resistant enterococci of about 25.4 % after ozonation. The results of our laboratory experiments supported this hypothesis since the median for inactivation of active cells of antibiotic resistant *E. faecium*

strains was higher (3.4 - 4.2 log-decades; Fig. 6.3b) compared to that of susceptible strains (1.7 log-decades; Fig. 6.3b). Thus, antibiotic resistant strains may reveal a higher sensibility against ozone. Luczkiewicz et al. (2011) also reported an increased percentage of erythromycin, ciprofloxacin and chloramphenicol susceptible *Enterococcus* strains after ozonation. They also observed a decreased percentage of isolates that were identified as *E. faecium* and *E. faecalis* (10.2 % and 25.2 % decrease, respectively), mainly in favor of *E. hirae* (36.3 % increase; Luczkiewicz et al. 2011). Händel et al. (2013) reported that *E. faecium* strains with ampicillin resistance invested the same maintenance energy as antibiotic sensitive *E. faecium* strains, whereas vancomycin resistant strains required extra energy. High-level vancomycin resistance (e. g. encoded by *van*B), associated with a metabolic burden, might result in a higher sensitivity against ozone compared to vancomycin susceptible strains in the absence of the antibiotic (Fig. 6.3b). *E. casseliflavus* and *E. gallinarum* strains intrinsically are low-level vancomycin resistant (*van*C1 and *van*C2-type). Comparing ozone susceptibility of isolates of the two *Enterococcus* species in laboratory assays, *E. casseliflavus* strains (median inactivation of living cells 1.5 log-decades; Fig. 6.3a) were more resistant against ozone than *E. gallinarum* strains (most sensitive *Enterococcus* species: median inactivation of living cells 3.8 log-decades; Fig. 6.3). Therefore, low-level vancomycin resistance seemed not to be “the” determining factor for ozone-susceptibility. Pigmentation of *E. casseliflavus* might be one possibility to explain this difference.

6.3.4 Inactivation of *Staphylococcus* isolates by ozone

Staphylococcus aureus isolates were less susceptible against ozone (median inactivation of active cells 2.1 log-decades) than e.g. *S. saprophyticus* (3.2 log-decades), *S. sciuri* (3.1 log-decades) or *S. xylosus* (2.7 log-decades; Fig. 6.4a). A possible explanation for this finding may be staphyloxanthin synthesis of *S. aureus* as a protecting substance. It is known that the orange-red triterpenoid carotenoid can function as an antioxidant and seems to play a role in the protection of *S. aureus* against oxidative stress (Clauditz et al. 2006). Clauditz et al. (2006) showed that staphyloxanthin, located in the cell membrane, scavenges free radicals with its conjugated double bonds and thereby probably protects primarily lipids. It might, however, also be involved in protecting proteins and DNA. Staphyloxanthin might also play a role in the defense against reactive oxygen species, whereas enzymes such as catalase and superoxide dismutase most likely contribute to a larger extent to the survival of cells during stress (Clauditz et al. 2006). Our results provide evidence for the assumption that pigments might confer an additional protection for *Staphylococcus aureus* as the tested non-pigmented isolates identified as *S. sciuri*, *S. epidermidis*, *S. xylosus* and *S. saprophyticus* were more sensitive against ozone (Fig. 6.4a). Highest tolerance against ozone coincided with a high content of carotenoides in *S. aureus* colonies but the level of resistance against ozone and the amount of staphyloxanthin were not always in line (data not shown), indicating that there must be also other factors responsible for resistance against ozone. The possession and expression of the *mecA* gene seemed to have no consequence for the susceptibility against ozone as median

inactivation of MRSA (methicillin-resistant *S. aureus*) and MSSA (methicillin sensitive *S. aureus*) were identical (Fig. 6.4b).

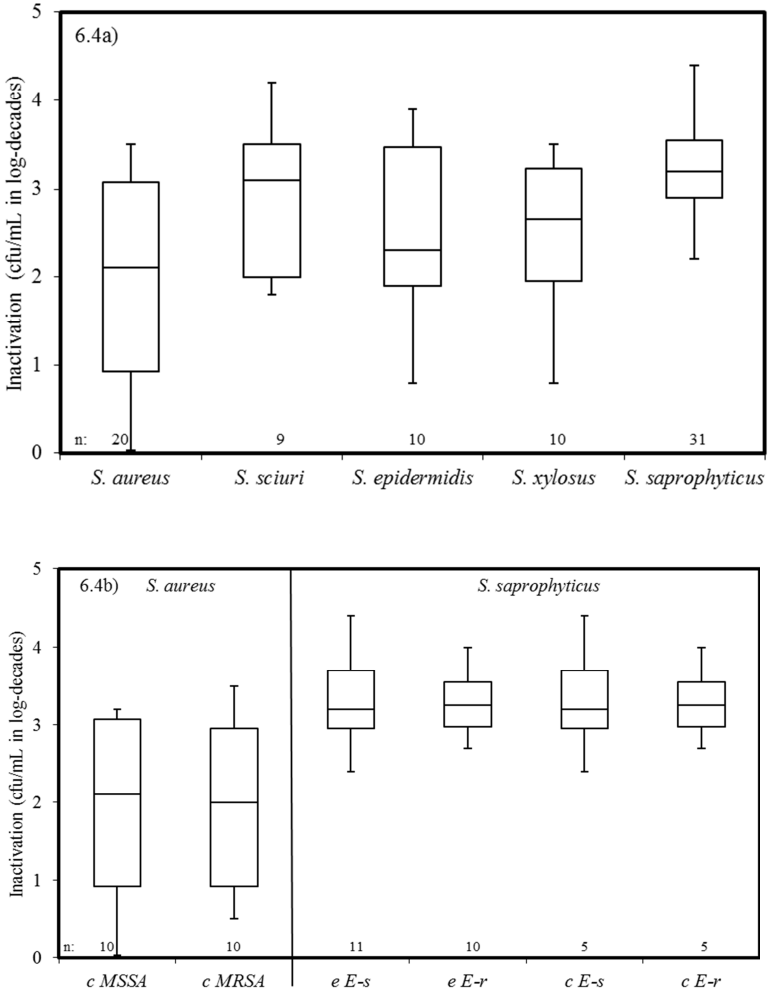

Fig. 6.4a, b: Inactivation of 80 *Staphylococcus* strains from environmental and clinical sources belonging to 5 different species (Fig. 6.4a) and of 20 *S. aureus* (splitted in

susceptible (MSSA) and resistant against oxacillin (MRSA)) and 31 *S. saprophyticus* strains (splitted in susceptible and resistant against erythromycin, Fig. 6.4b) by 4 mg/L ozone.

Number of tested isolates, n; isolates from the aquatic environment, e; clinical strains, c (all *S. aureus* strains were obtained from hospital); erythromycin susceptible strains, E-s; erythromycin resistant isolates, E-r. All *S. sciuri*, *S. epidermidis* and *S. xylosus* strains were susceptible against the tested antibiotics.

The median for inactivation of active cells of *S. epidermidis* isolates (2.3 log-decades) was only slightly higher compared to that of *S. aureus* (2.1 log-decades; Fig. 6.4a). *Staphylococcus epidermidis* is a well known biofilm-forming species (e.g. Cerca et al. 2005) and cells of an overnight culture already aggregated and formed visible flocs.

Although cell flocs in the *S. epidemidis* culture could be disaggregated to single cells by high shear forces, extracellular substances may have surrounded single cells and thus have protected cell walls or cell membranes from oxidation by ozone. The clearly higher resistance against ozone of cells embedded in biofilms has already been described and attributed to an extracellular polymeric substances (EPS) matrix (e.g. Hems et al. 2005). The same observation as for *S. epidermidis* was made with aggregating strains of *S. xylosus*, resulting in a median inactivation by ozone of 2.7 log-decades, whereas isolates of *S. sciuri* and *S. saprophyticus*, both of which neither produced pigment nor grew in aggregates were less resistant to ozone (Fig. 6.4a).

Regarding the inactivation of *S. saprophyticus* isolates of clinical and environmental samples by 4 mg/L ozone, there were no significant

differences with respect to their origin (Fig. 6.4b). The median for inactivation, independently of origin or antibiotic resistance e.g. against erythromycin was almost identical (Fig. 6.4b).

Taking the results of *Escherichia coli* strains, *Enterococcus* and *Staphylococcus* isolates from hospital sources, sewage and river water as a whole, then single isolates, that were resistant against at least one of the tested antibiotics, were not per se more resistant against oxidative stress by 4 mg/L ozone than antibiotic sensitive strains. Some species seemed to be (little) more "ozone-resistant" than others and thus might be "positively selected" by ozonation with the consequence that the percentage of such strains increases.

6.4 Conclusions

- Antibiotic resistance per se did not lead to a reduced sensitivity of *E. coli*, *Enterococcus* and *Staphylococcus* strains against 4 mg/L ozone.
- Pigment-producing *Enterococcus casseliflavus* and *Staphylococcus aureus* were little less sensitive against ozone compared to non-pigmented species of the respective genera.
- Within the same species, the susceptibility against ozone expressed as inactivation of active cells differed in a wide range up to 3.8 log-decades.
- Cell wall and membrane architecture seemed not to be "the" decisive structures that were responsible for survival in the presence of ozone. Several other factors may influence resistance against ozone within species or genera.

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7. Removal of micropollutants, facultative pathogenic and antibiotic resistant bacteria in a full-scale retention soil filter receiving combined sewer overflow

Wenn in Mischentwässerungssystemen beispielsweise infolge von Starkregenereignissen die Reinigungskapazität der Kläranlage und das Einstauvolumen von Regenüberlaufbecken überschritten werden, kann das mit Regenwasser verdünnte Abwasser fakultativ, bevor es in den Vorfluter abgeschlagen wird, über einen Retentionsbodenfilter geleitet werden. Im Rahmen des Projektes SchussenAktiv*plus* wurde die Effektivität des Retentionsbodenfilters in Tettnang hinsichtlich der Entnahme von Spurenstoffen und fakultativ pathogenen und Antibiotika-resistenten Keimen untersucht:

- Die Eliminationsleitung des Retentionsbodenfilters bezüglich *E. coli*, Enterokokken und Staphylokokken lag in der gleichen Größenordnung wie in einer dreistufigen Kläranlage. Bei diesem Vergleich bleibt allerdings zu berücksichtigen, dass bereits die Konzentration an fakultativ pathogenen Keimen im Zulauf zum Retentionsbodenfilter geringer war als im Zulauf zur Kläranlage bei Trockenwetter.
- Der Anteil resistenter *E. coli*- und Staphylokokken-Isolate nahm während der Passage durch den Retentionsbodenfilter ab, während sich der relative Anteil resistenter Enterokokken nicht veränderte. Projiziert man jedoch den jeweiligen prozentualen Anteil resistenter Isolate auf die ermittelten Lebendkeimzahlen pro Volumen, war die Konzentration Antibiotika-resistenter *E. coli*,

Enterokokken und Staphylokokken im Ablauf des Retentionsbodenfilters zwischen 2,1 und 2,9 log-Stufen (Mediane) geringer als in dessen Zulauf.

- Anhand des Anteils an *Staphylococcus*-Isolaten, die in die Sciuri-Gruppe gehörten beziehungsweise an der Abundanz von *Staphylococcus xylosus* innerhalb der Saprophyticus-Gruppe („Freiland-Marker"), war es möglich qualitativ die Anteile an Rohabwasser und Regenwasser/Oberflächenabfluss im Zulauf zum Retentionsbodenfilter abzuschätzen.

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Abstract

Combined sewer systems collect surface runoff as well as wastewater of industrial and domestic origin. During periods of heavy rainfall the capacity of the sewer system is exceeded and the overflow is discharged into receiving waters without any treatment. Consequently, combined sewer overflow (CSO) is considered as a major source of water

pollution. This study investigates the effectiveness of a retention soil filter (RSF) for the removal of micropollutants as well as facultative pathogenic and antibiotic resistant bacteria from CSO. The removal of organic group parameters like total organic carbon was excellent and the removal efficiency for micropollutants of the RSF and the wastewater treatment plant (WWTP), which treats wastewater of the same origin during dry and normal weather conditions, was comparable. Compounds of high environmental concern like estrogens or certain pharmaceuticals, e. g. diclofenac, were completely eliminated or removed to a high degree during RSF passage. RSF treatment also reduced the number of *E. coli*, enterococci and staphylococci by 2.7, 2.2 and 2.4 log-units (median values), respectively. Obviously, some *Staphylococcus* species can better adapt to the conditions of the RSF than others as a shift of the abundance of the different species was observed when comparing the diversity of staphylococci obtained from the RSF influent and effluent. RSF treatment also decreased the absolute number of antibiotic resistant bacteria. The percentage of antibiotic resistant *E. coli* and staphylococci isolates also decreased during passage of the RSF, whereas the percentage of resistant enterococci did not change. For *E. coli* ampicillin and for enterococci and staphylococci erythromycin determined the antibiotic resistance level.

The results demonstrate that RSFs can be considered as an adequate treatment option for CSO. The performance for the removal of micropollutants is comparable with a medium sized WWTP with conventional activated sludge treatment. The number of facultative pathogenic and antibiotic resistant bacteria was considerably decreased during RSF passage. However, as RSF effluents still contained antibiotic

resistance genes and traces of micropollutants; receiving waters may still be at risk from negative environmental impacts.

7.1 Introduction

Pollution of surface waters poses a threat for the aquatic environment. For this reason the European Union Water Framework Directive (EUWFD) was passed in 2000 to reduce acute and chronic toxicity for aquatic organisms, to minimize the accumulation of pollutants in ecosystems and to protect biological diversity and human health.[1] Environmental quality standards were established for an initial set of 33 priority substances, which must not be exceeded for a good surface water chemical status. In 2013 the list was extended by twelve new compounds. Additionally, three compounds (diclofenac, 17-β-estradiol and 17-α-ethinylestradiol) were put on a watch list, for which monitoring data are to be collected to support a decision on potential prioritization in the future.[2] Some chemicals listed in the EUWFD are supposed to be mainly discharged by point sources like wastewater treatment plants (WWTPs). This holds true not only for compounds on the EU list of priority substances, but also for numerous other chemicals as well as facultative pathogenic and antibiotic resistant bacteria which might also affect the aquatic environment or human health.

One successful approach to minimize the discharge of micropollutants and microorganisms (bacteria, viruses, protozoan pathogens) by point sources is upgrading WWTPs by additional treatment technologies like ozonation or powdered activated carbon filtration.[3] In Germany, however, two thirds of the sewer systems collect wastewater with domestic and industrial origin together with storm water runoff.[4,5]

During heavy rainfall up to seven times more water is collected in combined sewer systems than under dry weather conditions.[6] As a consequence, the capacity of the sewer system and of WWTPs can be insufficient during heavy rainfalls as most facilities have a maximum treatment capacity equivalent to twice the dry weather discharge.[4]

It is therefore assumed that 30 % to 50 % of the annual storm water runoff is discharged as untreated combined sewer overflow (CSO) into receiving waters,[5] including readily biodegradable or nonpolar micropollutants which would have been removed by microbial degradation or by sorption onto the sewage sludge under dry weather conditions. One measure to cope with high flows in the sewer system are storm water overflow basins (SOBs), which provide an interim storage capacity for excess water, that is transferred to the WWTP after the rain event. However, no microbial degradation of nutrients or micropollutants is achieved in the SOBs and when their storage capacity is exceeded, untreated SOB overflow is also a source for contamination with micropollutants and potentially pathogenic microorganisms. This chemical and bacterial pollution can be a threat to human health when water is used for drinking water production, recreational activities or irrigation. Antibiotic resistance genes within the released microorganisms even increase this health threat and may contribute to their dissemination to the autochthonous microbial community. Therefore, CSO is an important source of pollution for receiving waters as it may represent large loads of microbial and chemical contaminants.[7,8]

For combined sewer systems retention soil filters (RSFs) are a treatment technology that helps to safeguard WWTPs and receiving waters from hydraulic, chemical and hygienic stress caused by the feed of excess

water.[6] RSFs are soil filters with a vertical flow-through passage that also provide additional intermediate storage and retention capacity. Basically, RSFs are constructed wetlands that only have an intermittent inflow and receive overflows from combined sewers, storm water or highway runoff and not predominantly wastewater.[9] They provide water treatment by filtration, sorption and biological processes. In previous studies, RSFs showed excellent retention or removal efficiencies for nutrients like ammonium or phosphate as well as for suspended solids and oxidizable organic matter, expressed e.g. as chemical oxygen demand (COD) and biochemical oxygen demand after 5 d (BOD_5).[4.6]

The interest in retention soil filter technology is growing, but with many newly deployed filters there still is little experience with regard to long term performance. A survey among RSF operators in Germany (n = 83) revealed that only 21 % of all filters have been in service for more than five years.[10]

The RSF analyzed in this study treats CSO of two storm water overflow basins (SOBs) and discharges into the river Schussen, a densely populated catchment area in Southwest Germany. The Schussen is a tributary of Lake Constance, which is an important drinking water reservoir and a popular recreation area.[11] The elimination of nutrients, organic trace pollutants and facultative pathogenic bacteria by a RSF downstream of two storm water overflow basins is presented. For numerous micropollutants, facultative pathogenic and antibiotic resistant bacteria this study presents the first baseline data for their retention by RSF.

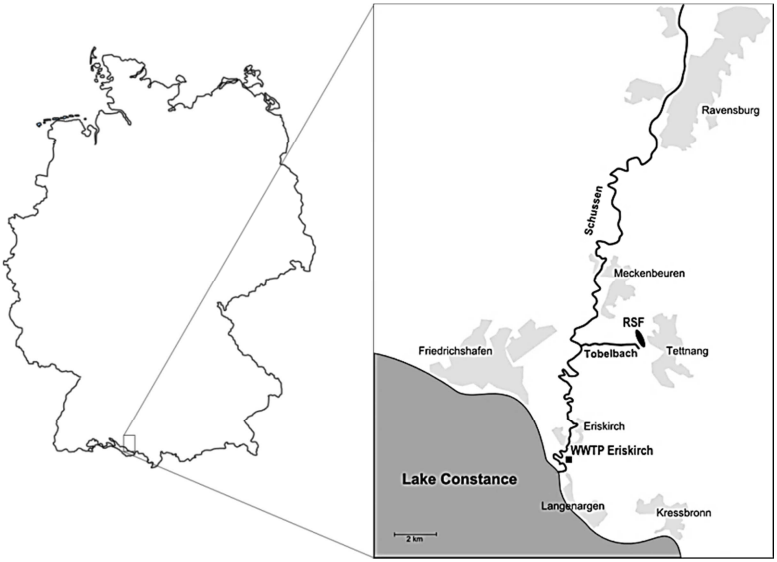

Figure 7.1: Map of Germany and scheme of the study area in the southwestern federal state Baden-Württemberg (by courtesy of Katharina Peschke, University of Tübingen, Germany. The map of the study area is based on OpenStreetMap. Mapdata: ©OpenStreetMap contributors, license: http://opendatacommons.org/licenses/dbcl/1.0/).

7.2 Materials and methods

7.2.1 Study site and sampling protocol

The RSF was put into operation in 2002. It receives the overflow of two SOBs, which serve the city of Tettnang in Baden-Württemberg, Germany. The effluent of the storm water overflow basins passes a cross-flow screen with a gap size of 5 mm. The wastewater solely originates from the two residential catchments of Tettnang (2,450 population equivalents) as the wastewater from the local hospital is

separately discharged to the wastewater treatment plant. The effluent of the RSF is discharged into the Tobelbach, which flows into the Schussen river, one of the main tributaries of Lake Constance. The RSF has a surface area of 2,000 m^2 which is planted with common reed (*Phragmites communis*). The formation of a colmation layer and the risk of surface clogging are reduced by the reed´s stable rootstock growth. At the study site no further measures against clogging are necessary. The rooting and intermittent flooding also enables the penetration of oxygen into the subsurface, which stimulates the microbial activity. Once a year the reed is mown and removed from the filter surface. The filter layer has a thickness of about 80 cm and rests on a gravel drainage system (40-60 cm). At its base the RSF is furnished with a 2 mm non-permeable lining. On average, 40 to 60 impounding events occur per year. In more than one third of the events the influent volume exceeds the capacity of the RSF of 2,000 m^3. Excess wastewater (stormwater) is discharged to the Tobelbach without treatment. The RSF's effluent discharge point is controlled by a throttle valve limiting the outflow to 10 L/s. Complete drainage takes about 60 h. The removal of group parameters, micropollutants, facultative pathogenic and antibiotic resistant bacteria presented here is based on the difference between concentration values determined for the influent of the RSF after SOBs and for the effluent after the throttle valve.

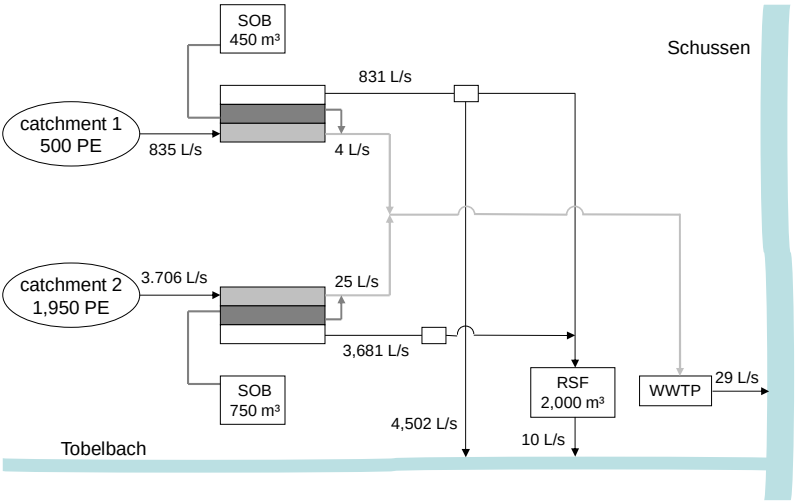

Figure 7.2: Flow diagram of the study site (adopted from Dr.-Ing. Jedele und Partner GmbH, Stuttgart, Germany).

The WWTP in Eriskirch which serves the two catchment areas depicted in Figure 7.2 was used for comparison of treatment efficiencies. This WWTP treats a population equivalent of 50,000 per day and the maximum dryweather flow is 17,300 m³/d. Treatment consists of a screen, sand trap and a primary clarifier, followed bybiological treatment comprised of zones of denitrification and nitrification combined with simultaneous phosphorus elimination. The water is then directed to a secondary sedimentation basin and iron (III) chloride sulfate is added as a precipitating agent. The flocs are removed by filtration through layers of sand and anthracite before the water is discharged into the Schussen river. Results presented here are based on

values determined for the influent of the WWTP after mechanical treatment and for the wastewater after activated sludge treatment.

At the RSF there were five sampling events between June 2012 and November 2013. The WWTP Eriskirch was sampled up to 11 times between May 2012 and August 2014. Though 187 micropollutants were measured, only a small number were defined as indicative parameters based on their nearly ubiquitous presence in WWTP influents and CSO and were measured more frequently. Some more hydrophobic micropollutants like polybrominated diphenyl ethers (PBDE) and polycylic aromatic hydrocarbons (PAHs) were only screened at the RSF site. Furthermore heavy metals and group parameters were measured.

During the sampling events, 250 mL of RSF influent were sampled every 3 min and for the effluent 250 mL were sampled every 10 m^3 until the required total sample volume for all analyses was collected. In case of the WWTP samples, 24 h composite samples were analyzed. All samples were cooled down immediately with icepacks and transported to the laboratories, where they were stored at 4 °C until sample preparation and analysis.

7.2.2 Analysis of micropollutants, heavy metals and water-chemical group parameters

For the analyses of micropollutants water samples were spiked with internal standards prior to extraction. Solid-phase extraction (SPE) or liquid-liquid-extraction (LLE) was used for pre-concentration. Gas chromatography or liquid chromatography were coupled to different

detector techniques ((tandem) mass spectrometry, nitrogen-phosphoros detection, diode array detection). Concentrations of micropollutants, heavy metals and group parameters were analyzed in influent and effluent waters of the RSF. A complete list of all parameters with their respective limit of quantification (LOQ) can be found in the supplementary material (Table S1). Standard methods used for analysis are listed in Table 7.1. Non-Standard methods are described briefly in the text below.

Pharmaceuticals and some of their metabolites, artificial sweeteners, iodinated X-ray contrast agents, benzotriazoles and antibiotics were pre-concentrated by SPE using SDB (J.T.Baker, Philipsburg, USA), Strata-X (Phenomenex, Aschaffenburg, Germany) or PPL Bond Elut (Agilent Technologies, Santa Clara, USA) polymeric adsorbent materials. The very polar antidiabetic drug metformin and its degradation product guanylurea were enriched with a cationic exchange adsorbent material (Strata-X-CW from Phenomenex) as described in Scheurer et al..[12]

Table 7.1: Standard methods used for the analysis of water samples. Non-standard methods are explained in more detail in the text.

Parameter	**Standard method**
Perfluorinated compounds	German standard method DIN 38407-42 [13]
Chelating agents	DIN EN ISO 16588 [14]
Metals	DIN EN ISO 17294-2 [15]
Chemical oxygen demand (COD)	ISO 6060 [16]
Total organic carbon (TOC) and dissolved organic carbon (DOC)	DIN EN 1484 [17]
Spectral absorption coefficient	German standard method DIN 38404-3 (C3) [18]

Sample pH, the water volume used for pre-concentration, elution solvents and the established liquid chromatographic methods were optimized for each substance group. The analytes were quantified using a 1290 HPLC system (Agilent Technologies) coupled to an API 5500 mass spectrometer (AB Sciex, Framingham, USA).

Polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs) and chlorinated insecticides were extracted by LLE with 20 mL cyclohexane. The organic solvent was evaporated to a final volume of about 0.5 mL and the compounds were measured by gas chromatography coupled to a tandem mass spectrometer (GC/MS/MS).

PBDEs were extracted with 25 mL cyclohexane. Residual water was removed with a sodium sulfate filled cartridge. The extract was evaporated to a volume of 0.2 mL and measured with GC/MS with negative chemical ionization.

Trialkylphosphates were enriched with a polymeric adsorbent (SDB) and cartridges were eluted with dichlormethane. GC/MS-MS was performed for separation and quantification using a TRACE GC Ultra gas chromatograph coupled to a TSQ Quantum XLS Ultra mass spectrometer (both Thermo Scientific, Waltham, USA).

For the SPE of phthalates self-packed glass SPE cartridges filled with Chromabond C18 Hydra material (Macherey Nagel, Düren, Germany) were used. Phthalates were analyzed using a Autosystem XL GC coupled to a Turbo Mass Gold MS (both Perkin Elmer, Waltham, USA).

Endocrine disrupting chemicals were also pre-concentrated by SPE with a polymeric adsorbent material (Strata-X from Phenomenex, Aschaffenburg, Germany). After elution of the analytes with acetone the extracts were evaporated to dryness and reconstituted with a derivatization mixture (MSTFA (N-methyl-N-(trimethylsilyl) trifluoroacetamide, trimethylchlorosilane and pyridine). After silylation (80 °C for 45 min) a keeper was added and the derivatization reagent was removed in a gentle stream of nitrogen. The residue was reconstituted in cyclohexane and measured by a Trace GC TSQ Quantum XLS Ultra GC-MS/MS (Thermo Scientific).

Pesticides were enriched using 1 g IST Isolute C18 adsorbent (Biotage, Uppsala, Sweden) and analyzed after elution with acetone by GC-MS using a 6890 5973 GC-MS system (Agilent Technologies).

Aliphatic amines were derivatized in the water sample with fluorenylmethyloxycarbonyl chloride (FMOC) and pre-concentrated using 200 mg LiChrolut EN adsorbent material (Merck, Darmstadt,

Germany). Measurements were performed with LC coupled to a fluorescence detector (both Agilent Technologies).

7.2.3 Analysis of microbiological parameters - Enumeration, isolation and identification of *E. coli*, enterococci and staphylococci

Depending on the probable contamination level, either the membrane filtration method (according to ISO 7704H) or direct plating for *E. coli*, enterococci and staphylococci was used.[19] *E. coli* were grown on ECD-agar (Merck, Darmstadt, Germany). After incubation at 37 °C for 20-24 h, under UV light blue fluorescent colonies on the membrane (cellulose nitrate, pore size 0.45 µm, Ø 50 mm (Sartorius, Göttingen, Germany)) or on the agar surface (resulting from the beta-glucuronidase activity that hydrolyzes MUG present in the media) were counted as *E. coli*. Presumptive *E. coli* isolates were further tested for tryptophanase activity with Kovac´s reagent.[20]

For *Enterococcus spec.*, membrane filters with appropriate dilutions were incubated on azide nutrient pads (Sartorius) for 40-48 h at 37 °C according to Slanetz and Bartley, followed by incubation on kanamycin-aesculin-agar (Merck) for 1 h at 44 °C.[21] Red, pink and reddish brown colored colonies with positive aesculin reaction were counted based on ISO 7899-2.[22]

For analysis of staphylococci Chapman-Stone agar containing 0.05 g/L sodium azide was used. After incubation for 48 h at 37 °C, colonies were counted and streaked on Mannitol-Salt agar.

For each sampling event, randomly selected colonies of the genera *Enterococcus* and *Staphylococcus* were selected for identification on species-level by their physiological reactions on Micronaut-Staph®- and Micronaut-Strep2®-microtiter plates (MERLIN Diagnostika GmbH, Bornheim-Hersel, Germany) according to manufacturer's instructions (for details: http://www.merlin-diagnostika.de/micronaut-identifizierung.html).

7.2.4 Antibiotic susceptibility testing

The agar diffusion test according to the German standard method DIN 58940 was performed with 50 enterococci to test the antibiotics ampicillin (10 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), erythromycin (15 µg).[23] To detect vancomycin resistant *E. faecium* and *E. faecalis* (VRE), the susceptibility of these isolates against vancomycin was tested as described by the Clinical and Laboratory Standards Institute (CLSI).[24] Intrinsic low-level vancomycin resistance of *E. gallinarum* and *E. casseliflavus* (vanC1- and vanC2-type) were not considered.

Susceptibility of 76 *E. coli* isolates was tested against the antibiotics ampicillin (10 µg), ciprofloxacin (5 µg), cotrimoxazol (1.25 µg trimethoprim / 23.75 µg sulfamethoxazol) with agar diffusion tests according to DIN 58940 and against cefotaxim (5 µg) using the clinical breakpoints listed by the European Committee on Antimicrobial Susceptibility Testing (EUCAST).[23,25] All ampicillin resistant isolates were additionally tested against ceftazidim (30 µg) and cefpodoxim

(10 µg) according to CLSI;[24] the inhibitory effect of clavulanic acid on the ß-lactamase was checked as described by Bradford.[26] According to the definition of the Robert-Koch-Institute, extended-spectrum-ß-lactamase producer phenotypically showed resistance against cefpodoxim as well as ceftazidim and/or cefotaxim and the inhibition of the enzyme by clavulanic acid.

Susceptibility of the 144 obtained *Staphylococcus* isolates against oxacillin (5 µg), ciprofloxacin (5 µg) and erythromycin (15 µg) was tested by the disc-diffusion test with Mueller-Hinton agar according to DIN 58940 and to clindamycin (2 µg) according to CLSI.[23,24]

7.2.5 Data analysis

Changes in the number of living *E. coli*, enterococci and staphylococci and in the percentages of antibiotic resistant isolates during passing the RSF were tested respective their statistical significance using bilateral t-test. Bacterial numbers represent the median values of five parallels. Removal of micropollutants was determined by calculating the average removal of all single sampling events. The variability of the data is expressed by the standard deviation. In the case of effluent values below the LOQ, the value half of the LOQ was used for calculation. Data are only presented if for at least half of the sampling events concentrations above the LOQ were measured.

7.3 Results and discussion

In the years 2012 and 2013, the volume of CSO treated in the RSF per month varied in a wide range between less than 100 m³ up to more than 12,000 m³. The same holds for the number of days when a measureable amount of drainage could be observed in the RSF´s effluent. The total number of drainage events at the RSF in Tettnang can be considered as rather high. A survey by Roth-Kleyer et al. showed that almost 60 % of RSFs in Germany had less than twelve events per year.[10] However, it has to be pointed out that such small numbers of events have to be regarded as contradictory to the establishment of reed vegetation. The intermittent loading of RSFs is important for the aeration of the subsurface. It has been demonstrated that sufficient oxygen supply in the soil filter enhances the removal of COD, ammonia and total nitrogen and that other factors like vegetation or temperature variations between 2 °C and 20 °C had only little effect on removal rates.[27]

7.3.1 Group parameters

DOC represents the total carbon concentration of all dissolved organic compounds. It is especially important for the depletion of oxygen and thus affects prevailing redox conditions and the degradation of inorganic nitrogen compounds like ammonia. The UV absorption of dissolved organic matter is measured by the parameter $SAC_{254\,nm}$. As mainly unsaturated organic compounds absorb radiation at 254 nm, the parameter is used as a proxy for aromatic dissolved organic matter. Both, DOC and $SAC_{254\,nm}$ can be used to calculate the specific UV absorption (SUVA) coefficient ($SAC_{254\,nm}$ divided by DOC). A high

SUVA indicates a high portion of aromatic compounds in the water. The good DOC-removal of more than 80 % (Figure 7.3) during a short residence time in the filter reflects a high portion of readily biodegradable dissolved organic matter in the influent of the filter. The average DOC concentration in the RSF influent of 6.9 mg/L is in a concentration range typically measured in the effluent of municipal WWTPs, where readily biodegradable DOC has already been assimilated during activated sludge treatment. The $SAC_{254\,nm}$ is reduced in the RSF by about 50 %. The more pronounced reduction of the DOC relative to the $SAC_{254\,nm}$ is expressed by an increase of the SUVA from 1.7 L/(mg*m) to 3.1 L/(mg*m). Therefore, it can be concluded that less UV light absorbing saturated organic compounds are preferentially degraded in the RSF.

In similar subsurface treatment processes like soil aquifer treatment (SAT) the same observations have been made.[28,29] For a SAT field in Israel a preferential degradation of non-aromatic organic compounds was reported as an increase of SUVA from the percolation basin to a vertically located observation well (detention time 1.5 months) was observed.[30] In contrast to the $SAC_{254\,nm}$, the $SSC_{254\,nm}$ takes the scattering of light due to suspended matter into account.[31] As expected, a higher removal rate of $SSC_{254\,nm}$ in the RSF Tettnang could be observed (Figure 7.3) due the removal of suspended particles in the RSF by physical filtration processes. Higher removal rates of TOC compared to DOC and the pronounced improvement in turbidity can also be attributed to the removal of suspended solids by the RSF.

The COD removal of 80±10 % is in the range found in literature for other RSFs in Germany. Mean removal rates from 40 % up to more than 90 % have been reported.[4,32]

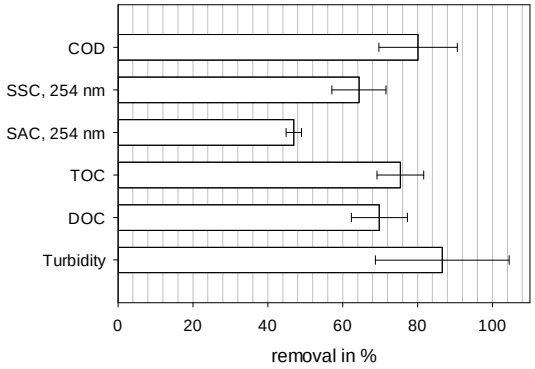

Figure 7.3: Mean removal of physical-chemical and group parameters by RSF treatment. Error bars represent standard deviation.

7.3.2 Micropollutants

Most of the 187 micropollutants analyzed were not detected in the RSF influent or effluent on a regular basis. Micropollutants with wastewater origin are diluted by storm water and consequently if present their concentrations often decreased below the respective LOQ.

Pharmaceuticals and their metabolites

From a total of 79 pharmaceuticals (including antibiotics and some metabolites and excluding X-ray contrast media) only 24 were regularly found in more than half of the WWTP influent samples analyzed within

this study. Only nine out of those 24 compounds were detected in the RSF influent in more than 50 % of the samples. Generally, the pharmaceutical residues with the highest concentrations in the WWTP influent were also detected in the RSF influent and are depicted in Figure 4. Pharmaceuticals additionally found in the influent of the WWTP Eriskirch belonged to several therapeutic classes with betablockers (atenolol, bisoprolol, sotalol) and antibiotics (clarithromycin, sulfamethoxazol, trimethoprim, ciprofloxacin, ofloxacin) as the most prevailing compound groups.

For most pharmaceuticals removal efficiencies were similar for activated sludge treatment in the WWTP and in the RSF. Pharmaceutically active compounds which are commonly readily biodegradable in WWTPs were also removed in the RSF to a high degree. Examples are paracetamol (98 %) and ibuprofen (94 %). For the latter substance good removal from 0.5 µg/L to a concentration below the LOQ has been reported for another RSF site.[33]

For diclofenac, metoprolol and 10,11-dihydro-10,11-dihydroxycarbamazepine, a metabolite of the antiepileptic drug carbamazepine, removal rates were slightly higher than in the WWTP. Varying removal rates have been reported for diclofenac.[34-37] The fact that diclofenac is not readily biodegradable is attributed to its chlorine group. Wastewater characteristics as well as treatment conditions also influence removal rates in WWTPs.[37] The mean removal of diclofenac in the WWTP Eriskirch was 14 % (n = 11) after activated sludge treatment and 37 % post sand filter at the end of the treatment train, whereas the RSF removed 89 % on average (all effluent values <LOQ).

This is in agreement with good diclofenac removal rates reported for other RSFs. In Altendorf (Germany) the mean concentration of diclofenac (0.14 µg/L) was reduced to below the LOQ.[33] Tondera et al reported a diclofenac removal of more than 70 % for two RSF in North-Rhine-Westphalia (Germany).[9,38]

The reported elimination for metoprolol in WWTPs is also highly inconsistent and ranges between 0 % and more than 80 %.[36,39-41] Sorption in water-sediment systems revealed negligible sorption tendency for metoprolol.[42] The removal of 36 % and 84 % in the WWTP Eriskirch and the RSF Tettnang, respectively should be attributed mainly to biodegradation although metoprolol is present in its cationic form at environmental pH values and might also be retained by negatively charged solid particles.

The negative elimination of carbamazepine (see Figure 7.4) can be presumably explained by the cleavage of glucuronide conjugates, which are formed in the human body, back to carbamazepine. The fact that carbamazepine is not removed at all may reflect the prevailing redox conditions in the RSF, as the compound is persistent in an aerobic environment but can be degraded under anaerobic conditions.[43]

For the antidiabetic drug metformin concentrations in WWTP influents up to 100 µg/L have been reported and as a rule removal rates are higher than 90 %.[12,44] This was also the case for the WWTP Eriskirch, but due to high influent concentrations still several hundred ng/L could be detected after biological wastewater treatment. However, as for most micropollutants no complete mineralization takes place during biological treatment, but degradation products are formed. In the case of

metformin, transformation into guanylurea (diaminomethylideneurea) takes place.[44,45] Consequently, a maximum concentration of 96 µg/L guanylurea was measured in the effluent of the WWTP Eriskirch. Removal of metformin was high and comparable to the WWTP Eriskirch, but again traces of metformin were detected in the RSF effluent. Interestingly, no guanlyurea was detected in the effluent of the RSF. The residence time and biological activity of the RSF appear to be sufficient to degrade or retain both metformin and its transformation product and therefore, the RSF has a better performance compared to conventional activated sludge treatment in WWTPs.

Benzotriazoles

The corrosion inhibitor 1H-benzotriazole is used in dishwashing liquids, anti-freezing and deicing fluids. Its elimination in the WWTP Eriskirch was incomplete (mean value 42 %) and an average value of 5.1 µg/L could still be measured in the treated wastewater. This is in accordance with the incomplete removal reported in literature, where effluent concentrations of several µg/L were detected.[46-48] The mean removal of 1H-benzotriazole in the RSF was better (66 ± 20 %) but not significantly different. The compound was found in production wells at river bank filtration sites after a residence of several months in the saturated subsurface.[49] However, sorption onto soils is negligible[50] and the decrease in concentration during RSF passage can most likely be attributed to a slow biodegradation. Greater persistence in aquatic environments has been reported for the 4-methyl analogue 4-methylbenzotriazole.[48] A decreasing 1H-benzotriazole/4-methylbenzotriazole ratio with increasing residence time was observed

in the partially closed water cycle in Berlin, Germany.[49] In comparison to 1H-benzotriazole, the removal for 4-methylbenzotriazole was lower at both sites, the WWTP and the RSF.

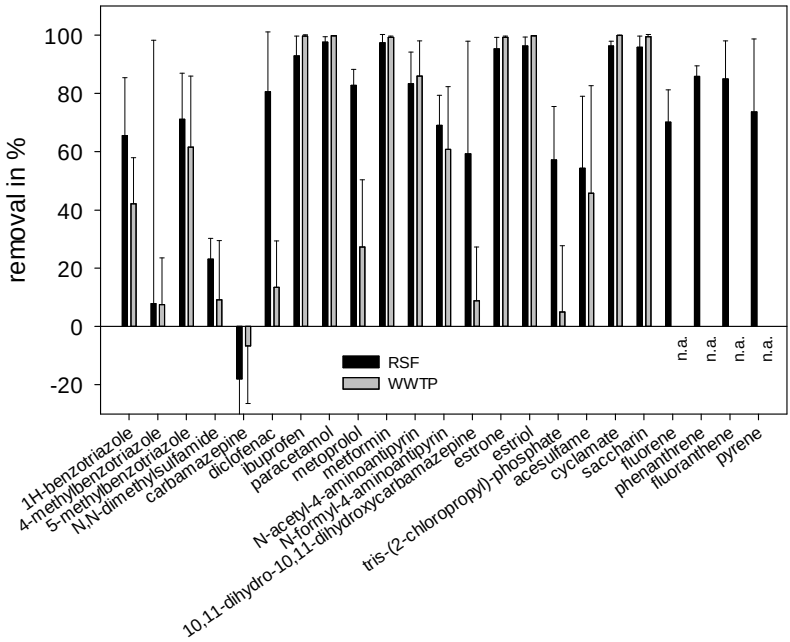

Figure 7.4: Comparison of removal rates of organic trace pollutants in a retention soil filter and a wastewater treatment plant after activated sludge treatment, n.a. = not analyzed. Error bars represent standard deviation of removal.

Estrogens

The main sources for estrogenic activity in wastewater are natural hormones and synthetic compounds like 17α-ethinylestradiol (EE2) and,

to a smaller extend, alkylphenols. The removal of such compounds during conventional wastewater treatment is usually good, but often incomplete. Both, biodegradation and sorption onto sludge contribute to the elimination of these compounds.[51] A good compilation of estrogenic compounds, their effects and fate during wastewater treatment can be found in Teske and Arnold.[52] Estrone (E1), 17β-estradiol (E2) and estriol (E3) were detected in every influent sample of the WWTP Eriskirch with mean concentrations of 140 ± 130 ng/L, 37 ± 16 ng/L and 180 ± 48 ng/L, respectively. The removal during wastewater treatment was in almost all cases complete and only once E1 was detected with 2.1 ng/L above the limit of quantification (LOQ = 0.5 ng/L) after activated sludge treatment. Only E1 and E3 were detected on a regular base in the RSF influent but due to dilution with lower concentrations of 22 ± 19 ng/L and 25 ±17 ng/L. In Figure 4 the removal of both compounds appears to be slightly lower than in the WWTP, but concentrations were lower than the detection limit. The apparent lower removal rate is simply due to lower influent concentrations, which were compared with the same effluent values as above (half of the LOQ).

Artificial sweeteners

For the artificial sweeteners saccharin and cyclamate, good removals were observed for both the WWTP and RSF. This is in accordance with high removal rates for both compounds during wastewater treatment reported in literature.[53] Acesulfame was retained with a mean value of 46 % in the WWTP Eriskirch. However, the removal rate varied over a wide range from -5 % to 90 % with no obvious trend or seasonal correlation like it was reported for some pharmaceuticals in the past.[54]

The results are against expectation as in previous publications acesulfame was reported to be recalcitrant and was only poorly removed in WWTPs.[53,55] An adaptation of microorganisms cannot be the only reason for the degradation as after sampling events with high removal a pronounced stability of acesulfame was observed again. However, recent studies have questioned the compound´s stability and report a partial degradation in certain environmental compartments like river bank filtration sites.[56] The reason for the heterogeneous stability of acesulfame is yet unknown. Tran et al. reported a poor removal of acesulfame in batch tests with nitrifying sludge, but removal was increased with increasing ammonium concentrations.[57] Maximum acesulfame concentrations after activated sludge treatment or in the effluents of the RSF were 19 µg/L and 1.3 µg/L respectively and thus among the highest values observed for micropollutants.

Antibiotics

Within this study 37 antibiotics comprising different classes (macrolides, tetracyclines, penicillins, sulfonamides, fluoroquinolones) were analyzed. Only two of them (sulfamethoxazole and trimethoprim) were detected once in the influent of RSF Tettnang with a maximum concentration of 0.12 µg/L for sulfamethoxazole. Beside these two antibiotics azithromycin, clarithromycin, roxithromycin, ciprofloxacin and ofloxacin were detected in the influent of the WWTP. With two exceptions the concentration levels were below 1 µg/L, but all antibiotics mentioned before could be found at least once above the LOQ after activated sludge treatment. No conclusions about the RSF performance in terms of the removal of antibiotics can be drawn as

values below LOQ can be a matter of dilution and are no proof for the complete absence of a certain compound. Assuming similar elimination processes and removal rates in the WWTP and the RSF, traces of antibiotics might end up in receiving waters. Other studies have pointed out that presence and long-term exposure even to rather low concentrations of antibiotics might be a reason for the selection of resistant bacteria and their spreading in the environment.[60,61]

The source of a compound is an important factor for its presence in combined sewers. If compounds which derive mainly from domestic wastewater are biodegradable, their absolute amount released to the environment will increase due to the absence of treatment of CSO overflow. However, there will be no change in the absolute amount of recalcitrant compounds.

In contrast, the absolute amount of micropollutants which are only re-mobilized, washed off or introduced by surface runoff during rain events will increase in any case.[62] Biodegradable micropollutants are discharged by CSO overflow, while recalcitrant substances are released by both CSO overflow and WWTP effluent to receiving waters.

One example for the appearance of a micropollutant in runoff is mecoprop which is used as pesticide but also as biocide, e.g. in roof sealings. As a consequence it can be washed off and released into the environment during rainfall events.[63] The compound was not detected in samples from the WWTP influent in Eriskirch. In contrast, in two RSF influent samples mecoprop was measured with a maximum concentration of 1.4 µg/L. However, mecoprop was efficiently removed during RSF passage to a final concentration below the LOQ.

Another compound group typical for the phenomenon of remobilization during rainfall events are polycyclic aromatic hydrocarbons (PAHs). Since their sorption tendency to particulate matter is high, it is of special importance for their elimination that the concentration of solids is reduced to a minimum during treatment. Filterable solids were reduced by more than 97 % in the RSF Tettnang. A 93 % retention was reported by Tondera et al. for a RSF six years in operation.[38] PAHs were analyzed only for the RSF site. When detected in the RSF influent removal efficiency was between 70-90 %.

In summary the performance of the RSF for the removal of micropollutants can be most likely attributed to a combination of sorption and biodegradation. Compounds not discussed above were not detected in the RSF influent or the calculation of their removal rate was not feasible due to the lack of frequent detection.

7.3.3 Removal of microorganisms

Concentrations of all microorganisms studied were significantly lower in the RSF effluent than in the influent (Figure 7.5). Removal rates were variable but comparable with those in WWTPs. The number of *E. coli* und enterococci decreased by 2.7 (median value; values for individual samples ranged from 2.1 - 3.2) and 2.2 log-units (median value; values for individual samples ranged from 0.9 - 2.8), respectively. The maximum elimination achieved was in a similar range as reported for other constructed wetlands planted with *Phragmites* spec..[33,64,65] The median elimination rate of fecal indicator bacteria (FIB) exceeded the

observations made by Tondera et al., who observed removal rates of 1.1 log-units for *E. coli* and enterococci.[9] Comparing the number of staphylococci in the influent and effluent of the RSF, their number significantly decreased by about 2.4 log-units (median value; values for individual samples ranged from 2.2 - 3.0 log-units). Faria et al. reported a removal of about 2 log-units in a conventional activated sludge treatment plant.[66] A higher retention of up to 4 log-units (median value) was detected by Heß and Gallert.[67] Comparing elimination efficiencies of WWTP and RSF, it has to be considered that the cell count of staphylococci was already lower in the influent of the RSF (10^3 CFU/100 mL, median value) compared to the influent of a WWTP (Faria et al.: 10^5 CFU/100 mL; Heß and Gallert: 2.7 x 10^4 CFU/100 mL (median value)).[66,67] In consequence, the removal expressed in log-decades has to be lower. Remarkably, the concentration of staphylococci in the effluent of the RSF (median value: 3.8 CFU/100 mL) was in the same order of magnitude or lower (Faria et al.: 10^2 CFU/100 mL; Heß and Gallert: 2.3 CFU/100 mL)[66,67] compared to the WWTP effluents.

Factors like retention time, characteristics of the filter material, organic matter concentration and biocenosis composition differ from site to site, however many authors suggested predation in the colmation layer or the subsurface of the RSF and adsorption as main factors for the retention of bacteria in RSFs.[65,68,69] Bacteria and viruses are attached to solids or in the free water phase[9] and like for hydrophobic micropollutants which are preferentially bound to particles, Passerat et al. observed a first-flush effect for *E. coli* in a CSO discharge caused by particle attached cells.[70] Based on the analysis of TOC and SSC, a considerable removal of suspended matter can be assumed and at least partial removal of bacteria

attached to particles by filtration is likely. However, Tondera et al. observed no correlation between the removal of total suspended solids and the removal of *E. coli* or enterococci.[9] Key factors and mechanisms governing removal processes comprise a complex combination of chemical, physical and biological factors, which was not accounted for in this study and should be investigated by further *in situ* studies. In this context, it would also be interesting to perform long-term experiments investigating whether the RSF sediment is likely to change from sink to source.

Nola et al. studied different soil columns with regard to their retention efficiencies.[71] The removal of staphylococci was in the order of 99.99 %. Comparing their results with those of the RSF in Tettnang is difficult because of higher staphylococci concentrations in the influent of the columns (2.9×10^7 CFU/100 mL; RSF, median value: 10^3 CFU/100 mL) with a retention efficiency of 6.7 log-units (mean value) and different construction and operating factors. Overall, the efficiency of the retention of particles depends on the construction and the operating factors of the RSF. Sidrach-Cardona and Bécares identified a subsurface-flow constructed wetland planted with *Phragmites* spec. as the best combination among several types of construction and reported a removal of total coliforms, *E. coli* and *Enterococcus* spec. between 2.5 and 3 log-units (mean value).[65] The construction of the treatment facility and the removal efficiency are similar to the results obtained in this study.

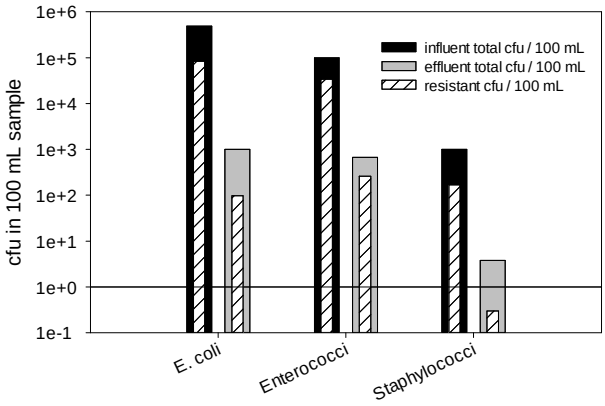

Figure 7.5: Colony forming units (cfu) (median values) of total and resistant *E. coli*, enterococci and staphylococci per 100 mL in the influent and effluent of the RSF Tettnang

7.3.4 Species diversity of staphylococci

Comparing the diversity of staphylococci obtained from the influent (71 isolates) and the effluent (73 isolates) of the RSF, a shift of the abundance of the different species can be observed: Whereas the percentage of isolates belonging to the Sciuri-group decreased from 59.3 % in the influent to 36.0 % in the effluent, the percentage of isolates clustered to the Saprophyticus-group remained stable (influent: 37.3 %; effluent: 36.0 %). However, within the Saprophyticus-group the abundance of the respective species changed: the percentage of *Staphylococcus xylosus* on isolates belonging to the Saprophyticus-group increased from 10.9 % in the influent to 66.7 % in the effluent. Obviously, some *Staphylococcus*-species can better adapt to the

conditions of the RSF than others. Coinciding with Faria et al., Heß and Gallert reported that species of the Saprophyticus-group were dominant in raw sewage (Faria et al.: 78.7 % of the isolates were representatives of the mentioned group; Heß and Gallert: 51.6 % of the isolates were species of the Saprophyticus-group and 31.8 % of them were identified as *S. xylosus*).[66,67] Furthermore, the study of Heß and Gallert showed that species of the Sciuri-group were more abundant in river water than in sewage and the percentage of *S. saprophyticus* ssp. *saprophyticus* drecreased in favor of *S. xylosus* in receiving water bodies.[67] Using the percentage of isolates belonging to the Sciuri-group as well as the percentage of *S. xylosus* on the abundance of isolates belonging to the Saprophyticus-group as "less-sewage derived" markers, a further characterization of the influent and the effluent of the RSF is possible: Based on diversity and abundance of the staphylococci, the influent of the RSF was a mixture of raw sewage diluted with a significant volume of surface runoff. The *Staphylococcus*-species diversity found in the effluent of the RSF was dominated by species rather associated with river water indicating that these species are better adapted to the condition of the RSF.

7.3.5 Antibiotic resistant *E. coli*, enterococci and staphylococci

The percentage of antibiotic resistant *E. coli* isolates (35 isolates obtained from the influent and 41 strains obtained from the effluent were tested) significantly decreased during passing the RSF from 17 % in the influent to 10 % in the effluent (level of significance 0.05). If an isolate was resistant, it was resistant against ampicillin. In the influent two

isolates were additionally resistant against cotrimoxazole, one of them also against cefotaxim classifying it as an extended spectrum β-lactamase (ESBL) producer. The fact that ESBL-producers can be detected by culture based approaches points out that they are present in respective numbers in sewage. In the effluent, one ampicillin resistant *E. coli* was additionally resistant to cotrimoxazole. Luczkiewicz et al. found a comparable resistance pattern for *E. coli* isolated from a municipal wastewater treatment plant, but overall the resistance level was lower.[72]

Similar to *E. coli*, the percentage of antibiotic resistant staphylococci (71 isolates obtained from the influent and 73 strains obtained from the effluent were tested) was significantly lower in the effluent of the RSF (6.8 %) than in the influent (16.9 %, level of significance 0.05). The percentage of isolates resistant to erythromycin determined the resistance level. None of the obtained staphylococci was constitutively resistant against clindamycin and/or ciprofloxacin. The fact that 5 isolates obtained from the influent were resistant against oxacillin showed that the antibiotic resistance gene *mec*A is present in sewage. The observed resistance pattern was comparable to that described for raw sewage,[66,67] but the overall percentage of resistant isolates was lower. A possible explanation for this observation is based on the species diversity. Some species were more frequently resistant than others: for instance, 24.7 % of the *S. saprophyticus* ssp. *saprophyticus* isolates, a species dominating in raw sewage, was resistant against one of the tested antibiotics, whereas “only” 10.8 % of *S. xylosus* isolates, which rather could be isolated from river water, was resistant.[67] Consequently, based on the found resistance levels of *E. coli* and

staphylococci as well as on the described *Staphylococcus*-species diversity, the influent of the RSF cannot be directly compared to the influent of a WWTP under dry weather conditions.

The percentage of resistant enterococci (32 isolates obtained from the influent and 18 strains obtained from the effluent were tested) did not significantly change during passing the RSF (influent: 34.4 %; effluent: 38.9 %). This observation might be an effect of the small number of isolates; a higher number is needed to confirm this result. Erythromycin resistance determined the resistance level of enterococci in the respective sample. Two isolates in the influent and one isolate obtained from the effluent were additionally resistant against ciprofloxacin. One *Enterococcus* isolate of the effluent was resistant against the last resort antibiotic chloramphenicol. None of the obtained isolates were high-level resistant against vancomycin and/or ampicillin. Luczekiewicz et al., who isolated enterococci from sewage of a municipal WWTP, also found the described resistance pattern, but as already seen for *E. coli* and staphylococci the overall percentage of resistant isolates was lower.[72]

Even if the resistance level of enterococci did not decrease, the absolute concentrations of antibiotic resistant *E. coli*, enterococci and staphylococci in the effluent of the RSF were about 2.1 and 2.9 log-units lower than in the influent. Nevertheless, antibiotic resistant bacteria and antibiotic resistance genes were released into the receiving Tobelbach. At the moment, the remaining risk and the effect on autochthonous microorganisms in receiving waters cannot be predicted. Some authors discussed the long hydraulic-retention time as well as the exposure to low (not inhibitory) concentrations of different antibiotics as promoting

factors for the spread of antibiotic resistance genes.[60,61] Comparing the detected resistance level of the influent and the effluent of the RSF in Tettnang, there were no indices at present for the spread of resistance determinants.

7.4 Conclusions

The effectiveness of a retention soil filter (RSF) for the removal of micropollutants, facultative pathogenic and antibiotic resistant bacteria from CSO was investigated. The removal efficiency for micropollutans was comparable to activated sludge treatment in a nearby WWTP. WWTP effluent still is an important source of water pollution with micropollutants. The conventional treatment in WWTPs is especially important for biodegradable mircopollutants or compounds which tend to adsorb onto suspended and settleable solids. First measures for upgrading WWTPs with ozonation or activated carbon treatment units on a full-scale are taken and will improve the overall removal efficiency. However, the whole treatment train is only successfully applied during dry weather conditions. Therefore, a complementary treatment option with an active microbial environment and a reliable adsorption capacity is needed for events of heavy rainfall, when WWTPs and sewage overflow basins cannot cope with the additional amount of water. The results of this study support the extension of the RSF technology as a low cost addition to overflowing combined sewer overflow basins to control the discharge of standard pollutants and micropollutants, and the number of facultative pathogenic and antibiotic resistant bacteria. Detailed investigation of the subsurface of full-scale RSFs should be

conducted for a better understanding of the basic processes involved in the removal of micropollutants, facultative pathogenic and antibiotic resistant bacteria.

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8. Konzentration und Resistenzniveau fakultativ pathogener und Antibiotika-resistenter Keime in der durch Einleitung von gereinigtem Abwasser belasteten Schussen

Um den Effekt der Einleitung von (gereinigtem) Abwasser auf das aquatische Ökosystem zu untersuchen, um dann beurteilen zu können, inwieweit durch zusätzliche Abwasserreinigungsstufen, wie sie beispielsweise in Kapitel 5 und 7 beschrieben wurden, dieser verringert werden kann, ist es wichtig, das Oberflächengewässer, in das die Kläranlagenabläufe abgeschlagen werden, zu charakterisieren. Deshalb werden in diesem Kapitel die (noch unveröffentlichten) Ergebnisse bezüglich der Konzentration an fakultativ pathogenen Keimen sowie deren Resistenzniveau beschrieben.

8.1 Mikrobiologische Charakterisierung der Schussen

Vier Probenahmestellen an der Schussen und eine Referenzstelle an der Argen (Abb. 2.2) wurden innerhalb der dreijährigen Projektlaufzeit achtmal zu unterschiedlichen Jahreszeiten und hydrologischen Verhältnissen beprobt und die Konzentration von *E. coli*, Enterokokken und Staphylokokken sowie deren Resistenzverhalten bestimmt.

8.1.1 *E. coli*-, Enterokokken- und Staphylokokken-Konzentrationen in der Schussen

Die nachfolgend abgebildeten box-plot-Diagramme (Abb. 8.1) zeigen, dass die Konzentrationen abhängig von den hydrologischen Verhältnissen, innerhalb einer log-Stufe schwankten, aber sich weder die Gesamtkeimkonzentration noch die Konzentration fakultativ pathogener *E. coli*, Enterokokken und Staphylokokken entlang der Fließstrecke durch die Einleitung von Kläranlagenabläufen aufsummierte.

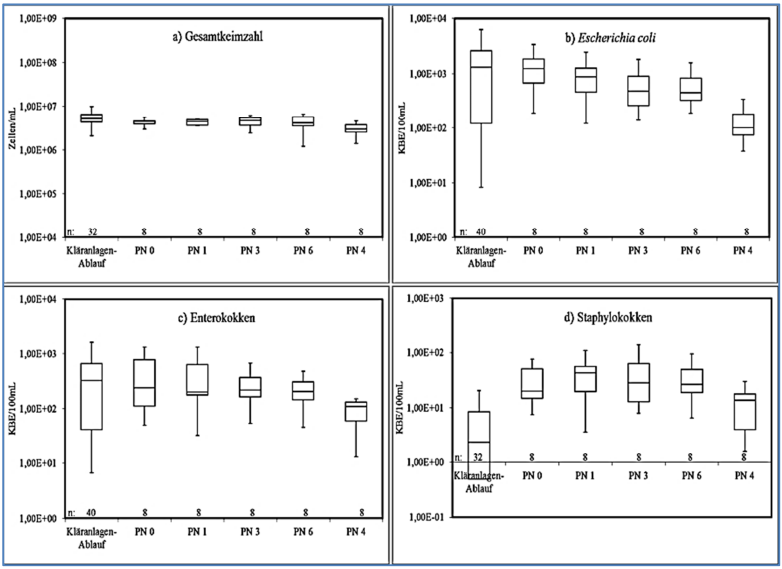

Abb. 8.1. Konzentrationen von Gesamtkeimen (a), *Escherichia coli* (b), Enterokokken (c) und Staphylokokken (d) an den einzelnen Probenahmestellen der Schussen (PN 0 bis zur Schussenmündung in den Bodensse PN 6) und Argen (PN 4) sowie im Kläranlagenablauf. (n: Anzahl der Probenahmen; die Konzentrationen von *E. coli* und Enterokokken wurden vom Institut für Seenforschung (LUBW) in Langenargen bestimmt)

Im Vergleich mit den dem fluviatilen Ökosystem zugeschlagenen Konzentrationen in den Kläranlagenabläufen lag die Konzentration von *E. coli* und Enterokokken in der Schussen in der gleichen Größenordnung, was in gewisser Weise das Konzept der fäkalen Indikatororganismen bestätigt. Die Konzentration der Staphylokokken lag im Kläranlagenablauf etwa eine log-Stufe unter der Konzentration im Freiland, was den Schluss zulässt, dass entweder eine andere Eintragsquelle existiert oder Staphylokokken, zumindest manche Spezies, natürlicherweise im fluviatilen Ökosystem vorkommen und sich dort auch vermehren können. Die Konzentrationen von *E. coli*, Enterokokken und Staphylokokken in der Argen waren 0,4 bis 0,7 log-Stufen geringer als die in der Schussen. Bei dieser Betrachtung bleibt allerdings zu berücksichtigen, dass der mittlere Abfluss der Argen mit 20,1 m^3/s fast doppelt so hoch ist wie der der Schussen (MQ: 11,8 m^3/s; Auerbach et al., 2009). Wenn man den Median der Konzentrationen auf den mittleren Abfluss projiziert, sieht man, dass die Frachten an fakultativ pathogenen Keimen, welche die Schussen und die Argen in den Bodensee eintrugen, in der gleichen Größenordnung lagen (Tab. 8.1; da die Argen nicht an ihrer Mündung in den Bodensee beprobt wurde, wurde für diese Betrachtung Probenahmestelle 4 herangezogen).

Tab. 8.1: Vergleich der Frachten von Schussen und Argen

	Fracht in KBE/s			
	Gesamtkeimzahl	*E. coli*	Enterokokken	Staphylokokken
Schussen (PN 6)	$5{,}0 \times 10^{13}$	$5{,}3 \times 10^{7}$	$2{,}4 \times 10^{7}$	$3{,}2 \times 10^{6}$
Argen (PN 4)	$6{,}0 \times 10^{13}$	$2{,}0 \times 10^{7}$	$2{,}2 \times 10^{7}$	$2{,}8 \times 10^{6}$

(KBE: Kolonie bildende Einheiten)

8.1.2 Speziesverteilung der Staphylokokken im Freiland

Die Speziesverteilungen der Staphylokokken waren an den 4 Probenahmenstellen entlang der beprobten Fließstrecke der Schussen sehr ähnlich (Abb. 8.2). Es dominierten Spezies, die in die Sciuri- und Saprophyticus-Gruppe gehören, während klinisch relevante Spezies, wie *Staphylococcus aureus*, *S. epidermidis*, *S. hominis* und *S. haemolyticus*, nur sporadisch isoliert wurden. Im Vergleich zum Kläranlagenablauf war der Anteil an Isolaten der Sciuri-Gruppe in der Schussen zu Lasten des Anteils an Mitgliedern der Saprophyticus-Gruppe höher. Innerhalb der Saprophyticus-Gruppe fand eine Verschiebung der Abundanzen der einzelnen Spezies zwischen Kläranlagenablauf und fluviatilem Ökosystem statt: Während im Kläranlagen-Ablauf *S. saprophyticus* ssp. *saprophyticus* die dominante Spezies innerhalb der Isolate, die in die Saprophyticus-Gruppe gehörten, darstellte, war im Freiland der Anteil an *S. xylosus*-Isolaten deutlich höher. In Kapitel 7 wurde schon erwähnt, dass ein relativ hoher Anteil an Isolaten der Sciuri-Gruppe sowie ein hoher Anteil von *S. xylosus* an Isolaten der Saprophyticus-Gruppe als „Freiland-Marker“ herangezogen werden kann, um beispielsweise die

qualitative Zusammensetzung von mit Niederschlag verdünntem Abwasser abzuschätzen.

Auch die Argen zeigte mit einem hohen Anteil an *S. xylosus*-Isolaten innerhalb der Saprophyticus-Gruppe (61%) und einem im Vergleich zum Kläranlagenablauf leicht höheren Anteil (5,5%) an Isolaten, die in die Sciuri-Gruppe gehörten, typische Merkmale einer Freilandprobe. Im Vergleich zur Schussen war der Anteil an Isolaten, die weder als Spezies der Sciuri- noch der Saprophyticus-Gruppe identifiziert wurden, mit 44,4% deutlich größer (die Anteile an Isolaten, die keine Spezies der Sciuri- und der Saprophyticus-Gruppe waren, lagen in den Schussen-Proben zwischen 18,1% und 24,3%). Möglicherweise sind diese Unterschiede in der Speziesdiversität zwischen Schussen und Argen in der unterschiedlichen Nutzung des Einzugsgebiets zu suchen: Die fäkale Belastung der Argen ist geringer und diffuse Einträge, beispielsweise aus der Landwirtschaft, könnten die Speziesverteilung der Staphylokokken stärker prägen.

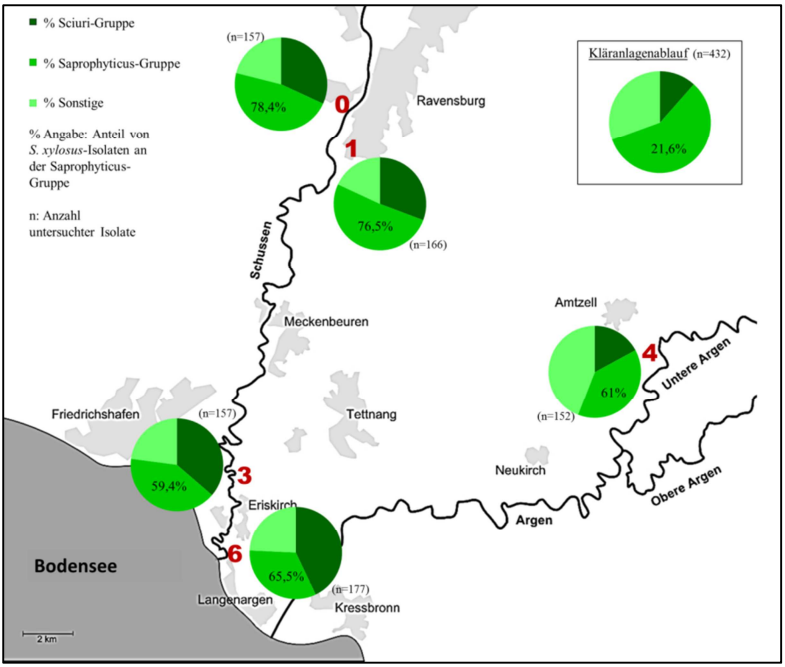

Abb. 8.2: Speziesverteilung der Staphylokokken entlang der Schussen sowie in der Argen und im Kläranlagenablauf

(Quelle der Karte: Kartharina Peschke, Universität Tübingen; Mapdata: ©OpenStreetMap contributors, license: http://opendatacommons.org/licenses/dbcl/1.0/)

Die Argen wurde vom NABU als „Flusslandschaft der Jahre 2014 und 2015“ ausgezeichnet und gilt als einer der letzten deutschen voralpinen „wilden“ Gebirgsflüsse (http://www.nabu.de/tiereundpflanzen/naturdesjahres/2014/16223.html). Wie bereits erwähnt, konnten die *Staphylococcus*-Isolate aus der Argen nur zum Teil einer bekannten Spezies zugeordnet werden. Eines dieser nicht „identifizierbaren“

Isolate ist eine neu entdeckte Spezies, die in den nächsten Wochen detailliert beschrieben werden wird.

8.1.3 Antibiotika-Resistenzsituation im Freiland

Der Trend eines mit zunehmender Fließstrecke ansteigenden Anteils resistenter Isolate war sowohl bei den beiden untersuchten Gattungen als auch der Spezies *Escherichia coli* erkennbar, wobei die Probenahmestelle 6 die höchste Belastung an resistenten Enterokokken und multiresistenten Staphylokokken (Isolate waren mindestens gegen zwei Antibiotika unterschiedlicher Klassen resistent) aufwies (Tab. 8.2). Die Probenahmestelle 3, die etwa 12 km unterstrom der Einleitung des Kläranlagenablaufs Ravensburg-Langwiese liegt, war insofern auffällig, da dort der höchste Anteil resistenter *E. coli* (24,4%) und Staphylokokken (15,3%) detektiert wurde. Interessanterweise war dort vor allem bei den Herbstprobenahmen der Anteil resistenter *E. coli* signifikant höher als an den anderen Freilandprobenahmestellen sowie dem Ablauf der Kläranlage Ravensburg-Langwiese. Möglicherweise ist diese Beobachtung auf die Düngung der angrenzenden Hopfenfelder zurückzuführen, von welchen eine Drainageröhre direkt in die Schussen führte.

Im Vergleich mit der Resistenzsituation im Kläranlagenablauf, lag das Resistenzniveau von *E. coli* in der Schussen (mit Ausnahme der Probenahmestelle 3) auf einem ähnlichen Niveau, während der Anteil resistenter Enterokokken und Staphylokokken in der Schussen im Vergleich zum Kläranlagenablauf bis zu 13,6% geringer war. Das

Resistenzniveau der Argen war vergleichbar mit dem der Schussen-Probenahmestelle 0. Ohne das natürliche Resistenzniveau in aquatischen Ökosystemen zu kennen, fällt auf, dass auch aus Argen-Proben Isolate mit Resistenzen gegen ausschließlich synthetisch hergestellte Antibiotika, wie zum Beispiel Cephalosporine der dritten Generation, gewonnen wurden, was auf eine anthropogene Belastung hinweist. Allgemein gesehen wurden die Resistenzen, die im Kläranlagenablauf detektiert wurden, auch in der Schussen gefunden, wobei Unterschiede im Verhältnis der Anteile der jeweiligen Resistenzen zwischen Kläranlagenablauf und Freiland erkennbar waren: So war beispielsweise im Freiland der Anteil induzierbar MLS_B-resistenter Staphylokokken an Erythromycin-resistenten Isolaten um 33,5% höher als im gereinigten Abwasser (Tab. 8.2c).

Tab. 8.2: Anteile resistenter *E. coli* (a), Enterokokken (b) und Staphylokokken (c), gegliedert nach den einzelnen untersuchten Antibiotika an den einzelnen Freiland-Probenahmestellen sowie im Kläranlagenablauf

a) *E. coli*

Probenahme-stelle	**n**	**% resistent**	**% multiresistent**	**% multiresistent an resistent**	**% AM-r**	**% ETP-r**	**% SXT-r**	**% CIP-r**	**% CTX-r**	**% ESBL**
Kläranlagen-ablauf	246	17,5	8,9	51,2	14,6	0	7,3	4,5	0,4	0,4
PN 0	100	14	8	57,1	13	0	5	5	2	2
PN 1	119	16,8	10,9	65,0	16,0	0	10,1	1,7	0,8	0,8
PN 3	86	24,4	15,1	61,9	22,1	0	11,6	5,8	0,0	2,3
PN 6	119	16,8	9,2	55,0	15,1	0	7,6	10,9	0,0	0,0
PN 4	91	12,1	8,8	72,7	12,1	0	7,7	1,1	1,1	1,1

b) Enterokokken

Probenahme-stelle	**n**	**% resistent**	**% multiresistent**	**% multiresistent an resistent**	**% AM-r**	**% CIP-r**	**% C-r**	**% E-r**	**% CC-r der E-r**
Kläranlagen-ablauf	203	29,1	9,8	33,9	7,9	5,4	1,0	26,1	79,2
PN 0	97	15,5	3,1	20	1	5,2	2,1	10,3	70
PN 1	125	16,8	3,2	19	2,4	4,8	1,6	13,6	82,4
PN 3	96	17,7	5,2	29,4	2,1	6,3	3,1	12,5	75
PN 6	91	26,4	4,4	16,7	4,4	4,4	0	24,2	95,5
PN 4	68	17,6	1,5	8,3	0,0	7,4	0,0	11,8	62,5

c) Staphylokokken

Probe-nahme-stelle	**n**	**% resistent**	**% multiresistent**	**% multiresistent an resistent**	**% MS_B**	**% $cMLS_B$**	**% $iMLS_B$**	**% Ox-r**	**% CC-r**	**% Cip-r**
Klär-anlagen-ablauf	432	23,1	6,9	30	16,2	3,0	3,4	0,7	0,9	0
PN 0	157	10,2	3,2	31,3	1,9	1,3	1,9	1,3	0	0,6
PN 1	166	12,0	4,8	40,0	3,0	0,6	5,4	0,6	0,0	0,0
PN 3	157	15,3	5,7	37,5	3,2	5,1	4,5	0,6	0	0
PN 6	177	14,7	9,6	65,4	3,4	1,7	6,2	0,6	1,1	0
PN 4	152	9,9	5,3	53,3	3,9	0	5,3	0	0	0,7

(n: Anzahl untersuchter Isolate; AM-r: resistent gegen Ampicillin; SXT-r: resistent gegen Cotrimoxazol; CIP-r: resistent gegen Ciprofloxacin; CTX-r: resistent gegen Cefotaxim; ESBL: Extended-Spectrum-ß-Laktamase-Bildner; C-r: resistent gegen Chloramphenicol; E-r: resistent gegen Erythromycin; CC-r: resistent gegen Clindamycin; MS_B: konstitutive Resistenz gegen Makrolide und Streptogramine B; $cMLS_B$: konstitutive Resistenz gegen Makrolide, Linkosamide und Streptogramine B; $iMLS_B$: induzierbare Resistenz gegen Makrolide, Linkosamide und Streptogramine B; Ox-r: resistent gegen Oxacillin. Keines der untersuchten Enterokokken-Isolate zeigte eine Resistenz gegen Vancomycin, wobei die intrinsische low-level Vancomycin-Resistenz von *E. gallinarum* und *E. casseliflavus* nicht berücksichtigt wurde.)

8.2 Einfluss von Niederschlagsereignissen

Um den Einfluss von Niederschlagsereignissen unterschiedlicher Charakteristika auf die Konzentration von fakultativ pathogenen und Antibiotika-resistenten Keimen zu zeigen, werden im Folgenden die Ergebnisse von drei verschiedenen Probenahmen einander gegenübergestellt (Abb. 8.3): Während es in der Woche vor der Probenahme O (09.07.2013) im Einzugsgebiet nicht geregnet hat (Trockenwetter-Probenahme), ist die Probenahme L (04.07.2012) durch einen mehrere Tage andauernden Landregen geringerer Intensität geprägt. Die Probenahme N (14.05.2013) wurde deshalb ausgewählt, weil dieser Probenahme ein Starkregenereignis vorausging, bei dem z.B. auch das Regenüberlaufbecken Mariatal in die Schussen abgeschlagen hat. Obwohl dieses Ereignis mehrere Tage zurücklag, waren die Auswirkungen auf das fluviatile Ökosystem noch erkennbar.

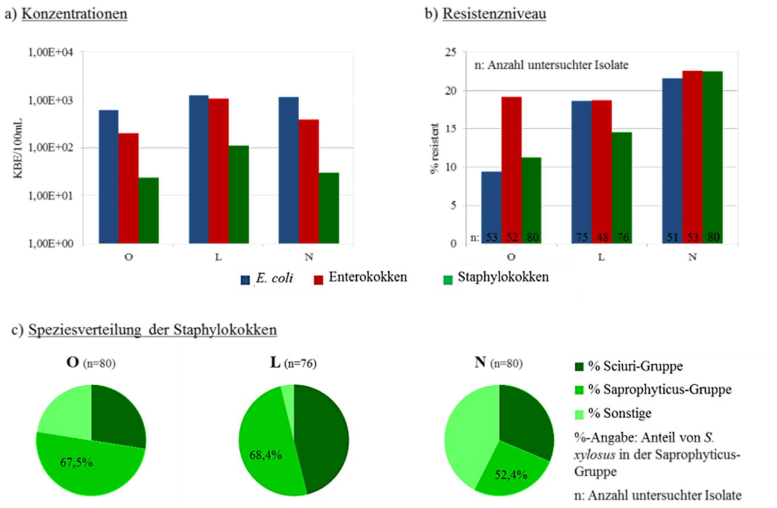

Abb. 8.3: Konzentration (a) und Resistenzniveau (b) von *E. coli*, Enterokokken und Staphylokokken sowie Speziesverteilung der Staphylokokken (c) bei Trockenwetter (O), Landregen (L) und nach einem Starkregenereignis (N)

(Die Konzentrationen von *E. coli* und Enterokokken wurden vom Institut für Seenforschung (LUBW) in Langenargen bestimmt.)

Bei Regenereignissen waren die Konzentrationen von *E. coli* und Enterokokken sowie Staphylokokken zwischen 0,3 und 0,7 log-Stufen höher als bei Trockenwetter, wobei die Differenz zwischen Trockenwetter und direkt nach dem Starkregenereignis, das der Probenahme N vorausging, wahrscheinlich noch größer war. Da es sich bei den Probenahmen O und L beides um Sommer-Probenahmen handelte, kann in diesem Zusammenhang ein jahreszeitlicher Einfluss ausgeschlossen werden.

Wenn man die drei Probenahmen vergleicht, fällt auf, dass die Staphylokokken-Konzentrationen in der Schussen bei den Probenahmen O und N nahezu identisch waren, während die Konzentration nach dem Landregen (Probenahme L) im Vergleich zur Trockenwetterprobenahme O um 0,7 log-Stufen höher lag. Diese Beobachtung stützt die bereits im Zusammenhang mit der um etwa eine log-Stufe höheren Staphylokokken-Konzentration im Freiland im Vergleich zum Kläranlagenablauf formulierten Vermutung, dass diffuse Einträge aus der Fläche zur Konzentration an Staphylokokken im fluviatilen Ökosystem beitragen. Wenn man einen hohen Anteil an Isolaten der Sciuri-Gruppe als einen „Freiland-Marker" heranzieht, bietet die Speziesverteilung der Staphylokokken einen weiteren Hinweis für diese Hypothese: Der Anteil an Isolaten, die als Mitglieder der Sciuri-Gruppe identifiziert wurden, war bei der Probenahme L mit 46,1% um 14,9 bzw. 18,6% höher als bei den Probenahmen N und O.

Dadurch dass manche Spezies weniger häufig gegen eines der getesteten Antibiotika resistent waren als andere, beeinflusste die Abundanz und die Speziesdiversität das Resistenzniveau. Bei der Probenahme N wurden 57,5% der 80 untersuchten Staphylokokken-Isolate nicht als Mitglieder der Sciuri- oder der Saprophyticus-Gruppe identifiziert; der Anteil an nicht identifizierten Isolaten bzw. an Isolaten, die als humanpathogen einklassifiziert werden, wie beispielsweise *S. epidermidis* oder *S. warneri,* war deutlich höher als bei der Probenahme L, was in einem um 8% höheren Anteil resistenter Staphylokokken resultiert. Auch bei den Enterokokken und *E. coli* war der Anteil Antibiotika-resistenter Isolate nach dem Starkregenereignis höher als bei den Probenahmen O und L. Während bei den Enterokokken bezüglich

des Resistenzniveaus zwischen Trockenwetter- und Landregen-Probenahme kein Unterschied festzustellen war, war der Anteil resistenter *E. coli* unabhängig von der Charakteristik des Regenereignisses mindestens doppelt so hoch.

8.3 Einfluss von Abwassereinleitungen auf die Verbreitung von Antibiotika-resistenten Bakterien im aquatischen Ökosystem

Die Unterschiede bezüglich der Konzentration und dem Resistenzniveau fakultativ-pathogener Keime sowie der Speziesdiversität von Staphylokokken bei Regen- und Trockenwetter verdeutlichen, dass die Schussen sowohl durch die Einleitung von gereinigtem bzw. verdünntem Abwasser als auch durch Oberflächenabschwemmungen beeinflusst wird. Die Speziesverteilung der Staphylokokken und das Resistenzniveau bietet einen Anhaltspunkt dafür, welcher Einfluss zum Probenahmezeitpunkt überwog, erlaubt aber, auch aufgrund der Wahl der einzelnen Probenahmestellen, keine quantitative Bilanzierung der Eintragsquellen.

Der Anteil resistenter *E. coli*, Enterokokken und Staphylokokken nahm mit zunehmender Fließstrecke, auf der mehrere Kläranlagen ihren Ablauf der Schussen zugeschlagen haben, zu. Akiyama & Savin (2010) berichteten von einem erhöhten Resistenzniveau coliformer Keime nach Einleitung von gereinigtem Abwasser und Czekalski et al. (2012) detektierten ein erhöhtes Resistenzniveau in Sedimentproben unterhalb der Einleitung von Kläranlagenablauf. Es wäre interessant zu untersuchen, ob sich langfristig die Konzentration entsprechender

Antibiotika-Resistenzgene unterstrom einer Kläranlageneinleitung in Sedimenten und Biofilmen erhöht und ob und in welcher Frequenz Resistenzgene auf autochthone Mikroorganismen, wie beispielsweise verschiedene Spezies der Genera *Bacillus* oder *Pseudomonas*, übertragen werden. Leider gibt es derzeit keine Untersuchungen, die das Resistom in einem Transekt, das sich über Probenahmestellen vor und nach der Einleitung eines Kläranlagenablaufs erstecken müsste, beispielsweise mittels qPCR-Analysen längerfristig analysieren und sich dabei nicht nur auf die fließende Welle beschränken. Daneben bleibt die Frage, wie sich klinisch relevante Stämme in der aquatischen Umwelt verhalten. Es wäre spannend über den Vergleich von Restriktionsmustern „bacterial source tracking“ zu betreiben und herauszufinden, ob diese aus Oberflächengewässern isolierten Stämme human- oder veterinärmedizinisch relevant sind.

9. Synopse

Durch erweiterte Abwasserreinigungstechnologien wie der in Kapitel 5 beschriebenen Ozonung ist es möglich, die Konzentration und damit letztendlich die Fracht an fakultativ pathogenen und Antibiotika-resistenten Keimen, die in die aquatische Umwelt eingetragen werden, um etwa eine log-Stufe zu reduzieren. Der keimeliminierende Effekt des Ozons überdeckte die teilweise detektierte relative Zunahme des Anteils resistenter *E. coli* und Staphylokokken, wobei Laborergebnisse gezeigt haben, dass Antibiotika-Resistenz per se nicht direkt mit einer geringeren Empfindlichkeit gegen Ozon gekoppelt ist (Kapitel 6). Vielmehr scheint letztere von mehreren Faktoren, unter welchen auch die Synthese von Pigmenten und extrapolymeren Substanzen zu nennen sind, determiniert zu werden. Nach der Passage durch Aktivkohle- oder Sandfilter wurde teilweise eine im Vergleich zum Zulauf höhere Konzentration an fakultativ pathogenen und Antibiotika-resistenten Keimen detektiert (Kapitel 5). Hier könnten detaillierte Untersuchungen helfen, mögliche Zell-Filtermaterial-Wechselwirkungen und Bedingungen für horizontalen Gentransfer, die auch Rückspülintervalle und die chemische Beschaffenheit und Konzentration zudosierter Flockungsmittel berücksichtigen, als Ursachen dieser scheinbaren Zunahmen zu identifizieren und in der Folge die Betriebsweise der Filter im Hinblick auf die Eliminationsleistung von fakultativ pathogenen und Antibiotika-resistenten Keimen weiter zu optimieren.

Die Untersuchungsergebnisse zum Retentionsbodenfilter haben gezeigt, dass in Mischentwässerungssystemen bei Starkregenereignissen, bei welchen die Reinigungskapazität der Kläranlage überschritten werden,

die Leitung des verdünnten Abwassers über eine Bodenpassage zu einer deutlichen Reduktion der Fracht an fakultativ pathogenen und Antibiotika-resistenten *Escherichia coli*, Enterokokken und Staphylokokken, die in den Vorfluter abgeschlagen werden, führt (Kapitel 7). Anhand der Speziesverteilung der Staphylokokken ist es möglich den Zulauf zum Retentionsbodenfilter qualitativ bezüglich seiner Zusammensetzung zu charakterisieren und weiterführend auch zwischen den Eintragsquellen „Abwasser“ und „Oberflächenabfluss“ im aquatischen Ökosystem zu differenzieren (Kapitel 7, 8).

Auch nach einer Ausstattung von Kläranlagen mit den derzeit getesteten vierten Reinigungsstufen beziehungsweise dem Bau von Retentionsbodenfiltern werden fakultativ humanpathogene Keime, die teilweise Antibiotika-Resistenzgene besitzen, in die aquatische Umwelt eingetragen werden. Im Vergleich zum Resistenzniveau in der Klinik ist zwar der Anteil Antibiotika-resistenter *E. coli*, Enterokokken und Staphylokokken, die aus der aquatischen Umwelt isoliert wurden, deutlich geringer (Tab. 2.4), dass allerdings auch dort klinisch relevante *mecA*-positive Staphylokokken, ESBL-produzierende *E. coli* sowie gegen das Reserveantibiotikum Chloramphenicol resistente Enterokokken über den gewählten kulturbasierten Ansatz nachgewiesen werden konnten, zeigt, dass sie in entsprechenden Konzentrationen in fäkal belasteten Oberflächengewässern vorkommen (Kapitel 8). Wenn man sich die Antibiotika-Resistenzmuster der aus dem aquatischen Ökosystem gewonnenen Isolate genauer ansieht, stellt man fest, dass die Abundanz von Resistenzen gegen bereits seit längerem in der Medizin eingesetzte Antibiotika, die auch in der Umwelt in messbaren Konzentrationen detektiert werden können, höher ist als gegen

antibiotische Substanzen, die erst kürzlich zugelassen bzw. als Reserveantibiotika eingesetzt werden. Als einen weiteren Hinweis für die anthropogene Beeinflussung des fluviatilen Ökosystems kann der Nachweis von Resistenzen gegen Antibiotika, die ausschließlich synthetischen Ursprungs sind, angeführt werden (Kapitel 3, 5, 7).

Beim Vergleich des Resistenzverhaltens von klinischen Staphylokokken und Isolaten, die aus der Umwelt isoliert wurden, fällt auf, dass letztere häufig keine für klinische Isolate typische high-level Resistenzen zeigen und Resistenzen häufig auch nicht konstitutiv exprimieren (Kapitel 3, 4, 8). Betrachtet man die *erm*-Gene für sich, so ist die Diversität der Gene und die phänotypische Expression der von ihnen kodierten MLS_B-Resistenz in der aquatischen Umwelt deutlich größer als diejenige, die für die Klinik beschrieben wird (Kapitel 3, 4). Möglicherweise stellt die feine Regulation der Expression von Resistenzgenen eine Anpassung an Umweltbedingungen dar: Während die Antibiotika-Resistenz klinischer Isolate, die mit inhibitorischen Antibiotika-Konzentrationen bekämpft werden, eine Art Schutzschild darstellt, sind Umweltisolate schwankenden, aber subinhibitorischen Konzentrationen ausgesetzt und benötigen keine high-level-Resistenz, deren Expression in ihrem Lebensraum eventuell mit energetischen Nachteilen verbunden wäre.

Aus dieser Beobachtung leitet sich die Frage ab, ob solche in aquatischen Ökosystemen präsenten Antibiotika-Resistenzen basierend auf klinischen Grenzwerten überhaupt detektiert werden und infolge dessen die Diversität und Abundanz von Antibiotika-Resistenzgenen in der Umwelt nicht unterschätzt wird. Bakterien, die diese Resistenzen exprimieren, mögen zwar auf den ersten Blick aufgrund ihrer relativ

niedrigen minimalen Hemmkonzentrationen zunächst keine Probleme bezüglich der Behandlung durch sie ausgelöster Infektionen darstellen, es ist allerdings unklar, wie diese sich verhalten, wenn sie inhibitorischen Antibiotika-Konzentrationen ausgesetzt werden, und was passiert, wenn diese Resistenzgene auf andere pathogene Spezies übertragen werden (Kapitel 4). Solche Gentransfer-Ereignisse könnten sowohl in der Klinik als auch im aquatischen Ökosystem – möglicherweise forciert durch subinhibitorische Antibiotika-Konzentrationen – stattfinden. Laborversuche haben gezeigt, dass subinhibitorische Antibiotika-Konzentrationen die Dissemination von Resistenzgenen fördern (Ohlsen et al., 2003) und zur Selektion Antibiotika-resistenter Bakterien führen können (Gullberg et al., 2011). Dass sogar subinhibitorische Antibiotikametabolit-Konzentrationen einen Einfluss auf die Induktion von Antibiotika-Resistenzen haben, konnte für Anhydroerythromycin gezeigt werden: Dieser Makrolid-Metabolit, der selbst eine vernachlässigbar geringe antimikrobielle Wirkung besitzt, induziert bereits nach wenigen Minuten Kontaktzeit eine Kreuzresistenz gegen drei verschiedene Antibiotika-Klassen, wofür picomolare Konzentrationen, die praktisch ubiquitär in der aquatischen Umwelt gemessen werden, ausreichen (Kapitel 3).

Die im Rahmen der vorliegenden Dissertation durchgeführten Untersuchungen und dargestellten Ergebnisse machen deutlich, wie wichtig es ist, Abwasser-reinigungstechnologien dahingehend weiter zu verbessern, dass der Eintrag sowohl von Antibiotika und deren Metaboliten als auch von Antibiotika-resistenten Keimen und Resistenzdeterminanten weiter minimiert wird, um zu verhindern, dass

auch die derzeit noch wirksamen Antibiotika in Zukunft für die Human- und Veterinärmedizin nicht mehr zur Verfügung stehen.

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Verfassererklärung

Kapitel 3


RESEARCH ARTICLE

Demonstration of staphylococci with inducible macrolide-lincosamide-streptogramin B (MLS_B) resistance in sewage and river water and of the capacity of anhydroerythromycin to induce MLS_B

Stefanie Heß & Claudia Gallert

Zitat: Heß, S., Gallert, C. (2014): Demonstration of Staphylococci with Inducible Macrolide-Lincosamide-Streptogramin B Resistance MLS_B in Sewage and River Water and of the Capacity of Anhydroerythromycin to Induce MLS_B. FEMS Microbiololgy Ecology 88, 48-59

Eigener Anteil: Experimentelle Arbeit, Schreiben des Manuskript-Entwurfs

Kapitel 4

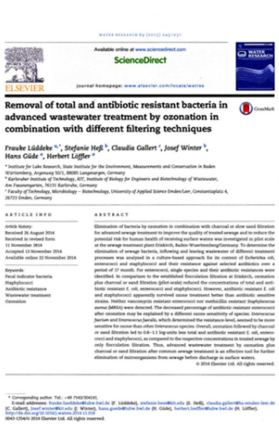

Resistance behaviour of inducible clindamycin-resistant staphylococci from clinical samples and aquatic environments

Stefanie Heß and Claudia Gallert

Zitat: Heß, S., Gallert, C. (2014): Resistance behavior of inducible clindamycin-resistant staphylococci from clinical samples and from aquatic environments. J. Med. Microbiol. 63: 1446–1453

Eigener Anteil: Experimentelle Arbeit, Schreiben des Manuskript-Entwurfs

Kapitel 5


Available online at www.sciencedirect.com
ScienceDirect
journal homepage: www.elsevier.com/locate/watres
Removal of total and antibiotic resistant bacteria in advanced wastewater treatment by ozonation in combination with different filtering techniques
Frauke Lüddeke, Stefanie Heß, Claudia Gallert, Josef Winter, Hans Güde, Herbert Löffler
ARTICLE INFO
ABSTRACT

Zitat: Lüddeke, F., Heß, S., Gallert, C., Winter, J., Güde, H., Löffler, H. (2015): Removal of total and antibiotic resistant bacteria in advanced wastewater treatment by ozonation in combination with different filtering techniques. Water Research 69: 243-251

Eigener Anteil von ≥ 50%: Bestimmung der Lebendzellzahl der Staphylokokken und Gewinnung von Isolaten; Identifizierung der Enterokokken- und Staphylokokken-Isolate; Untersuchung der *E. coli*, Enterokokken und Staphylokokken-Isolate hinsichtlich ihrer Antibiotika-Resistenz; Schreiben der entsprechenden Passagen im Manuskript. Ohne diese Arbeiten hätten die Ergebnisse nicht veröffentlicht werden können.

Kapitel 6

Zitat: Heß, S., Gallert C. (eingereicht bei "Journal of Water and Health"): Sensitivity of antibiotic resistant and antibiotic susceptible *Escherichia coli*, *Enterococcus* and *Staphylococcus* strains against ozone

Eigener Anteil: Experimentelle Arbeit, Schreiben des Manuskript-Entwurfs

Kapitel 7


Environmental Science Processes & Impacts

PAPER

Removal of micropollutants, facultative pathogenic and antibiotic resistant bacteria in a full-scale retention soil filter receiving combined sewer overflow†

Marcia Scheurer, Stefanie Heß, Frauke Lüddeke, Frank Sacher, Hans Güde, Herbert Löffler and Claudia Gallert

Zitat: Scheurer, M., Heß, S., Lüddeke, F., Sacher, F., Güde, H., Löffler, H., Gallert, C. (2015): Removal of micropollutants, facultative pathogenic and antibiotic resistant bacteria in a full-scale retention soil filter receiving combined sewer overflow. Environmental Science Processes&Impacts 17 (1): 186-196

Eigener Anteil von 33%: Bestimmung der Lebendzellzahl der Staphylokokken und Gewinnung von Isolaten; Identifizierung der Enterokokken- und Staphylokokken-Isolate; Untersuchung der *E. coli*, Enterokokken und Staphylokokken-Isolate hinsichtlich ihrer Antibiotika-Resistenz; Schreiben der entsprechenden Passagen im Manuskript.

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