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The ecological risks of antibiotic resistance in aquatic environments: a literature review

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ABSTRACT

Ecological risks of antibiotic resistance in surface waters: a literature review

Bacteria that are resistant to antibiotics occur in the aquatic environment, including sewage and surface waters. The consequences for ecosystems are however difficult to assess, RIVM concluded in a literature review ordered by the Centre for Water Management.

RIVM investigated the environmental risks of antibiotic resistance genes in aquatic environments. Resistance genes render bacteria insusceptible to antibiotics. In the Netherlands, for the treatment of humans and animals yearly about 40 and 508 tonnes antibiotics are used, respectively. After enteric bacteria have become resistant to these antibiotics, the bacteria and their resistance genes may enter the sewage or manure. Via DNA particles that may be easily transferred, these genes can be further spread to other bacteria.

Resistance genes present in enteric bacteria from waste water have been found in surface water downstream of sewage treatment plants, also when enteric bacteria were absent. Resistance is further favoured by different environmental conditions such as nutrients, chemicals and metals in the water. Recent Dutch research indicated that the use of antibiotics in pig farming leads to an increase diversity of bacterial resistance genes in the local aquatic environment. However, the studies did not conclude on the relationship between the presence of resistance genes and numbers of resistant bacteria. The review concludes that no research is available on the possible environmental effects. Also there is little information on the presence of resistance genes in unpolluted waters, which makes a thorough comparison impossible. RIVM recommends studying the presence and possible effects of resistance genes in such a way that also the absolute number of resistant bacteria can be compared between polluted and unpolluted sites.

Key words: antibiotic resistance, antibiotic resistance genes, environment, ecology, risk.

RAPPORT IN HET KORT

Ecologische risico's van antibioticaresistentie in oppervlaktewater: een literatuurstudie

Bacteriën die resistent zijn voor antibiotica verspreiden zich via het watermilieu, waaronder riool- en oppervlaktewater. De ecologische gevolgen zijn echter nog niet in te schatten, zo blijkt uit een literatuurstudie van het RIVM in opdracht van de Waterdienst. Het RIVM beveelt aan mogelijke effecten nader te onderzoeken.

Het RIVM onderzocht de informatie in de wetenschappelijke literatuur over de milieurisico's die optreden als resistentiegenen in het watermilieu zich verspreiden. Dit zijn genen in bacteriën waardoor deze ongevoelig worden voor antibiotica. In Nederland worden jaarlijks voor de behandeling van mens en dier respectievelijk 40 en 508 ton antibiotica gebruikt. Als darmbacteriën resistent worden voor antibiotica, komen deze bacteriën met hun resistentiegenen in rioolwater of in mest terecht. De genen worden op andere bacteriën overgebracht via genetisch materiaal dat wordt uitgewisseld.

Resistentiegenen van darmbacteriën in rioolwater worden teruggevonden in oppervlaktewater stroomafwaarts van de lozingspunten, hoewel de darmbacteriën daar niet overleven. Stoffen in het oppervlaktewater, zoals nutriënten, metalen en chemische stoffen, selecteren ook op resistentie bij bacteriën. Recente Nederlandse meetgegevens wekken de indruk dat door het gebruik van antibiotica bij de varkensbedrijven meer bacteriële resistentiegenen in het lokale watermilieu zitten. De studies leggen echter geen duidelijk verband tussen de aanwezigheid van genen en het aantal resistente bacteriën. Onderzoek naar effecten op het milieu ontbreekt vooralsnog. Omdat resistentiegenen van nature ook voorkomen, en gegevens over de aanwezigheid van resistentiegenen in 'schone' wateren schaars zijn, is het niet duidelijk of er sprake is van een ongewone situatie. Voor deze vergelijking is het ook belangrijk de absolute aantallen van resistente bacteriën te meten.

Trefwoorden: antibioticaresistentie, resistentiegenen, water, milieu, ecologie, risico

PREFACE

This desk study investigates the environmental behaviour, fate, and effects of antibiotic resistance (genes) in aquatic environments as currently available in the scientific literature.

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CONTENTS

UITGEBREIDE SAMENVATTING

Het gebruik van antibiotica bij mens en dier kan leiden tot antibioticaresistentie van bacteriën die met (afval)waterstromen, waaronder rioolwater en oppervlaktewater, over een groter gebied verder verspreid kunnen worden. Het RIVM heeft in opdracht van de Waterdienst van Rijkswaterstaat in de studie 'The ecological risks of antibiotic resistance in aquatic environments, a literature review', de ecologische gevolgen hiervan voor het watermilieu verkend. Er is hierbij voornamelijk gekeken naar de resistentiegenen. Uit ziekenhuizen en huishoudens komen deze genen via (gezuiverd) stedelijk afvalwater in het oppervlaktewater terecht. Vanuit landbouwhuisdieren kunnen de genen via mest en bodem in het oppervlaktewater terechtkomen. De belangrijkste conclusie van deze literatuurstudie is dát de belasting van het watermilieu met dergelijke resistentiegenen plaatsvindt en dat deze genen ook in van nature voorkomende (autochtone) bacteriën gevonden worden. Wat de effecten en risico's zijn van zulke genetische veranderingen op de structuur en functie van bacteriegemeenschappen en of er werkelijk sprake van ecologische schade is, valt nog niet te zeggen. Hier is experimenteel onderzoek voor nodig.

Jaarlijks gebruiken mensen en landbouwdieren naar schatting respectievelijk 40 en 508 ton antibiotica. Als hun darmbacteriën resistent worden voor antibiotica, komen deze bacteriën met hun resistentiegenen in het rioolwater of in de mest. De selectie op antibioticaresistentie van de darmbacteriën kan zowel in mens of dier plaatsvinden als daarbuiten, in rioolwater of mestkelder, bij de aanwezigheid van voldoende restanten antibiotica.

Effluenten van rioolwaterzuiveringen bevatten doorgaans substantiële hoeveelheden antibioticaresistente bacteriën. Dit ondanks het feit dat er in de rioolwaterzuivering een aanzienlijk verwijdering plaatsvindt van de darmbacteriën die drager zijn van resistentiegenen. Belangrijke emissiebronnen van antibiotica en resistente bacteriën in afvalwater zijn ziekenhuizen/verzorgingshuizen, huishoudens en slachthuizen. Resistentiegenen, bijvoorbeeld uit ziekenhuizen worden niet alleen teruggevonden in gezuiverd rioolwater, maar ook in het ontvangende oppervlaktewater nabij het lozingspunt en op grote afstand benedenstrooms daarvan. Dit gegeven suggereert dat de verspreiding van resistentiegenen over een grote afstand kan plaatsvinden, verder dan het verspreidingsgebied

van de dragende darmbacteriën. Ze worden mogelijk verspreid via DNA-deeltjes die gemakkelijk naar andere bacteriën worden overgedragen. Zo zijn resistentiegenen van klinische oorsprong (waaronder *van*A, een gen dat codeert voor resistentie tegen vancomycine) in het watermilieu geïdentificeerd bij afwezigheid van darmbacteriën.

Een andere verspreidingsroute is die via mest. Omdat resistente darmbacteriën van landbouwdieren als varkens, slachtkuikens en mestkalveren en restanten antibiotica in de mest terechtkomen, kan ook het gebruik van deze mest bijdragen aan de verspreiding van resistentiegenen in het milieu. Mest kan via uit- of afspoeling van bemeste akkers of graslanden in het water komen. In een verkennende studie naar de aanwezigheid van resistentiegenen in wateren van veeteeltgebieden in Nederland blijkt dat deze genen in een relatief hoge diversiteit in zowel water als sediment zijn aangetroffen; met name in wateren nabij varkenshouderijen.

Het gedrag van resistentiegenen in aquatische milieus is complex. Het aantal genen kan toenemen onder condities die hun expressie bevorderen, zoals de aanwezigheid van stoffen met een antibiotische werking. De eventuele effecten van de genen op de diversiteit en aantallen van bacteriële gemeenschappen hangen af van diverse factoren, zoals de populatiedynamica van autochtone gemeenschappen en de condities die de genoverdracht, de genexpressie en de verdere groei bevorderen. Belangrijke condities zijn bijvoorbeeld de aanwezige soorten bacteriën zelf, het voorkomen van hoge microbiële dichtheden zoals in biofilms, en de concentraties van vrij DNA. Wanneer stoffen met een antibacteriële werking een voldoende hoge concentratie hebben kunnen ze op (multi) resistente bacteriën selecteren, die zich kunnen vermeerderen ten kosten van de niet-resistente. Bedacht moet worden dat tot slechts 1% van de soorten bacteriën kweekbaar is in het laboratorium. Het beeld van de relatie tussen de mate van resistentie en de verspreiding van resistentiegenen in het aquatische milieu is daardoor nog verre van compleet.

Bacteriële levensgemeenschappen zoals biofilms bestaan uit vele soorten die op diverse manieren van elkaar afhankelijk zijn. De gemeenschappen zijn stabiel in termen van structuur en functie in de tijd, en zijn in staat zich aan te passen aan veranderende omstandigheden. Effecten van een verschuiving in de populaties van bacteriën die resistentiegenen dragen bestaan in eerste instantie uit veranderingen in structuur of in functionaliteit. In tweede instantie zou er een effect kunnen zijn op consumenten in deze biofilms, zoals protozoën en

nematoden. Echter, gezien het beperkte vermogen soorten en functies in kaart te brengen, is het onmogelijk alle ‘kleine’ veranderingen waar te nemen. Veranderingen in microbiële gemeenschappen kunnen daarom alleen bestudeerd worden aan de hand van de meest talrijke (of kweekbare) soorten en de meest dominantie processen.

De gevolgen van zulke veranderingen in bacteriële gezelschappen moeten nog worden aangetoond. Kwantitatief vergelijkend onderzoek hiernaar ontbreekt. Bij verder onderzoek is het vooral van belang dat methodes worden gevonden die antropogene resistentie van autochtone resistentie kunnen onderscheiden en waarmee zowel bacterieel verontreinigde milieus als meer natuurlijke milieus kunnen worden geanalyseerd. Deze informatie moet vervolgens in een ecologisch perspectief worden geplaatst. Relevante effectparameters zijn functionele parameters zoals het vermogen bepaalde substraten om te zetten, en structurele parameters zoals de verhouding tussen schimmels en bacteriën en de biomassa en diversiteit van consumenten (protozoën, nematoden).

1. Introduction

Recent concerns about the presence of antibiotic resistance in the environment refer to the resistance of bacteria to clinically relevant antibiotics. Direct contact with resistant bacteria may later prevent a proper medical treatment with antibiotics in case of hospitalisation, considering [30;38;63]:

(a) microbial pathogens resistant to more than one antibiotic, e.g., MRSA (methicillin-resistant *Staphylococcus aureus)*;

(b) microbial pathogens resistant to 'last resort' antibiotics like vancomycin.

The occurrence of antibiotics and antibiotic resistance in the environment has increasingly been reported [8;52;59;63]. Although publications suggest that the aquatic environment can be 'contaminated' with antibiotic resistance, there is almost no integral research on the effects on aquatic biocoenoses in particular.

1.1. Study objectives

The Centre for Water Management of the Directorate for Public Works and Water Management asked the National Institute for Puiblic Health and the Environment (RIVM) to give an overview of the risks to aquatic organisms by antibiotic resistance in the aquatic environment. In this way a better understanding could be gained of the ecological risks of antibiotic resistance in relation to sources of pollution with antibiotic resistance. This initiative follows from the results of a Dutch research that indicated that the use of antibiotics in pig farming leads to an increase diversity of bacterial resistance genes in the local aquatic environment (RIVM report 601500004) [46].

Antibiotic resistance is defined here, following Wikipedia, as follows: antibiotic resistance is the ability of a micro organism to withstand the effects of an antibiotic. It is a specific type of drug resistance. Antibiotic resistance evolves naturally via natural selection through random mutation, but it could also be engineered. Once such a gene is generated, bacteria can then transfer the genetic information in a horizontal fashion (between individuals) by plasmid

exchange. Antibiotic resistance genes (ARG) should be seen as the most relevant markers of antibiotic resistance in the environment (c.f. [31;35;39]). Resistance comes in different grades [82]. The Minimum Effect Concentration for inhibition is also the concentration where selection for further resistance starts [83].

The most recent data on the distribution of antibiotic resistance in the environment will be taken into account, as well as the factors that determine the environmental behaviour and fate of the genes. The role of antibiotics in the further spread of antibiotic resistance will be discussed.

1.2. Data collection

This report has been based primarily on publicly available scientific literature. The bibliographical databases Medline and Current Contents were searched for useful scientific literature from 2001-2006 (see search profile below).

No.	Records	Request
1	71299	antibiotic* or antimicrobial*
2	16060	vancomycin* or tetracyclin* or sulfonamide or methicillin*
3	642205	gene or genes or genetic material* or dna
4	281810	resist*
5	201966	(river? or lake? or sewage or effluent? or surface water? or groundwater? or ground water? or sludge? or wastewater? or waste water? or lagoon? or freshwater? or fresh water? or aquatic*) in ti ab kw mesh js
6	198	(#1 or #2) and #3 and #4 and #5
7	144430	(environment* or water? or fish) in ti
8	151	(#1 or #2) and #3 and #4 and #7
9	102	#8 not #6
10	22	(#1 or #2) and #3 and #4 and ((aquaculture) in ti ab kw mesh js)
11	8	#10 not (#6 or #9)

Based on the selection criteria in the profile above, 253 records were retrieved with an abstract. Based on these abstracts, 60 papers with were considered relevant for this study. In the process of reviewing this literature, another 69 records were additionally retrieved. Finally, 96 bibliographical sources have been referenced in this report.

1.3. Readers guide

The scientific backgrounds of antibiotic resistance are explained in chapter 2. They are about the relation between antibiotic resistance and bacteria in general (§ 2.1), and more specifically about the origin of antibiotic resistance (§ 2.2), the mechanisms responsible for the transfer of antibiotic resistance to other bacteria (§ 2.3), the requirements for such transfers to other bacteria (§ 2.4), and the requirements for the expression of the genes encoding for antibiotic resistance (§ 2.5). The persistence of antibiotic resistance in aquatic systems and the factors that influence its persistence are clarified in § 2.6. A general introduction to microbial ecology is given in § 2.7.

Transport may occur to other locations in the aquatic environment dependent on the environmental conditions. These transport routes will be discussed in chapter 3. The sources will be discussed in § 3.1 and some general aspects of the transport routes in § 3.2. The particular role of sewage treatment plants (STPs) and manured soils in the further distribution of antibiotic resistance in the environment will be discussed in § 3.3, and § 3.4, respectively.

The effects of antibiotic resistance and its risks to aquatic environments are discussed in chapter 4. These effects refer to (potential) changes caused by antibiotic resistance, whereas the environmental risks refer to the likelihood of such alleged effects taking the exposure into account.

Finally, conclusions will be drawn and discussed in chapter 5 and recommendations are made.

2. Scientific backgrounds of antibiotic resistance

2.1. Antibiotic resistance and bacteria

Resistance of bacteria in humans or husbandry animals to clinically relevant antibiotics is generally assumed to be initiated under clinical conditions [2;39]. High amounts of various antibiotics are applied to infected patients (40 tonnes in 1999 [45]) or animals (508 tonnes in 2004 [28]). Under such conditions gastro-intestinal bacterial communities may respond to high and frequent doses of antibiotics by a shift towards more resistant communities. Mutations may cause (slight) differences in the expression of proteins by genes. In case such a protein is able to de-activate the antibiotic or its residues, the bacterium is able to survive. Therefore, the high concentrations of antibiotics in the gastro-intestinal tract provide an environment that gives selection advantage to those bacteria that are able to withstand antibiotics [57]. In this way, they may proliferate at the cost of resistance trait missing micro-organisms that compete for the same sources.

The genetic information that encodes for proteins involved in de-activating antibiotics is found in the antibiotic resistance genes (ARGs). A schematic representation of a bacterium carrying an ARG is in Figure 1. Resistance can generally be effected through either efflux (pumps excreting antibiotics from the cell), target modification (proteins that protect the target of action of the antibiotic, or that replace the target by a less sensitive one), or destruction of the antibiotic [12]. These abilities of bacteria to withstand antibiotics are based on intrinsic mechanisms, or on changes in their genetic material (acquired resistance). Intrinsic resistance can, for example, be linked to cell wall properties that determine the transport of the antibiotic into the cell.

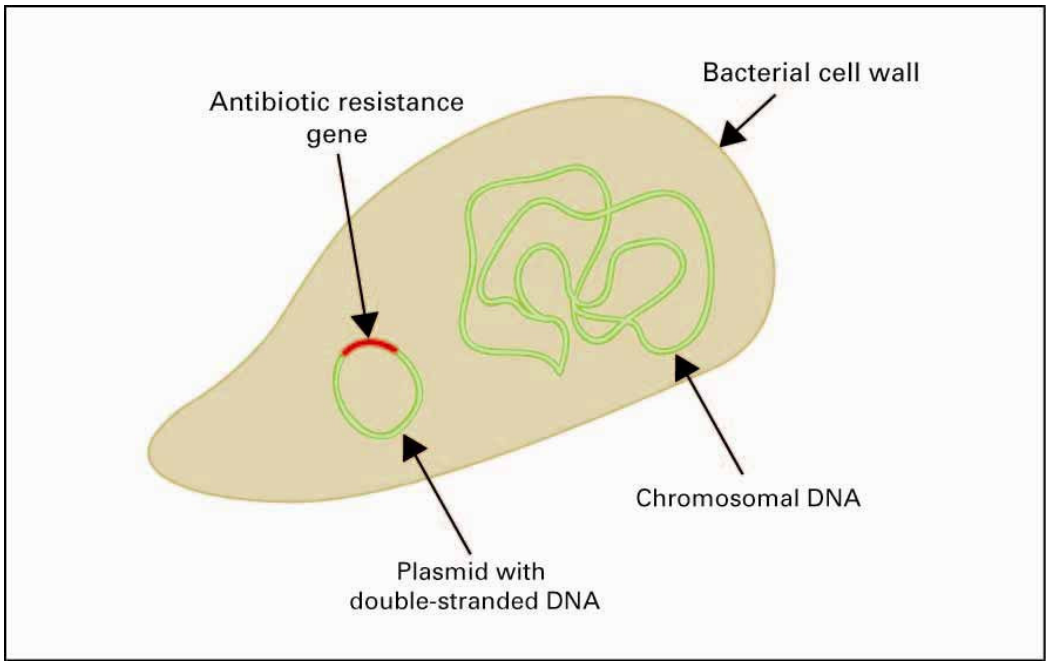

Figure 1. A bacterium, carrying an antibiotic resistance gene (ARG) on a plasmid.

A single mechanism of resistance can render bacteria resistant to a whole class of antibiotics, if members of this chemical class share a common cellular mode of action. The number of genetically-encoded resistance mechanisms is not completely known yet, as not all resistant bacteria have been sufficiently characterized. New resistances are discovered every year. For some classes, more than 40 different genes are already known, such as for tetracycline resistance [53;54].

Antibiotic resistance is here defined as the presence or activity of antibiotic resistance genes. It would have been less appropriate to focus only on the dissemination of bacteria that carry these antibiotic resistance genes, as the studies measuring the diversity and abundance of bacteria are often based on more traditional plating techniques which are not suited for most bacteria from environmental samples. Also, the antimicrobial resistance genes (ARGs) may not confine themselves to a known range of hosts. However, as the actual effect of resistance genes becomes apparent only when they are expressed, data acquired with plating may be useful. Therefore, plating data have been added, when deemed relevant. Bacteria with antibiotic resistance genes are abbreviated to ARBs, antibiotic resistant bacteria.

2.2. Origin of antibiotic resistance

When concerned about the origin of antibiotic resistance in the environment, the antibiotic resistance of bacteria can be divided into two groups of origin, *anthropogenic* and *autochthonous*:

(a) *anthropogenic*: the resistance present is the result of human activities. Enteric bacteria in humans and husbandry animals treated with antibiotics may develop and proliferate resistance to these substances [55]. Treatment with (man-made) antibiotics creates conditions where selection advantages will thrive. Human and animal faeces are the direct sources of this resistance.

(b) *autochthonous:* the resistance in situ is a natural phenomenon. Antibiosis to other micro-organisms to compete for particular niches is a common phenomenon in microbial communities present in terrestrial and aquatic environments. Various therapeutic antibiotics are derived from e.g. soil actinomycetes [12]. It is thought that most acquired resistance mechanisms originate in producers of antibiotics, such as *Streptomyces* ssp. or *Penicillium notatum*. Autochthonous bacteria can also be resistant to more than one antibiotic without direct contact with therapeutic antibiotics [63]. Various plasmids belong to a class of plasmids found in bacteria from human intestines which may show resistance to a wide variety of antibiotics [66].

It is necessary to discern in situ between a 'natural' background of ARGs and an 'added' anthropogenic level. Presence of resistance against man-made antibiotics as such is no conclusive proof on the origin. Chromosomal β-lactamases, antibiotic multi-resistance genes (AMRG), and some aminoglycoside inactivating enzymes are assumed to have an anthropogenic origin. However, D`Costa et al. [17] proved that fluoroquinolone resistance in soil was autochthonous, whereas it was previously assumed to have an anthropogenic origin only. Jones *et al*. [92] already found in 1985 that the incidence of antibiotic resistance in aquatic bacteria was lower than that in *Pseudomonas* spp. and *E. coli*, but higher than in coliforms and faecal streptococci in Lake Windermere. However, aquatic bacteria from two remote upland tarns, with hardly any anthropogenic influence (no sewage or other effluents) displayed comparative results to the lake bacteria. It was coined that nutrient poor conditions

associate with increased resistance. Pirnay et al. [51] found no real difference between *Pseudomonas aeruginosa* strains from clinical and environmental strains. The pattern of biodiversity within clinical samples, and aquatic samples from a Belgium river, resembled that of all global samples (clinical and environmental). Likewise, the origin of antibiotic resistance has been investigated in a Portuguese mesotrophic estuary affected by harbour facilities, industrial plants, domestic sewage inputs and aquacultures. This estuary was also used for recreational purposes. Henriques et al. focused on the genes encoding for β-lactamases, the enzymes that de-activate β-lactam antibiotics. It was shown that various bacterial DNA sequences extracted from the water samples were (almost) identical to β-lactamase sequences of enteric isolates. The patterns of molecular diversity of the bacterial DNA sequences, however, indicated that the origin of the β-lactam resistance in the estuary was mixed and that there were sequences of genes encoding for β-lactamases that were more ancient than the resistance genes clusters of enteric isolates, and therefore not of anthropogenic origin. This investigation showed that in situ antibiotic resistance in aquatic environments can be (partly) autochthonous, in spite of the anthropogenic pollution with antibiotic resistance [31]. It is expected that recent improvements in DNA-based analytical techniques and the combinations of these techniques will be helpful to discriminate the anthropogenic from the autochthonous contributions to resistance (see e.g., [61]). Indeed, Riesenfeld *et al.* [94] demonstrated that soil bacteria harbour many more resistance genes than known based on culturable bacteria or known primers for polymerase chain reaction (PCR) detection.

Oxytetracyclin (OTC) resistant bacteria (counted on plates) were favoured in marine sediments in the presence of high levels of unmedicated and sterilised fish feed; in spite of absence of oxytetracycline. The sediment layer depth was 6 cm; the feed layer was 16 cm. Under feed layers of 1-2 cm, no relative increase in OTC resistant bacteria as found [85]. This experiment shows that resistance can be developed by other stressors than antibiotics.

In marine water in China, resistance against chloramphenicol was measured both in indigenous estuarine or marine bacteria, and in potential human or marine animal pathogens, although chloramphenicol had been banned in China in 1999. The *cat*I gene in the marine bacteria had probably the same clinical origin as the gene in the *cat*-positive Enterobacteriaceae [87].

Other substances than clinical antibiotics might be involved in increasing antibiotic resistance in the environment. Therefore, within the context of this review, other substances with an antibiotic mode of action or antibiotics used for other purposes than clinical antibiotics are defined as antibiotics as well. Such substances may be biocides used for industrial or sanitary purposes as antimicrobial slimicides or disinfectants, but also agricultural pesticides. Examples of the former are quaternary ammonium compounds, used as disinfectants in the food and feed industries and for veterinary hygiene. Examples of the latter are streptomycin and kasugamycin used as fungicide in various crops and arboriculture. In this way other substances than clinical antibiotics may select for resistance. Also organic solvents and detergents have been discussed as possible stressors to select for mutant bacteria with higher expressions of multiple antibiotic resistance [6].

Cross-resistance of antibiotics or heavy metals is indeed possible under laboratory conditions, as the genes for resistance to these compounds may be located in close proximity and may therefore be transferred in tandem [55;69]. Also, the mechanism for resistance could be effective against multiple substrates. For instance, the bacterial cell pump from the human pathogen *Listeria monocytogenes* that excretes antibiotics has been shown to excrete heavy metals as well (Mata et al., 2000, cited in [6]). There is evidence for in situ cross-resistance to metals in the aquatic environment [55]. Recent experiments on the co selection for microbial resistance to metals and antibiotics in freshwater environments showed that exposure of bacterioplankton to cadmium or nickel resulted in co selection for antibiotic resistant strains [69]. Berg et al. [9] have shown that antibiotic resistance evolved in agricultural soils treated with copper, thus indicating co selection for terrestrial environments.

Pirnay et al. (2005) found one Multi Drug Resistant (MDR) clinical strain of *P. aeruginosa* in a Belgium river, among many other strains. The resistance level of this environmental strain was not as high as that of clinical strains in hospitals. However, this strain is hypothesized to have been selected for in the environment by noxious substances. Since all *P. aeruginosa* strains should be considered as potential pathogens, this observation shows that MDR strains can be disseminated through rivers and that the emergence of antibiotic resistance in the environment should be taken seriously [51].

2.3. Transfer mechanisms of antibiotic resistance

Many studies indicate that antibiotic resistance is transferred via the genes [6;39;63]. The basic transfer mechanisms for antibiotic resistance in aquatic environments are represented in Figure 2. Figure 2 depicts two transfer mechanisms. Transfer mechanism 1 refers to the horizontal gene flow (HGF), i.e., when a recipient bacterium acquires genetic material from a donor that is not its 'parent'.

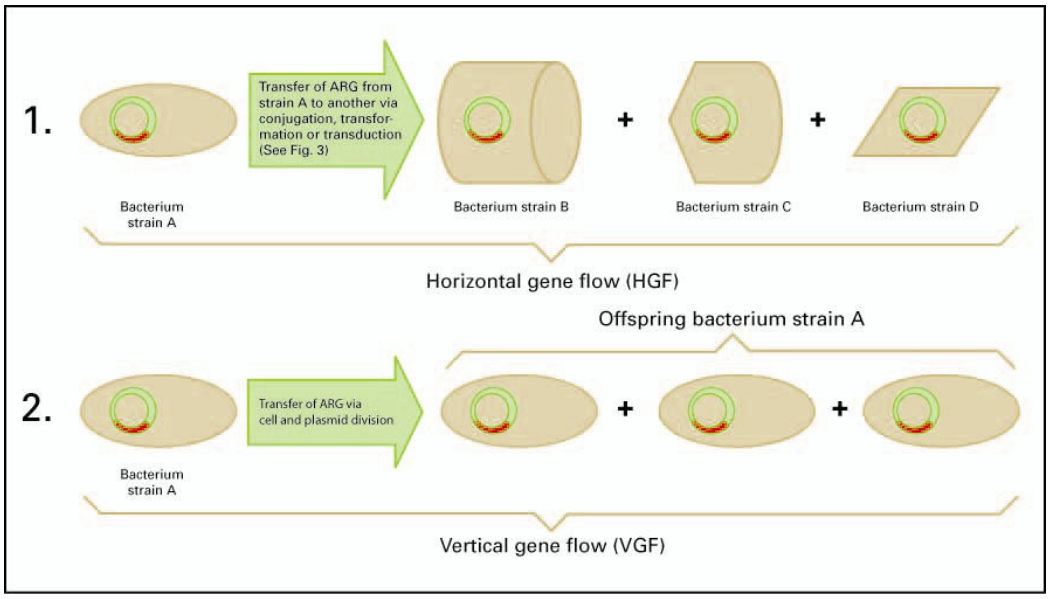

Figure 2. Transfer mechanisms for antibiotic resistance via the genome.

This horizontal gene flow (HGF) is achieved via conjugation, transformation[a] or transduction of mobile DNA fragments (plasmids[b], transposons[c], and integrons[d]). [21]. Figure 3 provides more details. HGF is very common within bacterial communities and most of the resistance

[a] Transformation is the transfer of extracellular DNA (e.g., after cell lysis) to a bacterium via inclusion. Conjugation is the transfer of plasmids or other mobile genetic elements from one to another bacterium via a conjugation bridge. Transduction is the transfer from a bacterium to another bacterium via viruses (bacteriophages). Conjugation is generally thought to be the most important transfer mechanism.

[b] Plasmids are double-stranded circular units of DNA that replicate within a cell, independently of the chromosomal DNA. They are generally found in bacteria.

[c] Transposons are DNA sequences that can shift to different genome locations in a cell by a process called transposition. Transposons often have antibiotic resistance genes. They may move within or between plasmids and chromosomes.

[d] Integrons are gene capture systems found in bacterial chromosomes, plasmids and transposons. They consist of genetic structures for acquiring and expressing gene cassettes. A gene cassette may encode for one or more genes, *e.g.*, those encoding for resistance against a particular antibiotic.

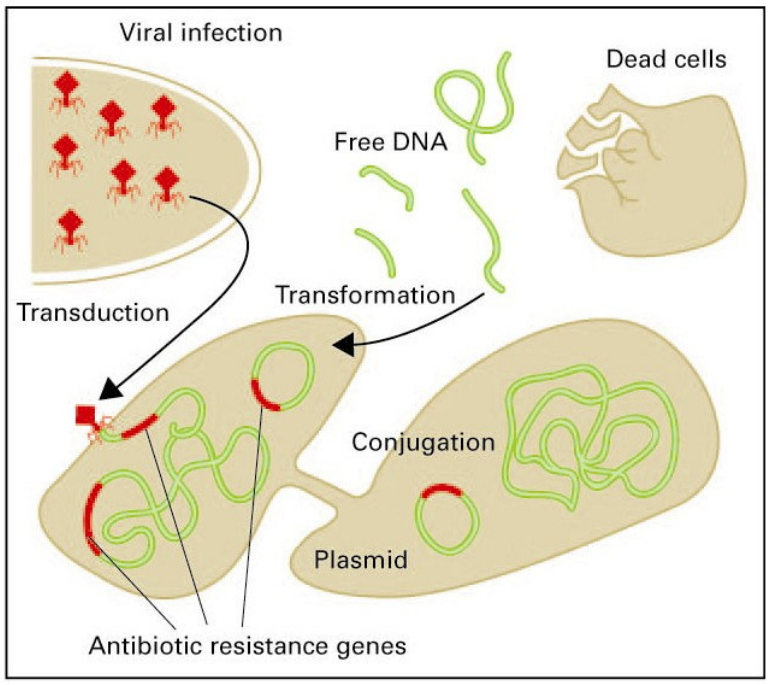

Figure 3. Transfer of ARGs via micro-organisms (by courtesy of dr. T. Schwartz).

present in a community is indeed assumed to be obtained by HGF [6]. Antibiotic resistance can be transferred by HGF from enteric bacteria to autochthonous bacteria. Since the latter may be much more resilient to the in situ environment than the bacteria originally carrying the resistance, the resistance can remain present while the donors do not persist.

Transfer mechanism 2 in Figure 2 is the parental or vertical gene flow (VGF), i.e. from bacterial 'parents' to 'offspring' by cloning, the regular process of cell and plasmid division. VGF will predominantly occur in case the microbiological conditions for bacteria in a particular niche are optimal, favouring proliferation.

Hotspots are physical locations where relatively high resistance transfer rates may occur. Such hotspots may be the guts of soil arthropods [33], manure [67], but also biofilms [60] and upper aerobic sediment layers with high microbial densities [46]. Biofilms may be present in various aquatic environments varying from industrial process water and surface water to drinking water systems. High microbial densities indicate favourable conditions for the growth and proliferation of bacteria.

In situ transfer rates of ARGs in the aquatic environment are needed for a more mathematical approach to quantify the dissemination and fate of antibiotic resistance. Under laboratory conditions, the transfer frequencies could be determined in mating experiments in which donors, e.g. from sewage, are inoculated into media with reference recipient bacteria (*cf.* [32]). Currently these data are lacking.

2.4. Requirements for the transfer of antibiotic resistance

The requirements for the transfer of antibiotic resistance between bacteria are not completely known [21;22]. Such transfer via mobile DNA fragments from one bacterium to another is dependent on various factors and should be seen in the context of complex, dynamic and adaptive microbial communities [23]. The transfer via conjugation may be genus specific, as has been described for bacteria in human intestines. For instance, the R100 plasmid, also known as NR1, carries several genes conferring resistance to antibiotics and has been shown to transfer itself between intestinal bacterial genera as *Klebsiella*, *Salmonella* and *Escherichia*, but not to *Pseudomonas* [12].

There are various laboratory studies showing gene transfers under predetermined conditions, indeed indicating very dynamic and adaptive systems [13;39]. As an example, eleven tetracycline resistant bacteria (*Acinetobacter* ssp.) were isolated from Danish fish farms and sewage [3]. In these experiments, genes were successfully transferred from one donor to three aquatic species (two from sewage, one from a fish farm). Laboratory experiments and in situ experiments with membrane filter chambers nearby the outlet of a Danish fish farm investigating the transfer of plasmids carrying oxytetracycline resistance showed that this transfer was dependent on the characteristics of the donor and recipient bacteria, the time-span and physico-chemical conditions [13]. De Gelder et al. [22] concluded that the bacterial host range can be particularly influenced by the plasmid donor in an activated sludge microbial community grown in the presence of antibiotics. Still, it is demonstrably difficult to extrapolate the results of such experiments to the dynamic and complex in situ microbial communities.

For an efficient uptake of free DNA in bacteria by transformation, the presence of high molecular weight DNA is needed at concentrations of approximately 1 µg/mL under

laboratory conditions. Similar concentrations of high molecular weight DNA are reached in the environment, even in more natural waters [pers. commun. H. Bergmans, RIVM/Bureau GGO]. Therefore the amounts of free DNA in Dutch aquatic environments are probably not a limiting factor for transformation in situ. Additional factors that may support gene transfer are the presence of metal ions as Ca^{2+} and Mg^{2+} [21]. The chance of transfer between and persistence in bacteria would very much depend on the genetic context. Transfer is greatly enhanced for instance, if the gene that is taken up is part of a transposon or integron.

2.5. Requirements for the expression of antibiotic resistance

Antibiotics, or substances with an antibiotic-like mode of action, are generally assumed to be the primary requirement for the expression of resistance genes. This is based on the empirical evidence from various studies that stressors select for species that are better adapted to that specific stressed environment. There is, however, a debate on the validity of this paradigm in relation to antibiotic resistance as it may not always explain the ubiquitous dissemination of antibiotic resistance [65].

Antibiotics or their residues can create an environment that is hostile to bacteria, susceptible to these antibiotics. Only a bacterial trait to deal with such a hostile environment may enable the bacterium's survival. The expression of an ARG is then vital for the survival of bacteria. A transfer mechanism like HGF will be of particular relevance in case the donors of the antibiotic resistance are not fit to withstand an in situ aquatic environment, whereas the recipients of the resistance, e.g., autochthonous bacteria, may be much more fit. On the other hand, to sketch some of the complexities of bacterial systems, the presence of antibiotic resistance genes in an antibiotic-stressed environment does not necessarily imply their expression [39]. An important question to answer is how low antibiotic concentrations can be to evoke an antibiotic resistance response in bacteria. Resistance development may already occur at the Minimum Effect Concentration at which bacterial growth is slightly reduced, which is approximately tenfold below the Minimum Inhibitory Concentration (MIC), the lowest concentration at which bacterial growth is completely inhibited [47]. Obst et al. [48] found in a bacterial bioassay with *Enterococcus faecium* B7641 *van*A+ that at vancomycin concentrations more than thousand times lower than the MIC for vancomycin of 32 mg/L a dose-related antibiotic resistance response was evoked (thus at circa 32 µg/L). This response

was the production of *van*A specific RNA by the enterococcal bacteria exposed to vancomycine. This may mean that a selection advantage may occur even at relatively low concentrations of antibiotics. In the Netherlands, concentrations of various antibiotics in surface water range from < 0.4 – 90 ng/L, whereas in hospital effluent and STP influent concentrations up to 30 µg/L and 0.5 µg/L can be found, respectively [58].

An example of a study in which genotypic changes in bacterial communities in polluted environments were investigated is given in chapter 3 (Heuer et al. [32]).

2.6. Persistence

As antibiotic resistance is commonly found in aquatic environments, its persistence seems obvious. The factors that determine the persistence of antibiotic resistance, however, are largely unknown [31]. It is clear that the presence of antibiotics, or substances with a likewise mode of action, may provide selection advantages for resistant bacteria. This will depend on the concentrations of antibiotics (see also § 2.5).

It is interesting to know whether the antibiotic resistance of such hotspots will decrease in time, in case the antibiotic concentrations decrease. One may expect that under such conditions the antibiotic resistance trait may 'get lost', especially if carried on a plasmid, but that seems not necessarily to be the case. While carriage of the resistance trait may theoretically decrease the bacterial fitness if no selection pressure is present, compensatory mutations may again increase the fitness without loss of the resistance element [20;57]. There are investigations showing that the 'costs' of recipient bacteria to acquire and maintain resistance are low [26]. Singer et al. [65] also emphasised that the persistence of antibiotic resistance in the environment is too complex to be attributed to the selection role of antibiotics alone.

Free or extracellular DNA, e.g., released after cell damage or death, may be taken up by other bacteria via transformation (see Fig. 3) or remain free-floating in the water. This will depend on the availability of suitable recipients, the time-span, and the physicochemical environmental properties. The persistence of extracellular DNA in water is limited: substantial degradation within 8-10 hours in freshwater, and 6-11 hours in marine water, both DNA determinations by colorimetry (Paul et al., 1987, 1989, both cited in [63]). In microcosm

studies simulating natural transformation, substantial DNA degradation in natural water occurred in 48-96 hours [10]. In STPs the degradation of extracellular DNA may be more rapid due to DNAse activity.

Extracellular DNA in soil may be more persistent than in water. DNA bound to mineral surfaces may be relatively persistent and even maintain its transformation ability (Nielssen et al., 1997, cited in [63]). Extracellular DNA applied to 'natural' soils was detectable by PCR up to 60 days after incubation in 'natural' soil (Romanowski et al., 1993, cited in [63]).

The disappearance of OTC resistance genes — as the sum of free and bacterial DNA — in aquatic microcosms has been studied by inoculating waste water from a US beef cattle waste water lagoon [25]. In these laboratory studies, the antibiotic resistance was measured via a total of *tet* activity by real-time PCR. The dissipation rates of tetracycline resistance were the highest in the presence of simulated sunlight, regardless of the concentrations of oxytetracyclines (25 and 250 µg OTC/L). First-order rate coefficients under a light regime over the first week were 0.75-0.84 d^{-1}, whereas under dark conditions this rate coefficient was 0.49 d^{-1}. Under light, the total *tet* amounts decreased by circa 10000 times within 29 days after inoculation, whereas under dark conditions, this decrease was circa 1000 times. It was indicated that the dissipation of OTC resistance on a short-term depends on ecological interactions — possibly via photosynthesis and primary production — rather than on OTC levels. In STPs, simulated sunlight was also shown to decrease the abundance of ARBs substantially [19].

The fate of antibiotic resistance of *Pseudomonas putida* containing RP4 plasmids in groundwater was investigated in experimental microcosms (test tubes) of sterilised soil from seven metres below the soil surface with groundwater [8]. This Swedish study indicated that plasmid-borne resistance was already gone in 80-90% of the microbial cells during the first day of incubation in these test tubes. Most of this reduction was attributed to the presence of soil particles as 70% of the resistance expression was retained in bacteria suspended in groundwater without soil. Also, in additional laboratory experiments, bacteria sorbed to soil particles had a lower frequency of expression of antibiotic resistance to tetracycline and kanamycin than suspended bacteria. The test result indicated that ARBs in aquifers may lose their antibiotic resistance quicker in the presence of soil particles.

2.7. Microbial ecology

Microbial ecology provides a scientific frame to investigate the effects of antibiotic resistance on microbial communities taking their function and structure into account.

Microbial life-forms are found in every imaginable habitat on earth, including all sorts of extreme environments. They are distributed from acidic lakes to the deepest ocean, from frozen environments to hydrothermal vents, and from permafrost soils of the Arctic Circle to termite guts in sub-Saharan Africa. They play a vital role in all biogeochemical cycles: the nitrogen cycle, the phosphorus cycle and the carbon cycle all depend on micro organisms in one way or another.

Their long evolutionary history, diversity and abundance have made microbes a highly heterogeneous group of organisms, covering all three domains of life (Prokarya, Archaea and Eukarya). Microbial ecology examines the diversity and function of micro-organisms and studies how these organisms interact with each other and with their environment.

Historically, our knowledge of microbial diversity and activity was derived from cultured microbes in laboratory experiments. However, our knowledge of microbial ecology has progressed enormously in the last 15 years as a result of technological developments. Particularly studies utilising molecular analysis of biomolecules (nucleic acids: DNA/RNA, fatty acids) and accurate measurements of isotopes have learned us that culture-based work is highly biased and that as-yet cultivable microbes comprise only 0.1-1% of the diversity actually present (depending on the environment). As a result, there are limited opportunities for studying the genetic, biochemical, and metabolic capacities of the vast majority of single-celled organisms. To circumvent this culturing problem, the last few years have seen efforts to large-scale sequencing of all the genetic information of all the millions of organisms present in a particular microbial ecosystem and use powerful computers to pick out the genes. This technology — metagenomics — has enabled the identification of genes from the full complement of microbes in certain environments. Obviously, metagenomics is an extremely laborious and costly enterprise and cannot be done on a routine basis.

Micro-organisms live and function in assemblages of multiple species: microbial communities. The microbial communities on solid surfaces are called biofilms. These

microbial assemblages are often highly complex and structured. Microbial communities such as biofilms are able to maintain great stability in structure and function over time. They are capable of recovering from and adapting to radical habitat alterations by altering community physiology and composition. More subtle habitat changes, such as chronic exposure to antibiotics, or to ARGs, will be difficult to assess. Laboratory studies on the effect on individual microbes can provide valuable background information. However, to investigate the effects of such compounds on microbial ecosystems, populations must be studied in situ. There are several — mainly molecular — techniques to study the spatial and temporal distributions of microbial diversities. But most microbial communities are comprised of high numbers of very diverse and largely unknown organisms (see above), and it is only possible to study clear changes of the most abundant members of a microbial ecosystem routinely. Subtle community changes due to mild exposure could be hard to identify, and will be difficult to reliably attribute to the presence of ARGs. The latter is a more general problem.

Although resistance genes in microbial populations are accessible for studies using molecular methods, the difficulty of cultivating micro organisms makes it difficult to establish a link between the occurrence of ARG and the presence a particular organism, i.e. to identify which bacterium carries the ARG. Also, establishing in situ changes of microbial communities requires good baseline data on composition and dynamics, which are usually not available.

3. Anthropogenic sources and transport

Sources will be discussed in § 3.1. Some general aspects of transport to the aquatic environment in § 3.2, some more detailed aspects of the role of STPs herein in § 3.3 and more details of the role of manured soil in contaminating the aquatic environment in § 3.4.

3.1. Anthropogenic sources

Various anthropogenic sources contribute to the spread of antibiotic resistance into the environment. The treatment of infected patients and husbandry animals is the most relevant in this respect. Waste streams that contain faeces and their residues may therefore contain antibiotic resistance genes. As manure used for fertilisation of grassland or arable land may contain antibiotic resistance genes as well, manure may contribute to the dissemination of resistance as well. The most relevant sources of antibiotic resistance in European Union countries have been listed in Table 1.

After emission, antibiotic resistance may be concentrated downstream of the source. In this way, some environmental compartments can be expected to function as sinks for bacteria and on a longer-term as a secondary source, re-emitting low amounts of antibiotic resistance. Such compartments may be STPs, sediments, suspended particles with sorbed DNA, and biofilms in natural systems, but also filtrates in coastal dune areas prior to final drinking water processing. There is an extensive amount of studies on the effects of antibiotic use and the spread of resistance in non-Dutch aquacultures (see e.g. [14;81;88]). Such open aquacultures, however, are not operational in the Netherlands. Also, policies for sustainable fish cultures, including the use of antibiotics, are under development in the Netherlands (based on e.g., [27]). There is, however, some concern about the use of antibiotics for aquarium cultures of tropical fish.

Table 1. Sources of antibiotic resistance in (Western) European environments, likely to be also relevant for the Netherlands (determinations based on genetically based tests such as PCR).

ANTHROPOGENIC SOURCES	***REMARKS***
a) Hospital waste water and waste water from other clinical settings	Waste water from hospitals in Gent (Belgium) and Rotterdam (The Netherlands) was shown to contain various ARG types in bacterial hosts [32]. Studies by Volkmann et al. in German hospital and municipal waste water and other studies [61] also indicated the occurrence of antibiotic resistance in wastewater [74]. The abundance of vancomycin resistant enterococci and streptococci was circa 25% in waste water of a German hospital (45% was resistant to imipenem and circa 45% to ampicillin) [48]. Biofilms in German hospital waste water (n = 5): the average percentage of antibiotic resistant bacteria is 6.8-71% [60]. Sewage systems in a German hospital area showed particular hotspots with high levels of vancomycin resistant enterococci and multiresistant pathogens [personal communication of T. Schwartz, ITC-WGT]. Water in a city lake by a hospital contained as much resistant bacteria (75%) as dairy farm canal water [95]. More data on the role of STPs in § 3.3.
b) Sewage from households	Sewage contains antibiotic resistance [74; 96] a.o. due to antibiotics for public health care but also used for private tropical fish cultures. Water of an artifical residential lake (receiving no discharge) contained only 1.5% resistant bacteria; whereas recreation park canal water contained 54.2% resistant bacteria [95].
c) Manure of industrial farms with husbandry animals, incl. farms for veal calves	All faecal samples of pigs (n = 12) from German commercial animal farms showed Enterococcus faecium resistant to quinupristin/dalfopristine 37;60. There is a relatively low number of Dutch veal calve farms and they generally discharge their waste water on prepurification plants, before entering the sewage. More details on antibiotic resistance in manure and the consequences of using manure are found in § 3.4.
d) Waste water of facilities in the feed and food industries (e.g., slaughterhouses)	Slaughterhouses are likely sources of antibiotic resistance as organic waste fluids of treated animals will be discharged via the sewer. Indeed, waste water from Portuguese poultry slaughterhouses contained a wide variety of antibiotic resistance [18]. No significant differences were found in the resistance to various antibiotics of inflow *versus* effluent of eight Portuguese waste water treatment plants of poultry slaughterhouses. All samples contained high percentages of resistant isolates (e.g., up to 86% for tetracycline, and up to 46% for erythromycin) [18].
e) (Industrial) facilities discharging antibiotics	No data available, therefore a potential source. There is debate on the role of biocides and heavy metals as pollutants that may co select for antibiotic resistance (cross-resistance) [11;55].
g) Application of antibiotics for crop protection	No data available, therefore a potential source. In the Netherlands kasugamycin and streptomycin are used for crop protection. Streptomycin is effective against fire blight (*Erwinia amylovora*) in apple and pear orchards. Streptomycin-resistant *E. amylovora* has been found in various areas in countries of the EU.

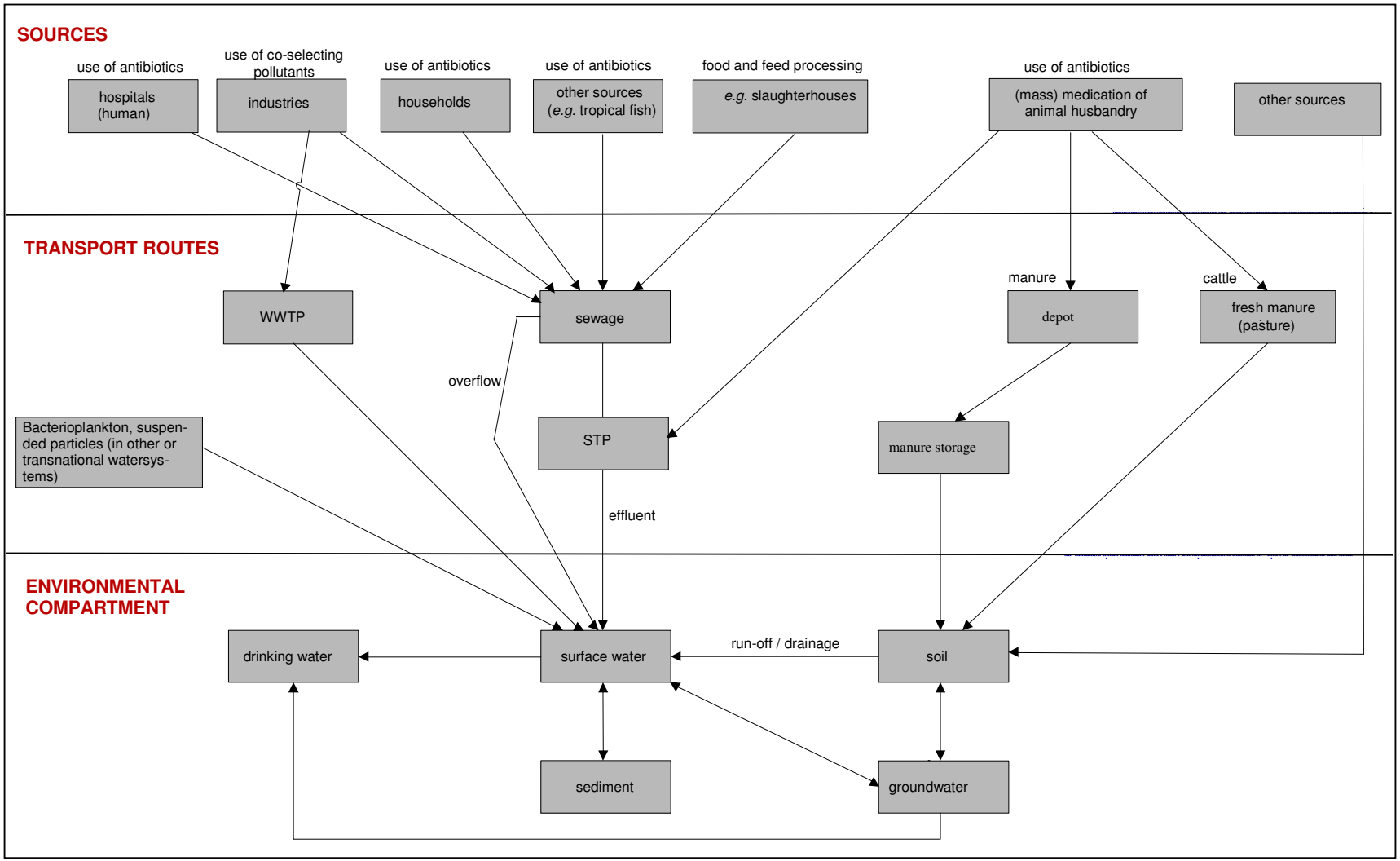

Figure 4. Transport routes for antibiotic resistance in the Netherlands.

3.2. Transport routes of antibiotic resistance

As antibiotic resistance in aquatic environments has been monitored only since a few years and on a modest scale [8;52], there is no clear understanding of its behaviour, fate and distribution, especially on a longer term. There is also no clear understanding of resistance transport routes in a quantitative way as such investigations are complex and require combinations of advanced DNA-based tracking techniques [68;76]. Therefore, the environmental behaviour and fate in the environment have been simplified in Fig. 4, showing the transport routes of antibiotic resistance in the environment. Transport routes and the relevant 'recipient' environmental compartments have been represented in this figure.

Transport in water may occur
(a) via free-floating ARB (bacterioplankton),
(b) via ARBs, sorbed to suspended particles that are subjected to drift,
(c) high molecular weight DNA, sorbed to suspended particles that are subjected to drift, and
(d) via free-floating high molecular weight DNA.
These routes are schematised in Figure 5.

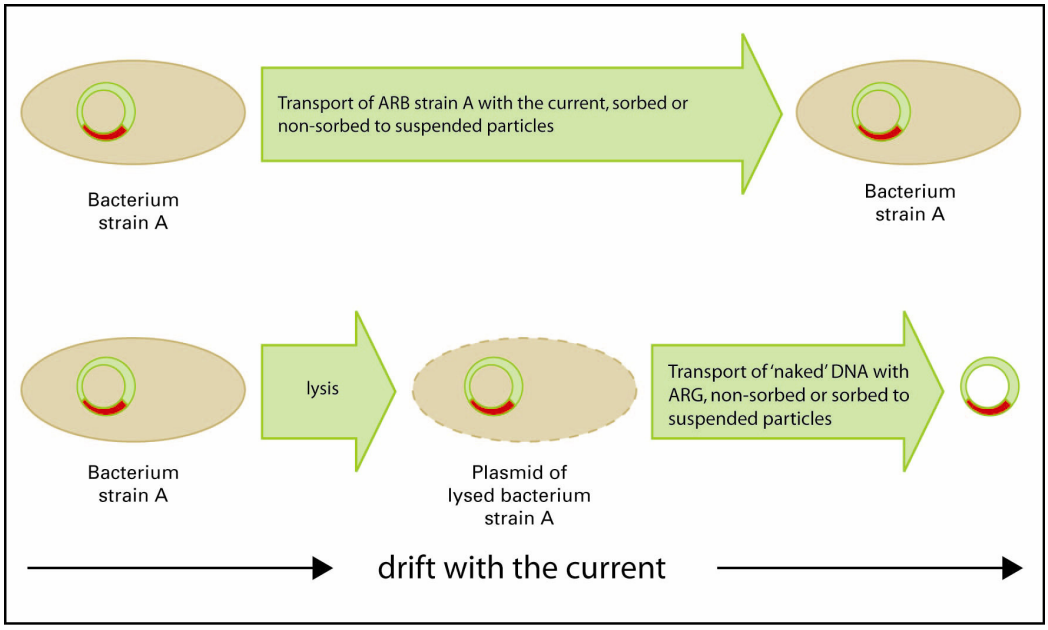

Figure 5. Transport of bacteria and extracellular ARGs.

After emission, ARGs may be degraded or redistributed in the sediment of surface water. Degradation will depend on the local microbial communities, their diversity and degrading capacities. Redistribution of resistance may occur via HGF (see § 2.4). High densities of micro-organisms are often found in the upper aerobic sediment layers thus creating micro-environments suitable for HGF. Increased antibacterial resistance in sedimentary bacteria is often one of the most sensitive indications of previous antibiotic use in aquaculture [39].

3.3. Transport via STPs

STPs and rivers play a major role in the spread of antibiotic resistance into the environment [19;29;35;61;86]. This may be via the discharges of effluent containing resistance from hospitals, but also via sewage overflows that may occur following periods of heavy precipitation [1]. In the latter case, sewage directly flows into surface water. As micro-organisms can accumulate in activated sludge, the transfers of mobile DNA to recipient micro-organisms could occur [39]. Gentamicin resistant bacteria have indeed been found in soil treated with polluted sewage sludge [32].

Da Costa et al. [18] found that substantial amounts of antibiotic resistant enterococci were able to pass Portuguese municipal STPs, in spite of an enterococci decrease of 0.5-4 log units. More than 4 million colony forming units of enterococci were counted per litre of STP effluent. Even in STPs equipped with UV disinfection, up to 61% of the sampled *Enterococcus* ssp. isolates were resistant to the clinically relevant rifampicin, although the absolute numbers of resistant isolates were the lowest of all STPs investigated. Other sources also report the passage of a part of the initial resistance [35]. Goni-Urriza et al. found comparable distribution patterns for riverine *Enterobacteriaceae* and *Aeromonos* ssp. downstream of an STP effluent discharge in Spain [29]. They concluded that urban effluent caused an increase in the percentages of resistant strains of both *Enterobacteriaceae* and *Aeromonos* ssp. over a downstream distance of 30 km. Ash *et al*. report ampicillin resistant bacteria downstream of several US cities [89]. Over 40% of the bacteria resistant to one or more tan one antibiotic had at least one plasmid. Ampicillin resistance genes were identified in 70% of the plasmids. The most common resistant bacteria were however Enterobacteriaceae or bacteria belonging to the genera Acinetobacter and Alcaligenes. Both genera are widespread in nature and in clinical and domestic areas.

Depending on the initial amount of resistance, the effectiveness and selectivity of the removal of micro-organisms, but also on the DNAse activity in the STP and the concentration of (co)selecting chemicals, ARGs or their carriers may persist. Indeed, both enteric ARBs and resistance genes may pass biodegradation and elimination in STPs, as has been confirmed by various studies [8;48;74;80]. Therefore, further transportation into the effluent receiving waters may occur under favourable conditions.

Rhodes *et al*. and Huys *et al*. report the occurrence of tetracycline resistance genes in both hospital effluent and aquaculture effluent in England and Ireland [90;91]. It was clear that tetracycline-encoding resistance genes have disseminated between different Aeromonas species and E. coli and between human and aquaculture environments in distinct geographical regions. Tolerance profiles in a specific environment do not necessarily reflect the corresponding tolerance profiles of the same type of environment in another country, mainly as a result of the unique taxonomic composition of each site.

Pruden et al. tracked *tet*O and *tet*W genes (coding for oxytetracycline resistance) in various compartments along the Poudre river in Colorado [52]. The genes were found in dairy lagoon water, sewage, river water, and filtrated drinking water. The research indicated that the abundance decreased from the anthropogenic 'sources' to the 'sinks' (i.e. the drinking water). The diversity of 10 different *tet* genes was compared between two Wisconsin (USA) STP influent and effluents, and two urban lakes (one in Wisconsin and one in Maine). The STPs influent sources were primarily domestic, with some industry. All STP samples contained more different types of *tet* genes (3 to 10), as compared to the lake water samples, which contained only *tet*A (*tet*Q was only detected with qPCR, which has a lower limit of detection than PCR). Gene copy numbers of *tet*G and *tet*Q in the samples were quantified via qPCR and normalised to both the volume of original sample and to the amount of DNA extracted per sample. Concentrations of *tet*Q were found to be the highest in wastewater influent while *tet*G concentrations were highest in activated sludge [80]. In a study from Illinois (USA), the *tet*O and *tet*W genes in cow rumens and pig faeces were identical [77]. A high level of sequence identity between the *tet*W genes found in bacteria of different genera isolated from different hosts (namely humans (UK, 1999), pig (Japan, 1974), sheep (Australia, 1996) and cows (UK, 1989; 1993), implies recent gene transfer events. Still, it is impossible to conclude from this evidence whether transfer of the gene has been from or to the human gut flora [79].

A high genetic diversity was found by Hamelin et al. (2006) among 308 environmental *E. coli* isolates, tested with DNA micro arrays [30]. These isolates were sampled in the Canadian recreational waters of the Great Lakes and not only analysed for antibiotic resistance but also for their pathogenicity. Fourteen % of these isolates showed one or more genes for antibiotic resistance. Relevant pollution sources in this respect were supposed to be various municipal STPs, discharging their effluents on these waters.

Heuer et al. determined in various European aquatic environments the presence or absence of clusters of gentamicin resistance. They found that clinically relevant isolates were predominant in sewage, faeces, and sewage polluted coastal water. Also, the diversity of the detected resistance gene clusters in these polluted environments was high, indicating different processes in different habitats. A higher proportion of mobile genetic elements was found, but not of gentamicin resistance. Mobile genetic elements (MGE), e.g. IncP-β plasmids which are extensively studied in clinical environments, were detected in a wide range of recipient bacteria in the aquatic environments. These recipients included CFB, α- and β-Proteobacteria which are phylogenetically very different from the original carriers in clinical environments. Additionally, laboratory mating experiments showed that donor bacteria from sewage were able to transfer gentamicin resistance to autochthonous bacteria at increased frequencies between 1×10^{-5} - 3×10^{-8} recipients per total number of (potential) recipients, possibly indicating selection pressure in the sewage inoculi. The increased abundance of relevant plasmids in the samples was probably an indication for increased selection pressure, though one may have expected a higher proportion of gentamicin resistant bacteria which was not the case. This investigation showed that in resistance-polluted environments the transfer of MGEs was increased thus changing the genetic diversity by increasing the diversity of resistance genes. Apparently the selection pressure was not so high that it changed the abundance of gentamicin resistant bacteria [32].

Sewage overflows, which can occur after heavy rainfall, are likely to spread antibiotic resistance in the environment. This was confirmed in a case study in which antibiotic resistance was frequently detected in mud samples from flooded areas after heavy flooding in 2002 of the rivers Elbe and Mulde in Germany [1]. In this study on the effects of facultative pathogenic micro-organisms high cell counts were found in bacterial isolates for multiple antibiotic resistance (MAR) in the river mud in flooded cellars, playgrounds and streets, one

year after the flooding, whereas such MAR cell counts for the river itself were much lower. The likely cause of the high cell counts was the overflow of nearby STPs.

Experimental research in Germany investigated the environmental fate of antibiotic resistance present in the waste water of hospitals [48;59-61]. It showed that following the influent and effluent of STPs, resistance genes were found not only in biofilms in the receiving surface water but also downstream in the river bank infiltrated drinking water. Sampling points in the house-branch connections of the water distribution system were 1-2 km from the waterworks where water was disinfected with UV. The river Rhine was upstream sampled. The waste water system and the hospital were in Mainz. Bacteria were cultivated and tested for their resistance, and their DNA was analysed by PCR and Southern Blot hybridisation or DGGE. The interpretation of the research is challenged by two facts: firstly, not all bacteria are culturable; and secondly; the DNA analysis is mainly qualitative and give a binary result: the genes are spotted or not. The method gives only a slight indication of the amount of genes present. The resistance genes *van*A (against vancomycin) and *amp*C (against beta-lactam) were found at every location based on the total DNA present; although the occurrence seems higher in the waste waters than downstream. In contrast, the gene against methicillin (*mec*A) was only found at the hospital waste water. The *van*A genes in the biofilms in the drinking water systems had a high degree of homology (96%) to the *van*A gene of the enterococcus *E. faecium* isolated from the hospital waste water. For the *amp*C gene, the degrees of homology to the *amp*C gene of the enterobacteriae *E. cloacae* and *Klebsiella pneumoniae* were 96% and 86%.

Are enteric bacteria present in the drinking water system biofilms, which could explain this homology? Separate analyses for Enterococci, Enterobacteriaceae, and heterothropic bacteria were performed. Within the Enterococci, the relative resistance decreased from 25% in the hospital waste water down to 1% in the river water. The researchers found also a decrease of the absolute number of Enterococci from hospital waste water to river bank filtered drinking water. The number of Enterococci in drinking water was nil. In short: resistant Enterococci are abundant in hospital waste water; and their numbers decrease downstream both in terms of total Enterococci, and in terms of the resistant fraction. There were no Enterococci in the biofilms in the drinking water systems. The picture for the Enterobacteriaceae is the same. The observed resistance genes in the drinking water are hence not attributable to the presence of Enterococci and Enterobacteriaceae. The percentage of antibiotic resistance among the culturable heterotrophic bacteria generally decreased from hospital waste water, activated

sludge, treated waste water and receiving river water (see table below, from Schwartz et al. [60]). However, these percentages in bank filtrated drinking water were higher. The variation between the samples was high. There is a difference in the percentages resistant bacteria in the different aquatic compartments (Table 2).

Table 2 Percentage of cultivated heterotrophic bacteria in biofilms with antibiotic resistance to selected antibiotics (Schwartz et al. [60]).

Compartment	**vancomycin**	**ceftazidime**	**cefazolin**	**penicillin**	**imipenem**
hospital waste water (n=5)	6.8±5.0	45±21	58±23	71±25	8.1±3.5
activated sludge (n=4)	11±3.8	44±17	39±16	30±8.0	2.8±0.2
treated waste water (n=5)	15±10	27±17	39±20	20±6.7	0.6±0.4
upstream river water (n=3)	2.3±0.5	11±1.6	8.1±0.0	31±3.3	0.4±0.1
drinking water (n=8)	20±10	5.1±2.4	48±27	43±26	0

Population shifts may explain the shift in these resistance patterns. Emtiazi et al. [24] found that DNA patterns of local bacterial communities differed between waste water, effluent, receiving and river bank filtrated water, and also in drinking water processed from this filtrated water showed a different pattern. However, despite the resistance these bacteria showed against these antibiotics, the resistance genes *van*A and *amp*C were not found in these culturable heterotrophes. This result indicates that :

- the homologue resistance genes *van*A and *amp*C found in drinking water biofilms are not located in Enterococci, Enterobacteriaceae (since these were absent), but also not on the culturable heterotrophic bacteria. Therefore other (non-culturable heterotrophic) bacteria or free DNA must account for the signal in the PCR.
- This presence of the *van*A and *amp*C resistance genes in the drinking water system biofilms may be the result of horizontal gene transfer from enteric bacteria to autochtonous aquatic bacteria, given the high degree of homology. This transfer may have happened on several occasions between the presumed source (waste water) and sink (surface water), although an autochthonous origin of this gene can not be excluded.
- the observed resistance in these culturable heterotrophic drinking water bacteria to selected antibiotics is apparenty not mediated via *van*A and *amp*C. Thus, this resistance is due to other mechanisms, for example other genes.

Keeping in mind that only up to 1% of all bacteria can be cultured, the overall contribution of the waste waters (possible sources) to the total resistance observed downstream (sinks) is

hence not competely revealed. There is insufficient information on the diversity of resistance mechanisms within the autochtonous bacterial communities (how many genes or other mechanisms are in place?), on the prevalence of these systems (what genes or systems are dominant?), and on the role the mechanisms play in the factual resistance potential of the bacteria (how much protection does the system offer?). The relevance of any anthropogenic addition or change cannot yet be assessed.

3.4. Transport via manured soil

Transport of resistance via animal faeces to the soil may occur (a) via in situ excretion of grazing cattle, but also following (b) application of organic manure onto, or (c) injection of manure into soil with grass or arable crops. Further dissemination to the aquatic environment may potentially occur (a) via drainage, runoff and erosion directly to surface water and (b) via leaching to groundwater. As surface water may be used for soil infiltration resistance may percolate into the soil as well, whereas resistance can flow via seepage from groundwater to surface water. There are no quantitative data available expressing the actual mass transport of resistance in these ways.

Large amounts of antibiotics are used for prophylactic or therapeutic purposes in animal husbandry in the Netherlands (broilers, pigs, veal calves). In animal husbandry 508 tonnes antibiotics were used in the Netherlands in 2005 [28]. A survey of the antimicrobial resistance and antibiotic use in farm animals in the Netherlands is published yearly (e.g., [42]). Although organic manure application is regulated by environmental standards for nutrients, the gross production of manure in the Netherlands is estimated to be 10 million tonnes (fresh weight) per year, indicating an average of circa 5000 kg manure per hectare [44]. This total weight of the manure is dependent on, amongst others, the husbandry animal, its feed, and water loss during storage. In view of the large amounts of manure applied on soil, these transport fluxes of antibiotic resistance to the soil may be one of the largest.

Only a slight effect of manure application on resistance in soil-borne bacteria has been detected in Danish field studies [36]. Other researchers found via plating that the application of manure increased the proportion of countable tetracycline resistant bacteria [62]. However, in five months, the tetracycline resistance declined to levels comparable to a non-manured soil,

and there was no significant change in macrolide and streptomycin resistance. In the same soils, the tetracycline resistance gene *tetM* showed elevated concentrations directly after manuring, and proportions were higher in agricultural soils than in garden soils [5]. Manure and residential soil fertilised with manure contained 95% and 70%, respectively, resistant bacteria. Of 200 culurable strains, 29% contained plasmids accounting for resistance. Plasmids with tetracycline resistance accounted for 65% of the plasmid pool [95]. Macrolide resistance in soil bacteria has been found to be related to the use of tylosin as feed additive by Onan and LaPara [50], who found that both the percentage of resistant bacteria increased and the types of resistant bacteria shifted from *Streptomyces*-like bacteria to α- and β- Proteobacteria. The local antibiotic usage pattern and soil properties might thus also contribute to the resistance observed at a given site. Recently, the mobility of resistance genes when connected to mobile genetic elements such as transposons has gained attention. Tetracycline resistance genes found in soil bacteria were often linked to class 1 integrons. These may harbour several resistance genes, and can be transferred to zoonotic pathogens such as *E. coli* [4].
Animal manure may be high in copper. In laboratory experiments, copper has been shown to select resistance mechanisms that also work against antibiotics [9]. Therefore, the emission of antibiotic resistance via the co-emission of copper cannot be excluded.

Bacterial pollution from manure in surface water enters the water by grazing cows or by spreading the manure onto the soil [71;75]. These bacteria are transported via water (through or over the soil) and therefore the precipitation surplus, i.e. the amount of precipitation minus evaporation and transpiration by plants, is relevant. Transport of these bacteria is also dependent on the soil type and hydrological conditions. Transport specifically occurs after rainfall [49;64]. It has been indicated that grazing cattle cause more bacterial pollution of drainage water than the application of liquid manure [43;73].

There are data on the effects of manure on the persistence of resistance in terrestrial environments that might be useful for aquatic environments. For instance, some publications have explicitly dealt with the hypothesis that manure enhances the frequency of occurrence of antibiotic resistant bacteria in soil. Exposure of soil to (high levels of) tetracycline resulted in a significant increase in the concentration of tetracycline resistant soil bacteria. Removing the selective pressure resulted in phenotypic shifts that returned the microbial community to initial conditions within 1 month [78]. In another study, the influence of antibiotics as selective agents was tested in microcosms against the inflow of resistant bacteria with manure [56]. It was

found that even unrealistically high concentrations of tetracyclines did not increase the diversity of tetracycline resistance genes or plate counts of resistant bacteria, but that manure amendment leads to the introduction of tetracycline resistance genes in soil [56]. Further, when soils were spiked with tetracycline-resistant enterococci instead of manure, the *tetM* gene could also be detected after 152 days, while its bacterial host reached levels below the detection limit after 90 days. This indicates that the persistence of genes is possibly longer than the persistence of suspected bacterial carriers of resistance.

Resistance has been found in Dutch surface water and sediments nearby industrial pig farms. These may have been emitted via manure and subsequent runoff or drainage. Montforts et al. [46] reported this in a qualitative research on the presence of veterinary antibiotics and antibiotic resistance in ditches and larger surface waters in the vicinity of animal husbandry farms in the Netherlands. They reported low concentrations of antibiotics in water and sediment: an order of magnitude of tenths of nanogrammes/L in water up to hundreds of nanogrammes/kg in sediment. There was a rough correlation between the presence of antibiotic residues and the development of resistance as determined by PCR. These results indicated that the use of antibiotics in pig farms, the use of pig manure nearby the farm and the increased presence of antibiotic resistance in nearby surface water were correlated. A causal relation, however, could not be established in view of the preliminary character of the study. The investigation also indicated that the contamination with antibiotic resistance nearby industrial pig farms appeared to be larger than in the vicinity of dairy farms.

There are no data available on the occurrence of antibiotic resistance in Dutch groundwater. Should ARGs be found in groundwater, these may have originated from percolation, because the selection pressure in groundwater will generally be low given the fact that antibiotics are rarely found in groundwater [48]. This was confirmed by in situ sampling and analysing groundwater in the US down to 13.5 m [15], indicating that the antibiotic resistance had been transported down, rather than in situ induced by percolated tetracyclines. Particularly in the latter study, tetracycline resistance genes had been dispersed into the environment (via manure storage and treatment in waste lagoons of industrial pig farms). Groundwater samples in the same area revealed various tetracyclines and their metabolites at concentrations $\leq$0.4 µg/L over the period 2000-2004 [41]. A three-year monitoring study revealed that seven *tet*-genes were continually detected in groundwater. At one site, an elevated detection frequency and concentration of *tet*-genes were observed in the wells located down-gradient of

the lagoon. Comparative analysis of *tet*W sequences revealed gene sequences almost identical (99.8% identity) to those in the lagoon, but these genes were not found in background libraries. Novel sequence clusters and unique indigenous resistance gene pools were also found in the groundwater. Thus, antibiotic resistance genes in groundwater are affected by swine manure, but they are also part of the indigenous gene pool [93].

Substantial transport of ARGs via subsoil drains to surface water or via percolation to groundwater may occur as shown by Aminov et al. [7]. In two large industrial pig farms in the US, the pigs consumed tetracycline containing feed, so the monitoring focused on the environmental behaviour and fate of tetracycline resistant genes (TRGs). The types of TRGs that were already present in the animal feed were not identical to the types in pig faeces, indicating no simple transfer route from feed to pigs. Several TRGs were identified in the waste lagoons nearby the pig farms that were used for manure storage and treatment. Some genes, namely *tetC* and *tetH*, were still identified in groundwater 250 m downstream of the lagoon, but not upstream of the lagoon. These monitoring results clearly indicate a further dispersion of most of these ARGs into the environment. The origin of the resistance genes in the pig feed was not reported.

4. Ecological effects and risks

When assessing the ecological risks of anthropogenic antibiotic resistance in aquatic environments one needs to:
(a) discern between anthropogenic and autochthonous levels, see § 2.2;
(b) identify and quantify the sources, emissions and the in situ environmental exposure to antibiotic resistance, see chapter 3;
(c) identify the aquatic organisms to be exposed, and possibly affected, see this chapter 4;
(d) estimate the adverse ecological effects of antibiotic resistance on exposed organisms, preferably in a quantitative way, see this chapter;
(e) assess the risks to biotic communities by assessing the likelihood of such adverse effects in relation to the exposure, see this chapter.

This whole process from (a) to (e) is generally referred to as the environmental risk assessment [72].

Respecting (a), it has been explained in § 2.2 that in various scientific studies it has been difficult to separate the autochthonous resistance from the anthropogenic resistance as genetic mobile elements encoding for the resistance may be transferred to autochthonous bacteria. It is, however, necessary to make this distinction if one wants to assess the anthropogenic contribution in particular.

Respecting (b), various studies on the sources of resistance, the emissions and the in situ environmental exposure indicate that antibiotic resistance is ubiquitous in aquatic environments, although quantitative data are generally lacking. Anthropogenic sources of resistance genes are the enteric bacteria in faeces from humans and husbandry animals treated with antibiotics. The data indicate that the genes reach aquatic environments via households, hospitals, slaughterhouses and municipal waste water or STP-effluents, and via manured soils. The behaviour and fate of antibiotic resistance genes in aquatic environments are complex. A part of the initial resistance in sewage is likely to pass STPs, its further distribution into water

systems and fate is much less clear. Their abundance may increase under conditions that favour their expression.

Respecting (c), it seems obvious that in view of the scientific backgrounds of antibiotic resistance, including the mechanisms of transfer and expression, autochthonous bacteria in exposed aquatic environments are the primary targets of antibiotic resistance. Environmental conditions may select further for unique resistant strains.

Respecting (d), there are no studies available that focus on the effects of antibiotic resistance on autochthonous microbial communities and higher organisms. There have been few investigations into the functioning of bacterial communities in antibiotic exposed media. For example, due to treatment with antibiotics, the sulphate reduction rate of marine sediment bacteria decreased; but this rate recovered together with the increase of number of resistant bacteria (determined by plating) [84]. This literature review shows that microbial ecology provides a scientific frame to investigate the effects of antibiotic resistance on microbial communities taking their function and structure into account. In spite of substantial progression in the field of microbial ecology, studying the effects of antibiotic resistance on bacterial communities is complicated because bacterial communities are complex and adaptive, and the replacement of species or isolates by others for vital functions is a common phenomenon (redundancy) [12]. Also, other aspects will have to be taken into account that may affect the population dynamics of local biocoenoses (e.g., the of other stressors like nutrients, temperature, other pollutants).

The contamination of surface water with ARGs may change the genotype of autochthonous bacteria as has been shown in various studies [31;46;48;51;52]. These genetic changes, however, will only be expressed in the presence of substances that act as antibiotics. At the same time it is unclear above which concentrations of antibiotics such expression will be evoked. Only the *expression* of resistance genes by the bacteria may have an effect — whether adverse or not — on the structure and function of autochthonous bacteria, because only then autochthonous bacteria that are not able to withstand antibiotic substances will be eliminated and resistant bacteria will prevail. Quantitative studies on the effects of antibiotic resistance genes on the structure and function of microbial communities and its ecological consequences are lacking.

Respecting (e), the risks of antibiotic resistance to exposed organisms in aquatic environments cannot be assessed. In view of the available data and information it is not possible to assess the likelihood that structural and functional effects on autochthonous bacterial communities occur.

5. Conclusions and recommendations

5.1. Discussion and conclusions

Exposure of surface water to antibiotic resistance genes from humans and husbandry animals treated with antibiotics seems evident. This exposure occurs primarily by waste water of hospitals, slaughterhouses and by municipal sewage, even after treatment. Manure from husbandry animals may enter surface waters via soils as well. Literature revealed that resistance genes present in enteric bacteria have been found in treated sewage and in water and sediment (biofilms) downstream of sewage treatment plants. Stressors in the environment, like nutrients, metals and chemicals, may select for (multiple) resistance. Recent Dutch research indicated that the use of antibiotics in pig farming leads to an increase diversity of bacterial resistance genes in the local aquatic environment. However, the studies did not conclude on the relationship between the presence of these resistance genes and numbers of resistant bacteria. Also, there is little information on the presence of resistance genes in unpolluted waters. Keeping in mind that only up to 1% of all bacteria can be cultured in the laboratory, the overall contribution of the waste waters and manure (possible sources) to the total resistance observed downstream (sinks) is hence not competely revealed. There is insufficient information on the diversity of resistance mechanisms within the autochtonous bacterial communities (how many genes or other mechanisms are in place), on the prevalence of these systems (what genes or systems are dominant), and on the role the mechanisms play in the factual resistance potential of the bacteria (how many and what concentrations of antibiotics can the bacteria tolerate). The relevance of any anthropogenic addition or change cannot yet be assessed.

The main conclusion of this study is that, in particular, communities are exposed nearby waste water or effluent outlets and nearby manured soil, and that the genetic constitution of local bacterial communities is affected. The effects of these genotypic changes on the function and structure of these bacterial communities and the ecosystem in general can only be hypothesised, since antibiotic resistance is also a natural phenomenon in autochthonous bacterial communities. There are no comparative experimental quantitative studies between

polluted and unpolluted sites into these potential differences in structure and function of bacterial communities.

In conclusion, the ecological effects and risks of antibiotic resistance in aquatic environments cannot be assessed in view of the available information that is often limited and not useful for risk assessment.

5.2. Recommendations

Changes in the diversity, abundance and functions of bacterial communities are hypothesised to be the primary (potential) effects of shifts in resistance gene populations in aquatic environments. The consequences of such shifts could be further analysed, whereby the natural variability in space and time of microbial communities should be taken into account.

The following recommendations are made. Quantitative measurements of antibiotic resistance are recommended (e.g., by real-time PCR with an appropriate detection system) in order to put the effects in a quantitative perspective. The choice of sampling locations is of importance. Monitoring of the molecular diversity of the resistance genes is recommended:

1. in surface water and sediment in the vicinity of sewage or waste water outlets;
2. in (small) water systems in rural areas, where emissions can be expected via manured soil (runoff, drainage, erosion);
3. comparable measurements in natural aquatic environments are advised as reference points (‘negative’ control; background levels).

This quantitative information is needed to determine the magnitude and diversity of the possible differences between locations, and to attribute these differences to anthropogenic activities. The information should then be placed in an ecological perspective. Relevant effect parameters could encompass functional parameters like the ability to degrade substrates, and structural parameters like the ratio between bacteria and fungi, and the biomass and diversity of consumer populations (nematodes, protozoans).

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ABBREVIATIONS AND DEFINITIONS

AMRG antibiotic multi-resistance gene (a gene encoding for resistance to more than one antibiotic)

ARB antibiotic resistant bacterium

ARG antibiotic resistance gene

autochthonous bacteria: (within the context of this study) bacteria which have no clear anthropogenic origin, contrary to enteric bacteria which are discharged via faeces

carrier the bacterium that carries the ARG(s)

DGGE denaturing gradient gel electrophoresis

enteric bacteria: intestinal bacteria, i.e. from the digestive tract

HGF horizontal gene flow

incl including

kbp kilobase pairs

MAR multiple antibiotic resistance

MDR multi drug resistance

MGE mobile genetic element

MRSA methicillin-resistant *Staphylococcus aureus*

om organic matter

OTC oxytetracycline

PCR polymerase chain reaction

qPCR quantitative PCR

STP sewage treatment plant

TRG tetracycline resistance gene

TO target organism

VGF vertical gene flow

WWTP waste water treatment plant