Vitenskapskomiteen for mattrygghet
Norwegian Scientific Committee for Food Safety

VKM Report 2017:29

The link between antimicrobial resistance and the content of potentially toxic metals in soil and fertilising products

Opinion of the Panel on Biological Hazards of the Norwegian Scientific Committee for Food Safety

Report from the Norwegian Scientific Committee for Food Safety (VKM) 2017:29

The link between antimicrobial resistance and the content of potentially toxic metals in soil and fertilising products

Opinion of the Panel on Biological Hazards of the Norwegian Scientific Committee for Food Safety
13.10.2017

ISBN: 978-82-8259-286-4
Norwegian Scientific Committee for Food Safety (VKM)
Po 4404 Nydalen
N – 0403 Oslo
Norway

Phone: +47 21 62 28 00
Email: vkm@vkm.no

www.vkm.no
www.english.vkm.no

Suggested citation: VKM (2017). The link between antimicrobial resistance and the content of potentially toxic metals in soil and fertilising products. Opinion of the the Panel on Biological Hazards of the Norwegian Scientific Committee for Food Safety. VKM Report 2017:29, ISBN: 978-82-8259-286-4, ISSN: 2535-4019. Norwegian Scientific Committee for Food Safety (VKM), Oslo, Norway.

The link between antimicrobial resistance and the content of potentially toxic metals in soil and fertilising products

Authors preparing the draft opinion

Yngvild Wasteson (Chair), Eystein Skjerve, Siamak Yazdankhah (VKM staff)

Assessed and approved

The opinion has been assessed and approved by Panel on Biological Hazards. Members of the panel are: Yngvild Wasteson (Chair), Karl Eckner, Georg Kapperud, Jørgen Lassen, Judith Narvhus, Truls Nesbakken, Lucy Robertson, Jan Thomas Rosnes, Olaug Taran Skjerdal, Eystein Skjerve, Line Vold

Acknowledgments

The Norwegian Scientific Committee for Food Safety (Vitenskapskomiteen for mattrygghet, VKM) has appointed a working group consisting of both VKM members and external experts to answer the request from the Norwegian Food Safety Authority. The project leader from the VKM secretariat was Siamak Yazdankhah. The members of the working group, Yngvild Wasteson and Eystein Skjerve, are acknowledged for their valuable work on this opinion. The Panel on Biological Hazards is acknowledged for comments and views on this opinion. VKM would like to thank Jan Thomas Rosnes, member of the Panel on Biological Hazards, and Kaare M. Nielsen, member of the Panel on Genetically Modified Organisms, for comments on this opinion.

Competence of VKM experts

Persons working for VKM, either as appointed members of the Committee or as external experts, do this by virtue of their scientific expertise, not as representatives for their employers or third party interests. The Civil Services Act instructions on legal competence apply for all work prepared by VKM.

Table of Contents

Summary

Potentially toxic metals (PTM), along with PTM-resistant bacteria and PTM-resistance genes may be introduced to soil and water through sewage systems, direct excretion, land application of biosolids (organic matter recycled from sewage, especially for use in agriculture) or animal manures as fertilisers, and irrigation with wastewater or treated effluents.

The Norwegian Food Safety Authority (NFSA) asked the Norwegian Scientific Committee for Food Safety (Vitenskapskomiteen for mattrygghet, VKM) for an assessment of the link between antimicrobial resistance (AMR) and potentially toxic metals (PTM) in soil and fertilising products.

The NFSA would like VKM to give an opinion on the following question related to the influence of potentially toxic metals on antimicrobial resistance:

- Can the content of arsenic (As), cadmium (Cd), chromium (CrIII + CrVI), copper (Cu), lead (Pb), mercury (Hg), nickel (Ni), and zinc (Zn) in soil and fertilising products that are relevant for Norway play a role in the development, spreading, and persistence of bacterial resistance to these elements, as well as cross or co-resistance to antimicrobial agents?

VKM appointed a working group, consisting of two members of the Panel on Biological Hazards, to prepare a draft Opinion document and answer the questions. The Panel on Biological Hazards has reviewed and revised the draft prepared by the working group and approved the Opinion document "The link between antimicrobial resistance and the content of potentially toxic metals in soil and fertilising products".

In this report we assess the following PTM: arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu), nickel (Ni), mercury (Hg), lead (Pb), and zinc (Zn), because of their possible presence in fertilisers and their potential to induce AMR in bacteria.

This assessment is based on internationally published data. There is no systematic monitoring for toxic metals in soils in Norway, and the levels are expected to be highly variable depending on the input sources, previous and current agricultural practices, and the characteristics of the soil. Data on PTM in fertilising products added to soil are also fragmented and limited. Fertilising materials, in the form of sewage sludge or livestock manure, will add toxic metals to the existing levels in soil, and in areas of intensive agriculture, the levels will be expected to be highest. The additive effect of toxic metals in fertilising materials must be assessed from a long-term perspective, as these metals accumulate in the environment.

Development of AMR can be partly regarded as a dose- and time-dependant response to exposure to different drivers for resistance. There is an indication that the presence of

potentially toxic metals is a driver for development of AMR in exposed bacteria, but the dose and time exposure most likely to cause this effect is not known. Investigation of PTM-driven co-selection of AMR in environments impacted by agriculture and aquaculture should focus especially on Cu and Zn, which are added to animal feed, and on Cd because of its high concentration, in comparison with other PTM, in inorganic fertilising products. The naturally occurring background resistance in environmental bacteria complicates the estimation of the effect of PTM exposure on development of resistance. In addition, it is difficult to distinguish between the natural resistome and an elevated abundance of AMR in environmental samples.

Spreading of resistance towards the PTM evaluated in this assessment involves cross- and co-resistance to antimicrobial agents used in prophylaxis and therapy in animals and people. Most important are those cases where toxic metal resistance is coupled to resistance towards *highly important* and *critically important* antibiotics. This has been described in some of the published articles included in this assessment. We do not fully understand the mechanisms behind persistence of AMR, and removing drivers for development and spread of resistance may result in a decrease in the levels of resistance, but not necessarily full disappearance.

There is lack of knowledge regarding links between the level and concentration of PTM in fertilising products and soil and development of resistance in bacteria. Data regarding the routes and frequencies of transmission of AMR from bacteria of environmental origin to bacteria of animal and human origin were lacking in the published articles reviewed here. Due to the lack of such data, it is difficult to estimate the probability of development, transmission, and persistence of PTM resistance in the Norwegian environment. More research is needed to explain the relationship between development of resistance against potential toxic metals and resistance toward antimicrobial agents in bacteria.

Key words: VKM, risk assessment, Norwegian Scientific Committee for Food Safety, potentially toxic metals, heavy metals, antimicrobial resistance

Sammendrag på norsk

Potensielt toksiske metaller (PTM), potensielt toksisk metall-resistente bakterier og potensielt toksisk metall-resistensgener, kan bli overført til jord og vann gjennom kloakk eller dyregjødsel, og gjennom bruk av gjødsel i landbruket.

Mattilsynet ba Vitenskapskomiteen for mattrygghet (VKM) om å vurdere sammenhengen mellom antimikrobiell resistens (AMR) og innholdet av potensielt toksiske metaller, populært kalt tungmetaller, i jord og gjødselprodukter, som kloakkslam og husdyrgjødsel.

Mattilsynet ønsket VKMs vurdering av følgende spørsmål:

> Kan innholdet av arsen (As), kadmium (Cd), krom (Cr), kobber (Cu), bly (Pb), kvikksølv (Hg), nikkel (Ni) og sink (Zn) i jord og gjødselprodukter som brukes eller er relevant å bruke i Norge spille en rolle i utvikling, spredning og persistens av resistens mot disse tungmetallene hos bakterier, og kryss eller co-resistens overfor antimikrobielle midler?

VKM satte ned en arbeidsgruppe bestående av to medlemmer fra Faggruppe for hygiene og smittestoffer for å utarbeide et utkast til uttalelse og for å svare på spørsmålet. Faggruppen har gjennomgått, revidert og godkjent uttalelsen.

I denne rapporten vurderer vi følgende potensielt toksiske metaller: arsen (As), kadmium (Cd), krom (Cr), kobber (Cu), nikkel (Ni), kvikksølv (Hg), bly (Pb) og sink (Zn). De er vurdert på grunn av deres mulige tilstedeværelse i gjødsel og deres potensiale til å utløse antimikrobiell resistens hos ulike bakteriearter.

Uttalelsen er hovedsakelig basert på internasjonalt publiserte artikler. Potensielt toksiske metaller i jord i Norge blir ikke systematisk overvåket men basert på tilgjengelige data, anser VKM det at nivåene av potensielt toksiske metaller er svært variable, avhengig av tilførsel av potensielt toksiske metaller, tidligere og nåværende landbrukspraksis og egenskaper ved jorden.

Data om potensielt toksiske metaller i gjødselprodukter overført til jord er også fragmentert og begrenset. Basert på tilgjengelige data, vil gjødselprodukter føre til atamengden av potensielle toksiske metaller i jord øker. I områder med intensivt landbruk vil nivåene forventes å være høyere. Mengden av potensielt toksiske metaller i jord må vurderes i et langsiktig perspektiv, fordi disse metallene akkumuleres i miljøet.

Utvikling av antimikrobiell resistens kan delvis betraktes som en dose- og tidsavhengig respons for ulike antimikrobielle midler. Mye tyder på at potensielt toksiske metaller er en drivkraft for utvikling av metallresistens hos bakterier, men dose- og tidseksponering som kan bidra til denne effekten, er ikke kjent.

For å undersøke resistens forårsaket av potensielt toksiske metaller fra landbruk og akvakultur, bør man rette særlig oppmerksomhet på kobber og sink som tilsettes dyrefor, og på kadmium på grunn av relativt høy konsentrasjon i uroganisk gjødsel. Det er imidlertid vanskelig å skille mellom resistens mot tungmetaller hos bakterier som skyldes potensielt toksiske metaller som finnes naturlig i miljøet, eller resistens som skyldes tilførsel via landbruk og akvakultur.

Resistens mot potensielt toksiske metaller innebærer også kryss- og ko-resistens mot antimikrobielle midler som brukes for å forebygge og behandle sykdom hos dyr og mennesker. Det gjelder spesielt når resistens mot potensielt toksiske metaller er koblet mot "viktige" og "kritisk viktige antimikrobielle midler". Denne koblingen er blitt beskrevet i flere av de publiserte artiklene som er vurdert.

VKMs har begrenset kjennskap til mekanismene bak resistens mot potensielt toksiske metaller og deres kobling mot antimikrobieller midler. Selv om eliminering av potensielt toksiske metaller kan føre til nedgang i resistensnivåene, vil det ikke nødvendigvis føre til at bakterier som er resistente mot toksiske metaller forsvinner.

Det er behov for mer kunnsakap om sammenhengen mellom nivået og konsentrasjonen av potensielt toksiske metaller i gjødselprodukter og jord og utvikling av resistens hos bakterier. Data om eksponeringsveier og om sannsynligheten for at metallresistente bakterier kan overføres fra miljøet til bakterier hos dyr eller mennesker, er mangelfull i de publiserte artiklene som denne vurderingen er basert på. På grunn av mangelen på slike data er det vanskelig å estimere sannsynligheten for utvikling, overføring og spredning av PTM-resistente bakterier i norsk miljø. Det er behov for mer forskning for å finne sammenhengen mellom utvikling av resistens mot potensielt toksiske metaller og resistens mot antimikrobielle midler hos bakterier.

Nølkkelord: VKM, riskovurdering, potensielt toksiske metaller, tungmetaller, antimikrobiell resistens

Abbreviations and/or glossary

Abbreviations

AMR	Antimicrobial resistance
ARB	Antimicrobial resistant bacteria
ARG	Antimicrobial resistance genes
ECDC	European Centre for Disease Prevention and Control
EPS	Extracellular polymeric substance
EUCAST	European Committee for Antimicrobial Susceptibility Testing
HGT	Horizontal gene transfer
MCC	Minimum metal co-selective concentration
MDR	Multidrug resistant
MIC	Minimum inhibitory concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
NFSA	Norwegian Food Safety Authority
NORM	The Norwegian monitoring programme for AMR in human pathogens
PTM	Potentially toxic metals
RND	Resistance-nodulation-cell division protein family
ROS	Reactive oxygen species
ToR	Terms of reference
VKM	Norwegian Scientific Committee for Food Safety
VRE	Vancomycin-resistant enterococci
WHO	World Health Organization

Glossary

Acquired resistance: Resistance to a particular antimicrobial agent to which the microorganism was previously susceptible. The change in resistance level is the result of genetic changes in a microorganism due to mutation(s), the acquisition of foreign genetic material, or a combination of both mechanisms.

Antibiotics: Traditionally refers to natural organic compounds produced by microorganisms that act in low concentrations against other microbial species, mostly bacteria. Today "antibiotics" also includes synthetic (chemotherapeutic) and semi-synthetic compounds (chemically modified antibiotics) with similar effects.

Antimicrobial agents: A general term for the drugs (antibiotics), chemicals, or other substances that either kill or inhibit the growth of microbes. The concept of antimicrobials applies to antibiotics, disinfectants, preservatives, sanitizing agents, and biocidal products in general.

Antimicrobial resistance: A property of microorganisms that confers the capacity to inactivate or exclude antimicrobials, or a mechanism that blocks the inhibitory or killing effects of antimicrobials.

Biofilm: Microbial biofilms are populations of microorganisms that are concentrated at an interface (usually solid/liquid) and typically surrounded by an extracellular polymeric slime matrix. Floccs are suspended aggregates of microorganisms surrounded by an extracellular polymeric slime matrix that is formed in liquid suspension.

Chemotherapeutics: Compounds with antimicrobial effect that are synthesized in the laboratory and that have no natural reserve in the environment. In modern popular literature, chemotherapeutics and antibiotics are commonly referred to as "antimicrobials".

Conjugation: Transfer of genetic material between different bacterial cells by direct cell-to-cell contact.

Co-regulation: The phenomenon called co-regulation arises when toxic metal exposure alters the expression of some antimicrobial resistance encoding genes, thus affecting the phenotype of the bacteria (Yu et al., 2017).

Co-resistance: Resistance occurring when the genes specifying different resistant phenotypes are genetically linked, for example by being located together on a mobile genetic element (e.g., a plasmid, transposon, or integron).

Cross-resistance: Resistance occurring when the same or similar mechanism(s) of resistance applies to different antimicrobials.

Fertilising product: A substance, mixture, microorganism, or any other material, applied or intended to be applied, either on its own or mixed with other material, on plants or their rhizosphere for the purpose of providing plants with nutrienst or improving their nutritional efficiency.

Heavy/potentially toxic metal: Naturally occurring elements that usually have a high atomic weight and a density at least 5 times greater than that of water.

Heavy metal resistance: Bacteria are considered to be resistant to heavy metals when: 1) a strain is not killed or inhibited by a concentration to which the majority of strains of a organism are susceptible, or 2) when bacterial cells are not killed or inhibited by a concentration acting upon the majority of cells in that culture.

Indicator bacteria: Bacteria that are used to measure the hygienic conditions of food, water, processing environments, etc. Indicator bacteria are not usually pathogenic, but their presence indicates that the product or environment tested may be contaminated with pathogenic bacteria, often originating from the same reservoirs as the indicator organisms.

Integron: Integrons are assembly platforms - DNA elements that acquire open reading frames embedded in exogenous gene cassettes and convert them to functional genes by allowing expression through a shared promoter.

Intrinsic resistance: A natural property of an organism resulting in decreased susceptibility to a particular antimicrobial agent.

Isolate (bacteria): A bacterial isolate is a single isolation in pure culture from a specimen.

Microbiome: The genes and genomes of the microbiota, as well as the products of the microbiota. This can also be referred to as the metagenome of the microbiota

Microbiota: Collective term for microflora (i.e., any type of microorganism) that may be found within a given environment.

Minimum Inhibitory Concentration (MIC): The lowest concentration of a given agent that inhibits growth of a microorganism under standard laboratory conditions.

Minimum co-selective concentration (MCC): Heavy metal concentration, which correlates with a detection of increased bacterial antibiotic resistance (the minimum **co-selective concentration** (MCC) of a metal) (Seiler and Berendonk, 2012).

Normal flora: Indigenous microbiota of human/animal external and internal surfaces like the skin, mouth, and gastrointestinal tract, and the upper respiratory tract. The normal flora contains numerous bacterial species, and numerous variants within each species.

Resistome: The collection of genes that could contribute to a phenotype of antimicrobial resistance.

Sanitizer: A chemical agent that reduces microbiological contamination.

Selection (bacteria): A process by which some bacterial species or strains in a population are selected for due to having a specific growth or survival advantage over other microorganisms. Antibacterial substances may provide a more resistant sub-population with such an advantage, enabling them to increase their relative prevalence.

Sterilization: The process of destroying all microorganisms (including spores).

Strain (bacteria): A strain is really a laboratory construction, and not a natural phenomenon, and is a subset of a bacterial species that differs from other bacteria of the same species by some minor, but identifiable, difference.

Susceptibility: Describes the response or vulnerability of a target microorganisms to an antimicrobial agent.

Sub-inhibitory concentration: A concentration that is below one capable of inhibiting detectable growth and replication of a microorganism within a defined time period.

Transduction: Transfer of genetic material from one bacterial cell to another via bacteriophages (viruses that infect bacteria).

Transformation: Direct uptake from the environment of fragments of naked DNA and their incorporation into the bacterial cell's own genome.

Transposon: A segment of DNA that is capable of moving into a new position within the same or another chromosome or plasmid. Also called jumping gene.

Background as provided by the Norwegian Food Safety Authority

There are three Norwegian regulations regulating the maximum levels of potentially toxic metals in fertiliser products. In both the national regulation on marketing of fertilisers and liming material and the Norwegian regulation implementing the EU Fertilisers Regulation (EC) No 2003/2003 there is a limit value for cadmium (Cd) in phosphorus (P) fertilisers. The limit value is 100 mg Cd/kg P. There are no maximum levels for other potentially toxic metals in these regulations.

When it comes to organic products, the products are divided into four categories based on the content of potentially toxic metals as shown in Appendix I. There are use restrictions coupled with the different categories. Category III products are, for example, not allowed to be used in agriculture. Products in categories I and II can only be used in agriculture if the heavy metal content in the soil is below the maximum levels given in § 26 (https://lovdata.no/forskrift/2003-07-04-951/§26-Forskrift 4. juli 2003 nr. 951 om gjødselvarer mv. av organisk opphav).

As part of the focus on a circular economy, the European Commission released a draft template for a new regulation on fertilising products in March 2016. The draft is currently under discussion, so it is still uncertain what will be the outcome. Several changes in the maximum levels for potentially toxic metals have been suggested in the discussions. The maxium levels suggested differ between product categories, as well as subcategories. The maximum levels suggested are based on maximum levels that are common to find in European fertiliser regulations today. Which levels that are achievable for the industry are also taken into account. The exception is cadmium (Cd), for which some risk assessments have recently been conducted and are used as a basis for the suggested maximum levels.

In the draft regulation, there is no requirement to label the content of potentially toxic metals, except for copper (Cu) and zinc (Zn), since these elements also are micronutrients. This makes it difficult to couple EU fertilising products with restricted use on the basis of the content of potentially toxic metals. The labelling requirement for organic fertilisers and soil improvers for Cu is 200 mg/kg dry matter and for Zn 600 mg/kg dry matter.

The Norwegian Food Safety Authority (NFSA) would like to request a risk assessment of the link between antimicrobial resistance and potentially toxic metals in soil and fertilising products. There is a need to know more about such metals in soil and fertilising products, and their influence on antimicrobial resistance.

Like all natural elements, the potentially toxic metals arsenic (As), cadmium (Cd), chromium (CrIII + CrVI), copper (Cu), lead (Pb), mercury (Hg), nickel (Ni), and zinc (Zn) are present in soil. These elements are also found as contaminants in fertilising materials.

In recent years, there has been increasing focus on antimicrobial resistance and it is known that some potentially toxic metals in soil and fertiliser products, like Cu, Zn, and Cd, can play a role in the development of resistance and cross- or co-resistance in bacteria. These resistant bacteria and the resistance genes can possibly be spread, both to those who handle the manure and to the environment. In addition, the genes associated with resistance may possibly spread further to those who handle the manure and to the environment.

Terms of reference as provided by the Norwegian Food Safety Authority

The Norwegian Food Safety Authority would like VKM to give an opinion on the following questions related to the influence of potentially toxic metals[1] on antimicrobial resistance:

Can the content of arsenic (As), cadmium (Cd), chromium (CrIII + CrVI), copper (Cu), lead (Pb), mercury (Hg), nickel (Ni), and zinc (Zn) levels in soil and fertilising products that are relevant for Norway play a role in the development, spread, and persistence of bacterial resistance to these elements, as well as cross or co-resistance to antimicrobial agents?

[1] We use the term "potentially toxic metals" (PTM) rather than heavy metals in this assessment.

1 Introduction

In the last decade, we have witnessed a dramatic increase in both the proportion and absolute number of bacterial pathogens presenting multidrug resistance to antimicrobial agents. Organizations such as the US Centers for Disease Control and Prevention (CDC), the European Centre for Disease Prevention and Control (ECDC) and the World Health Organization (WHO) consider those infections caused by multidrug-resistant (MDR) bacteria as threatening global disease and major public health concerns (Roca et al., 2015).

In environmental ecosystems, potentially toxic metals (PTM)/heavy metal contaminants may interact with native microorganisms residing in in the same ecosystems. These organisms have developed resistance mechanisms that allow them to survive and, in some instances, to remove/reduce the concentrations of contaminants in their environments. The co-occurrence of antimicrobial resistance (AMR) and metal resistance in bacteria has been reported in many review articles (Baker-Austin et al., 2006; Knapp et al., 2011; Nies, 1999; Seiler and Berendonk, 2012; Yazdankhah et al., 2014). This co-occurrence is caused by cross- and co-resistance phenomena. Cross-resistance occurs when the same mechanism simultaneously reduces the susceptibility to metals and antimicrobial agents used in therapy, and co-resistance occurs when separate resistance genes are situated on the same genetic element (Baker-Austin et al., 2006). Some studies suggest that metal contamination in natural environments could have an important role in the maintenance and proliferation of AMR (Alonso et al., 2001; Summers et al., 1993). This is of particular concern, considering that PTM/heavy metals of anthropogenic origin, such as agricultural and aquacultural practices, are currently found at several orders of magnitude greater than levels of pharmaceutically produced antimicrobials (Stepanauskas et al., 2005). Unlike pharmaceutically produced antimicrobial agents, metals are not subject to degradation and therefore represent a long-term selection pressure. Thus, there are concerns regarding the potential of metal contamination to maintain a pool of AMR genes in both natural and clinical settings.

After use, antimicrobials, including PTM, along with antimicrobial-resistant bacteria (ARB) and antimicrobial resistance genes (ARGs), including genes encoding resistance against heavy metals, may enter soil and water through sewage systems, direct excretion, land application of biosolids (organic matter recycled from sewage, especially for use in agriculture, included in Norway) or animal manures as fertilisers, and irrigation with wastewater or treated effluents. The presence of active antimicrobial compounds and their metabolites and toxic/heavy metals in environmental compartments may also select for resistance in environmental bacterial communities or microbiota.

Organic and inorganic fertilisers

A "fertilising product" is a substance, mixture, microorganism, or any other material, applied or intended to be applied, either on its own or mixed with another material, on plants or their rhizosphere (the area near the roots of the plants) for the purpose of providing plants with nutrients or improving their nutritional efficiency (http://www.mattilsynet.no/om_mattilsynet/engelsk_hoveddokument.22441/binary/Engelsk%20hoveddokument).

Commercial phosphate (P) fertilisers and "agricultural liming materials" contain low concentrations of PTM/heavy-metal contaminants. Animal manures and sewage sludge (biosolids), both treated and untreated, are the main organic fertilisers that may contain heavy metal contaminants, whereas inorganic fertilisers mainly contain Cd. PTM in biosolids may be found in the inorganic form or may be organically complexed, which could affect their toxicological profile and stability, and their chemical reactions in soil. These PTM may accumulate in soil with repeated fertiliser applications (Mortvedt, 1996).

Heavy metals OR toxic metals

According to the International Union of Pure and Applied Chemistry, the term "heavy metal" is a "meaningless term" because there is no standardized definition of a heavy metal (https://www.iupac.org/publications/ci/2001/november/heavymetals.html). This link lists all current definitions of the term "heavy metal" that the author (John H. Duffos) has been able to trace in scientific dictionaries and other relevant literature. It should be noted that the term is frequently used without an associated definition, presumably by authors who assumed that there was consensus about the meaning of the term. The list shows that this assumption is wrong and explains some of the confusion in the literature and in related policies and regulations. Some light metals or metalloids are toxic, but some high-density metals are not. For example, cadmium is generally considered a heavy metal, with an atomic number of 48 and specific gravity of 8.65, whereas gold is typically not toxic, but has an atomic number of 79 and a specific gravity of 18.88. For any given metal, the toxicity varies widely, depending on the allotrope or oxidation state of the metal. Most heavy metals are naturally occurring elements, usually with high atomic weight and a density at least 5 times greater than that of water.

Because of confusion regarding the term "heavy metals", we use the term **"potentially toxic metals" (PTM)** rather than heavy metals throughout this assessment.

Metals can be classified into four major groups based on their health importance (Kochare and Tamir, 2015):

- Essential: Cu, Zn, Co, Cr, Mn, and Fe. These metals also called micronutrients and are toxic in organisms when taken in excess of requirements.
- Non-essential: Ba, Al, and Li.
- Less toxic: Sn and Al.
- Highly toxic: Hg, Cd, As[2].

[2] As is a metalloid with both metallic and non-metallic properties, but will be included under the PTM group in this document.

Some heavy metals have been used as antimicrobial agents since antiquity, but their modes of action differ from those of classical antimicrobial agents. Among these metals, Zn, Ni, Cu, and Cr are toxic metals with high to moderate importance as trace elements and As, Cd, Hg, Pb have no beneficial functions in this context and should be considered entirely as toxic (Nies, 1999).

In this report we assess the following PTM: arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu), nickel (Ni), mercury (Hg), lead (Pb), and zinc (Zn), because of their possible presence in fertilisers and their potential to induce AMR in bacteria.

2 Literature

2.1 Literature search strategy

For **review articles**: the search was conducted in PubMed using the terms: Heavy metals, AND Antimicrobial resistance AND Review using the Advanced Search Builder provided in PubMed (www.ncbi.nlm.nih.gov/pubmed), limited to the period 1999-2017. This resulted in 156 hits (20. January 2017).

For **original articles**: the search was conducted in PubMed using the terms: different heavy metals listed in Table 1 [Title/Abstract] AND Antimicrobial resistance AND sewage or manure using PubMed (www.ncbi.nlm.nih.gov/pubmed). This resulted in 89 (sewage=65, manure=24) hits for all toxic metals assessed in this report (20. January 2017).

For this further search, the terms used were: different potentially toxic metals/ heavy metals, AND Antibiotic resistance or antibiotic resistant AND organic fertiliser/fertilizer" or "waste” or “effluent”. Only 12 citations that had not previously been identified and fulfilled the criteria for inclusion were obtained. In addition to the articles obtained by the primary searches, a few other relevant articles, were found referenced in the initially identified articles and were also included.

Table 1 shows the output of all PubMed search conducted in PubMed resulting in 107 citations (05.04.2017).

2.1.1 Inclusion criteria

For review articles, we limited our search to the period 1999-2017. For original articles, due to limited numbers of articles identified, all articles from the search strategy were included.

2.1.2 Exclusion criteria

Articles describing development of resistance in microorganisms other than bacteria, such as viruses, fungi, and parasites, were excluded, as these were not part of the mandate. Articles that were not in English or a Scandinavian language (Swedish, Danish, and Norwegian) were also excluded.

2.1.3 Papers considered

The titles and abstracts of all literature sources identified were screened by one person and those that did not relate to the terms of reference were excluded. For articles of potential relevance, the full text was obtained and assessed for relevance to this Opinion (Table 1).

Review articles

Review articles that focused on bacteria with reduced susceptibility against arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu), nickel (Ni), mercury (Hg), lead (Pb), or zinc (Zn) were included in this assessment. These review articles were used mainly to present information regarding **mode of action and mechanisms of resistance**.

Original articles

When using the search terms "Antimicrobial resistance" AND "heavy metals" AND "fertilisers", no articles were identified. Therefore, we used the search terms "manure" or "sewage sludge", which include organic fertilisers.

We did not identify any reports/studies of the Norwegian environment relevant for this assessment.

Table 1. Numbers of screened, excluded, and included articles identified through All PubMed searches.

Articles	PubMed n	Excluded n	Included n
Review	245	210	35
Original	196	155	41*
Total	441	365	76

n: number.

*Most of the articles included more than one, and up to 10, PTM.

3 Hazard identification

Hazard identification is implicit in the title of this opinion and in the terms of reference (ToR). The issue of AMR is addressed either as a **direct hazard** or as an **indirect hazard**.

- The direct hazard is antimicrobial-resistant pathogenic bacteria resulting as a direct outcome from exposure to PTM.
- The indirect hazard arises through resistance forming in a non-pathogenic bacterium that can subsequently act a source of resistance after horizontal gene transfer (HGT) into a pathogenic bacterium. In this case, the hazard is the potential for transfer of resistance genes.

In some cases, both hazards may be relevant.

4 Hazard characterization

The key factors contributing to the identified hazard is illustrated in **Figure 1**.

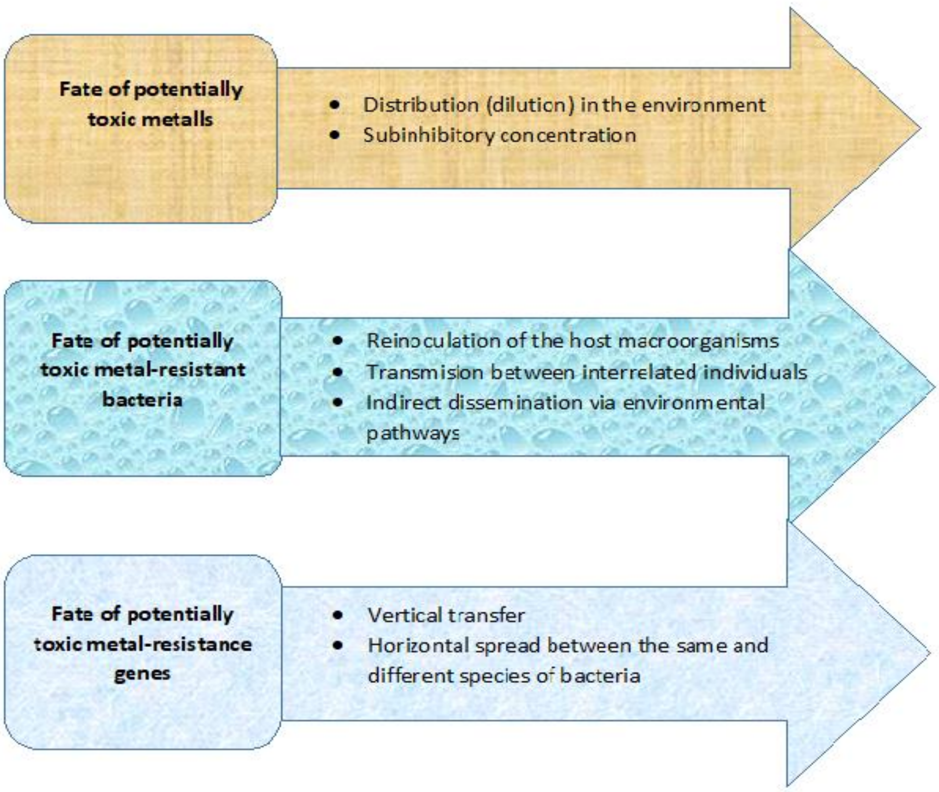

Figure 1. The development and dissemination of resistance is influenced by the release and stability of potentially toxic metals, the fate of bacteria with newly acquired resistance and their pathogenicity as well as the potential for HGT of resistance genes. Figure adapted from (da Costa et al., 2013).

4.1 Mode of action of toxic metals

In a metal, atoms readily lose electrons to form cations that are surrounded by delocalized electrons. This behaviour is responsible for the conductivity and antimicrobial effects of metals (Fraise et al., 2012). Metals may be toxic to bacteria, and this microbial toxicity may be due to their chemical affinity for thiol groups of macro-biomolecules, but also depends on the solubility of the metal compounds under physiological conditions (Lemire et al., 2013). Several possible modes of action of toxic metals have been reported:

a- Protein dysfunction
b- Production of reactive oxygen species (ROS) and antioxidant depletion
c- Impaired membrane function
d- Interference with nutrient uptake
e- Genotoxicity

These modes of action are illustrated in **Figure 2** and potentially toxic mechanisms related to specific metals are described in **Table 2**.

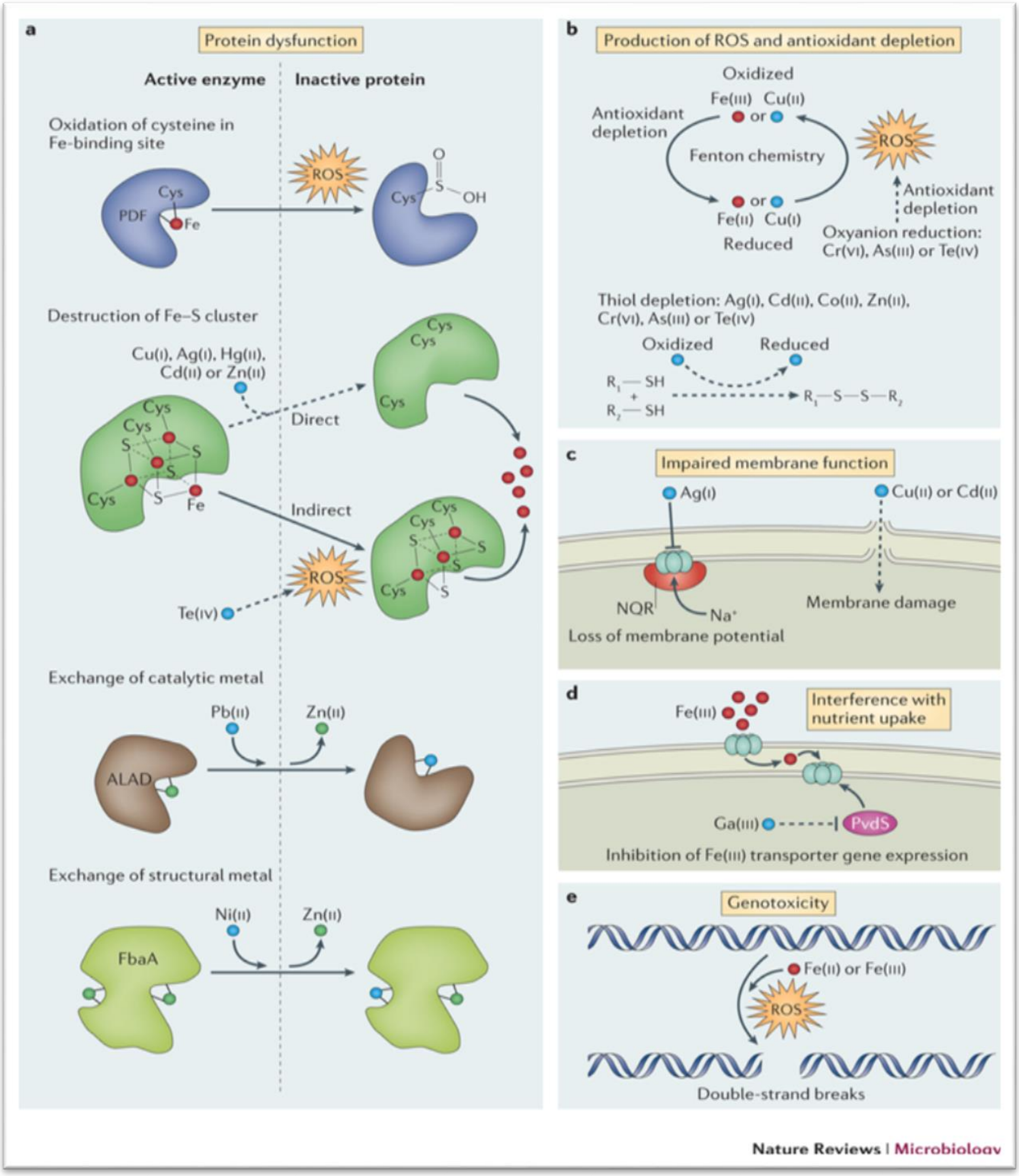

Figure 2. Exemplified mode of action of heavy metals (Lemire et al., 2013). These mechanisms of toxicity are specific to particular metal species. a) Metals can lead to protein dysfunction. b) They can also lead to the production of ROS and depletion of antioxidants. c) Certain metals have been shown to impair membrane function. d) Some can interfere with nutrient assimilation. e) They can also be genotoxic. Solid arrows represent pathways in which the underlying biochemistry has been elucidated, whereas dashed arrows represent a route of toxicity for which the underlying biochemical mechanism is unclear. ALAD, δ-aminolevulinic acid dehydratase; FbaA, fructose-1,6-bisphosphate aldolase; NQR, NADH:quinone oxidoreductase; PDF, peptide deformylase; PvdS, a σ-factor (σ24) from *Pseudomonas aeruginosa*. With permission from Nature Publishing Group.

Table 2. Mechanisms of action of potentially toxic metals in bacteria.

Toxic metal	Mechanisms of action
Arsenic*, **	As is a toxic metalloid that exists in two major inorganic forms: arsenate and arsenite. Arsenite disrupts enzymatic functions in cells, while arsenate behaves as a phosphate analogue and interferes with phosphate uptake and utilization (Kaur et al., 2011).
Cadmium**	Cd is the most toxic heavy metal, especially against microorganisms. The effects may be summed up under the general headings: "thiol-binding and protein denaturation", "interaction with calcium metabolism and membrane damage", "interaction with zinc metabolism", and "loss of protective function". The *dsbA* encoding gene for a product required for disulphite formation, leads to Cd resistance in Gram-negative bacteria (Nies, 1999).
Chromium***	Cr is a micronutrient metal and may be toxic when its concentration exceeds requirements. As a transition metal, it exists in different valency states, ranging from –II to +VI, with Cr(VI) and Cr(III) being the dominant species in the environment. Out of two commonly occurring states, Cr(VI) is toxic to biological systems due to its strong oxidizing potential that invariably damages the cells (Kotas and Stasicka, 2000). Cr(VI) is known to be harmful to all forms of living systems (Wise et al., 2004), including microorganisms (Ackerley et al., 2006).
Copper***	Cu interacts readily with molecular oxygen. Its radical character makes Cu very toxic. Cu toxicity is based on production of hyperoxide radicals and on interaction with cell membranes (Nies, 1999).
Lead	Pb has a low biological available concentration due to its low solubility. Thus, Pb is not extraordinarily toxic to microorganisms (Nies, 1999). Some forms of lead-salt, like lead acetate or nitrate, induce mutagenicity and DNA breaks at a toxic dose in some bacterial species (Tchounwou et al., 2012).
Mercury**	Hg toxicity has been attributed to the inactivation of enzymes and interference with other protein functions by the tight binding of mercuric ions to thiol and imino nitrogen groups in these, or the displacement of other metal cofactors from enzymes. Mercuric ions also bind to nucleotides and lipids, interfering with DNA function and contributing to lipid peroxidation. Mercuric ions and organomercurials have the ability to pass rapidly through biological membranes, and organomercurials are highly lipid soluble (Clarkson and Magos, 2006).

Toxic metal	Mechanisms of action
Nickel	Four mechanisms of Ni toxicity have been proposed: 1) Ni replaces the essential metal of metalloproteins; 2) Ni binds to catalytic residues of non-metalloenzymes; 3) Ni binds outside the catalytic site of an enzyme to inhibit allosterically; and 4) Ni indirectly causes oxidative stress (Macomber and Hausinger, 2011). Oxidative stress to Ni toxicity in microorganisms is known and some studies have shown that cells subjected to oxidative stress exhibit enhanced DNA damage, protein impairment, and lipid peroxidation, along with increased titres of oxidative stress defence systems; reviewed by (Imlay, 2003).
Zinc**	Zn ions inhibit multiple activities in bacterial cells, such as glycolysis, transmembrane proton translocation, and acid tolerance (Phan et al., 2004). Trace elements like Zn may be toxic to bacteria and this may be due to their chemical affinity to thiol groups of macro-biomolecules, but may also be dependent on the solubility of the metal compounds under physiological conditions; reviewed by (Yazdankhah et al., 2014).

* Arsenic is not a true metal, but a semimetal (a semimetal or metalloid is a chemical element that has the properties of both metallic and non-metallic elements)

** As, Hg, Cd are considered non-essential elements in living organisms.

*** Cu, Zn, and Cr are also essential metals in living organisms.

4.2 Methodology and terminology

Antimicrobial susceptibility testing with phenotypic methods is based on the measurement of the minimum inhibitory concentration (MIC) with the use of defined clinical breakpoints to categorize the test organism as susceptible, intermediate, or resistant. Phenotypic antimicrobial susceptibility testing requires an agreement on breakpoints and a rigorous standardization of methods and materials (Kahlmeter, 2015). Standardization of methods and materials for antimicrobial agents used in therapy and prophylaxis is performed by the European Committee for Antimicrobial Susceptibility Testing, EUCAST (http://www.eucast.org) in Europe, and by Clinical Laboratory Standard Institution, CLSI (http://clsi.org/m100/) in USA. The standardization includes many experimental parameters, such as preparation of media, inocula, inoculation of agar plates, application of antimicrobial discs, incubation of plates, examination of plates after incubation, measurement of inhibition zone diameters, and interpretation of results, and quality controls (Matuschek et al., 2014). Such standardized methods for determination of MIC-values for **toxic metals** have still not been established, although official methods for the determination of toxic metals in feed and food exist (https://ec.europa.eu/jrc/en/eurl/heavy-metals/legislation). Some limitations regarding determination of toxicity of toxic metals to bacteria have been discussed elsewhere (Rathnayake et al., 2013).

Our knowledge regarding the activity of toxic metals against the different bacterial species present in fertilising products and in soil are limited. Data regarding the biological effects of sub-inhibitory concentration of toxic metals, which for some antimicrobials are known to induce resistance in different bacterial species at the laboratory level, have not been identified in publications included in this assessment.

Terms such as "resistance" and "tolerance" have acquired specific technical meanings in the field of antimicrobials. The current terminology in microbiology distinguishes between clinical and microbiological antimicrobial resistance, particularly for antimicrobials used for therapy and/or prophylaxis. **Clinical resistance** is present when phenotypic testing of a microbe/antimicrobial combination against a clinical breakpoint indicates that therapeutic failure is likely, even with maximal dosing. **Microbiological resistance** is defined by the presence of an acquired or mutational resistance mechanism to the drug in question, in comparison with a fully susceptible "wild-type", and may be assessed by genetic analysis or phenotypic testing against a wild-type cut-off value. The clinical resistance scenario is clearly not applicable in the case of biocides/toxic metals, so, to avoid ambiguity, it is desirable to avoid using "resistance" in relation to the activity of these agents. However, in this document we use the term "resistance" since other terms have not been established, yet. Similarly, the non-specific use of the term "tolerance" is discouraged. The preferred terminology of many authors concerning variation in the effects of biocides/toxic metals upon bacteria is "reduced/increased susceptibility", or variants thereof (Wales and Davies, 2015).

In 2012, Seiler and Berendonk introduced the **minimum co-selective concentration** (MCC) of a metal (Seiler and Berendonk, 2012). MCC is defined as the minimum toxic metal concentration that correlates with detection of increased bacterial antibiotic resistance in co-regulation of a bacterial community/environment.

See Glossary for other terminology like co-selection, co-resistance, and co-regulation.

Most data regarding other antimicrobial agents, like biocides and toxic metals, are obtained from studies using planktonic phase microorganisms rather than microorganisms in more natural conditions, such as in a biofilm. Notably, gene expression in microorganisms living in a biofilm differs from that in planktonic cells, and the concentration of a compound needed to kill microorganisms in biofilms may be 10-500 times higher than in the planktonic phase (Yazdankhah et al., 2006).

4.3 Mechanisms of resistance

In order to avoid cellular toxicity from elevated exposure to potentially toxic metals, bacteria have evolved mechanisms of metal resistance. The mechanisms of resistance to toxic metals are presented in detail in the review article of Seiler and Berendonk (2012). The authors concluded that, like antimicrobial agents, toxic metals might promote the spread of AMR via co-selection.

Resistance mecahnisms for PTM may be divided into the following three groups:

1- **Complex formation** or sequestration of toxic metals (Silver and Phung, 1996). By selective binding with macromolecules, the concentration of the free toxic ions in the cytoplasm is reduced. Biosorption of toxic metals is known from cell membranes, cell walls, and extracellular polymeric substance (EPS) of biofilms (Harrison et al., 2007). For example, the EPS matrix and the polysaccharides contained in biofilm have been reported to bind toxic metals (Teitzel and Parsek, 2003). Thus, the metal tolerance of bacteria belonging to that biofilm is enhanced.
2- **Detoxification** through reduction of intracellular ions (Nies, 1999). A well-understood example is mercury reductase, encoded by the *merA* gene. The MerA protein reduces Hg^{2+} ions to the less toxic Hg^0 (Schiering et al., 1991). Hg^0 will then diffuse out of the cell, due to its low evaporation point (Nies, 1999).
3- **Excretion of toxic ions** by efflux systems (Nies and Silver, 1995). The cation/proton antiporter Czc, known, for example, from *Alcaligenes eutrophus*, mediates resistance to the metal ions Cd^{2+}, Zn^{2+}, and Co^{2+} by removal of metals from the cytoplasm though the inner and outer membranes to the surrounding environment (Silver and Phung, 1996).

These mechanisms are considered in greater detail for the PTM assessed in this report in **Table 3**

A database of antibacterial biocide- and metal-resistance genes has been established, based on an in-depth review of the scientific literature, by Pal et al (2014). The BacMet database (http://bacmet.biomedicine.gu.se) contains 470 experimentally verified resistance genes (Pal et al., 2014). In addition, the database also contains 25 477 potential resistance genes obtained from public sequence repositories. All resistance genes in the BacMet database have been organized according to their molecular function and induced resistance phenotype. This collection of genes enables correlations between metal resistance and antimicrobial resistance to be made, by investigating how often old and new emerging strains carrying either one of both type of resistances simultaneously.

Many of the toxic metals assessed in this report are among the metals with the highest number of known resistance genes reported by (Pal et al., 2014) (**Figure 3**). The BacMet database may facilitate research to improve our understanding of co- and cross-resistance of biocides and metals to antibiotics within bacterial genomes, as well as in complex microbial communities (metagenomes) from different environments (Pal et al., 2014).

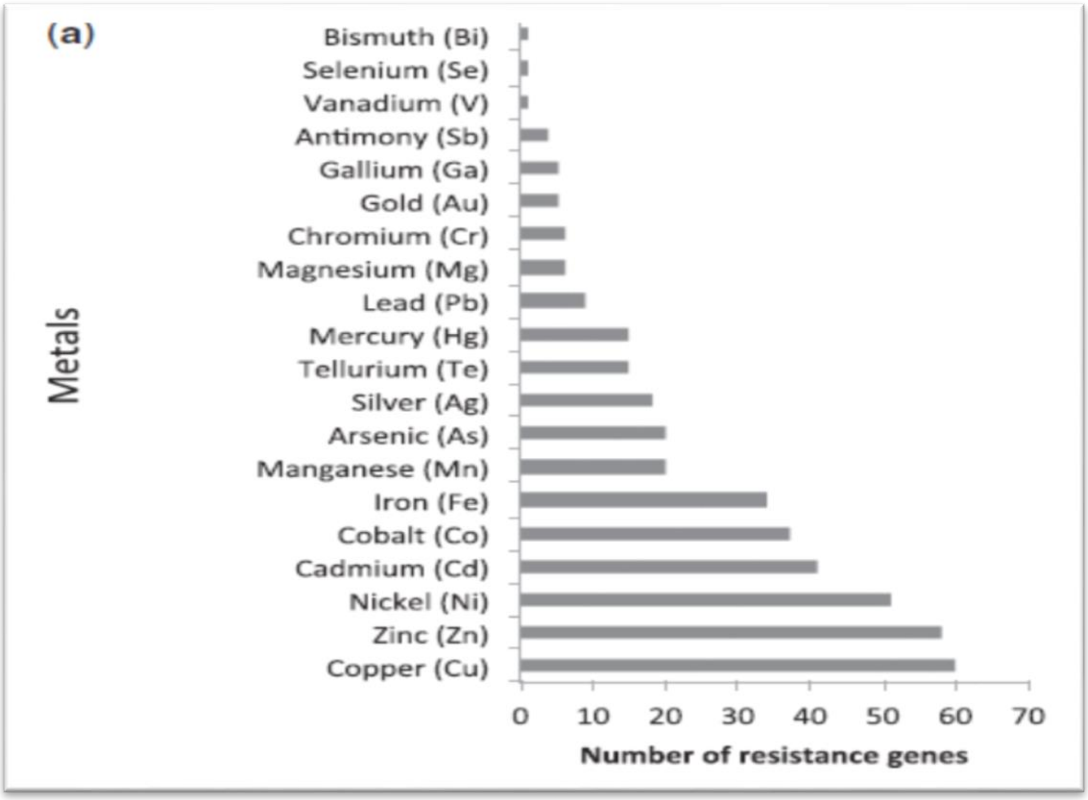

Figure 3. Summary of top 20 resistance genes for metals in the experimentally confirmed database. Some of the igenes are represented in more than one category. The figure reflects the most well studied compounds, although the actual substrate range is likely to be much broader for many genes (Pal et al., 2014).

Table 3. Mechanisms of resistance against different toxic metals assessed in this report.

Toxic metal	Mechanisms of resistance
Arsenic	As tolerance in bacteria is usually mediated by the gene products of the widespread extensively studied *ars* operon (Carlin et al., 1995; Rosen, 2002). Although the organization of the *ars* operons varies greatly between strains, there are some core genes that are almost always present: the simple gene set conferring basal resistance consists of the three-gene operon *arsRBC* as present in the *E. coli* genome (Carlin et al., 1995) and on *S. aureus* plasmid pI258 (Silver, 1998). Mechanisms of resistance against As in bacterial species have been reviewed by (Kruger et al., 2013) and (Hobman and Crossman, 2015). The main cross-resistance between As and antimicrobial agents may be activation of efflux pumps.

Toxic metal	Mechanisms of resistance
Cadmium	Resistance against Cd in bacteria is based on Cd efflux. In Gram-negative bacteria, Cd seems to be detoxified by an RND-driven system like Czc, which is mainly a Zn exporter, and Ncc, which is mainly a Ni exporter. Resistance against Cd in *S. aureus* and other Gram-positive bacteria is associated with a CdA pump or other CdA-like proteins (Nies, 1999).
Chromium	Both prokaryotic and eukaryotic microorganisms respond to Cr(VI) challenge by combining cellular networks acting at several levels, such as the reducing power generated by basal energy metabolism, iron and sulphur acquisition and homeostasis, protein oxidative stress protection, DNA repair, efflux pumps like *chrA*-encoding efflux pump orthologues, detoxification enzymes (Viti et al., 2014).
Copper	Resistance to Cu has been reported, both in bacteria isolated from humans and animals, and in bacteria of environmental origin. Resistance against Cu may be linked to resistance against several antimicrobials, for example macrolides including erythromycin (*erm*) (Amachawadi et al., 2011; Freitas et al., 2011; Jacob et al., 2010) or glycopeptides such as vancomycin (*van*) (Aarestrup et al., 2002) in enterococci. Resistance towards Cu is frequently encoded by genes located on plasmids and transposons, and is often transferable between bacterial species. Such resistance genes may be transferred to other bacteria and co-selection may occur.
Lead	To diminish its high toxicity, microorganisms have developed several mechanisms that allow them to survive exposure to Pb(II). The main mechanisms of Pb resistance involve adsorption by extracellular polysaccharides, cell exclusion, sequestration as insoluble phosphates, and ion efflux to the cell exterior (Jaroslawiecka and Piotrowska-Seget, 2014; Naik and Dubey, 2013).
Mercury	In Gram-negative enteric bacterial species, Hg-resistance genes are often found on plasmids and are associated with transposons/integrons (Foster, 1987; Khesin and Karasyova, 1984; Silver and Phung L, 2005). Similar mobile units are found in *S. aureus* and enterococci (Foster, 1987; Zscheck and Murray, 1993). More recently, oral streptococci and other oral genera have been shown to have reduced susceptibility to Hg, although, in general, the mechanisms of resistance have not been identified (Summers et al., 1993).

Toxic metal	Mechanisms of resistance
Nickel	Ni efflux pumps are best characterized in organisms exhibiting hyper-resistance to this metal, typically isolated from soils. Two examples of Ni-resistant organisms obtained from metal-contaminated industrial sites are *Cupriavidus* (formerly *Wautersia, Ralstonia,* or *Alcaligenes*) *metallidurans* and *Alcaligenes* (or *Achromobacter*) *xylosoxidans*. Ni-efflux pumps also are present in non-extremophiles, as exemplified by *E. coli* and *H. pylori*. Although Ni efflux is widely used by cells to protect against elevated concentrations of this metal, several other mechanisms are utilized by various microorganisms, and have been reviewed by Macomber and Hausinger (2011).
Zinc	Resistance against Zn has been reported, both in Gram-positive bacteria like MRSA (Cavaco et al., 2011) and Gram-negative bacteria like *E. coli* (Bednorz et al., 2013). Resistance to Zn, which is mainly associated with the *czrC* gene, has been reported in bacteria isolated from humans, animals, and from the environment. Resistance against Zn may be linked to resistance against methicillin in *S. aureus* (Cavaco et al., 2011) and Zn supplementation in animal feed may increase the proportion of multi-resistant *E. coli* in gut microbiota (Bednorz et al., 2013).

4.4 Horizontal gene transfer

Whereas AMR properties in bacteria are transferred from one generation to the next by vertical gene transfer within the same bacterial species, horizontal gene transfer (HGT) may occur both within the same species and between different bacterial species, including unrelated bacterial species. HGT may occur within and between bacterial species by conjugation, transformation, or transduction (see glossary), as has been described extensively in a review article by (Huddleston, 2014).

All uses of antimicrobials, including biocides and toxic metals, in human and veterinary medicine, including aquaculture and agriculture, may be drivers for the development of AMR in bacteria. The spread of AMR does not necessarily respect phylogenetic or ecological borders (Nielsen et al., 2014). Resistance to a certain antimicrobial agent can be selected, even by the use of other agents like antimicrobials, sanitizers, and some metal-containing compounds (for examples see Table 3). The mobility of these AMR genes is attributed to their residence on mobile genetic elements – plasmids, transposons, and integrons (IFT, 2006).

4.5 Potentially toxic metals in fertilising products and soil in Norway

4.5.1 Sewage sludge

In 2009, VKM published a risk assessment of contaminants in sewage sludge applied onto Norwegian soils (VKM, 2009). The data collected in that assessment were based on the levels of toxic metals in sewage sludge reported by Statistics of Norway (Statistisk Sentralbyrå (SSB, 2007) since the early 1990s and a report from Amundsen et al. (2001) that covered the main potentially toxic elements, namely As, Cd, Cr, Cu, Hg, Pb, Zn, and Ni. For Cd, Pb, Hg, and Cu the decreases in the concentration levels in the period 1993-2006 were 20-40%, while for Zn, Ni, and Cr there have been only minor changes in the concentration levels. The highest concentrations belonged to Zn and Cu, and the lowest to Hg, with the following order: Zn>Cu>Cr>Pb>Ni>Cd>Hg.

4.5.2 Soil

In the previous risk assessment of sewage sludge (VKM, 2009), background metal concentrations in agricultural soil were obtained from a study performed by Esser (1996). Three regions were defined, Sør-Trøndelag, Hedemark, and Østfold/Akershus/Vestfold (VKM, 2009) and the average concentrations of metals in soil samples from these regions were used as background levels for agricultural soil. The mean soil concentrations of toxic metals in Norway were found to be in the following order: Zn (71.1 mg/kg-^{1}>Cr (27.1 mg/kg^{-1})>Pb (23.9 mg/kg^{-1}) > Ni (21.1 mg/kg^{-1}) >Cu (19.2 mg/kg^{-1})> Cd (0.6 mg/kg^{-1}) > Hg (0.047 mg/kg-1). There is high variability in the concentrations in different regions of Norway, and it is important to be aware that the number of soil samples analysed is low.

For more information regarding concentrations of toxic metals in sewage sludge and soil in Norway, see APPENDICES – PART A, Appendix A1 (VKM, 2009) (http://vkm.no/dav/2ae7f1b4e3.pdf).

4.5.3 Livestock manure

Although there are some reports regarding the content of PTM (Cd, Cr, Cu, Hg, Pb, Ni, and Zn) in livestock manure in Norway (Serikstad et al., 2012), data are generally scant. Large variations in the concentrations of different PTM in livestock manure are found, depending on the animals sampled and the geographical areas in Norway (Appendix III). The highest concentrations are found for Zn and Cu (VKM, 2014), which are related to the addition of these trace elements to animal feed.

Sludge produced from hatcheries may also contain high concentration of heavy metals like Zn, Cd (Nofima 2016).

Based on the available data, which are incomplete, it seems that toxic metals, like Cu and Zn, occur in high concentrations in organic fertilising products in Norway. Cd is the predominant toxic metal in inorganic fertilising products (Mortvedt, 1996).

4.5.3.1 Toxic metals in animal feed in Norway

Cu and Zn are the toxic metals most commonly used in large quantities in Norway, mainly as feed additives for pigs and poultry. The data on Zn and Cu in complete compound feed in Norway indicate exposure of pigs and poultry to Zn and Cu concentrations of at least twice the required amount, and most often several times higher. The approximate total amount of complete compound feed produced for pigs in Norway in 2012, based on the reported production from the three main industry organizations, was 469,000 tonnes. The corresponding total amount of compound feed for poultry in 2012 was 428,000 tonnes. The concentrations of Zn and Cu in the complete compound feed, as reported by the different producers, are fairly similar (VKM, 2014). In complete compound feed for pigs in Norway in 2012, the estimated total amounts of Zn and Cu were 66,733 kg and 10,886 kg, respectively. In complete compound feed for poultry in Norway in 2012, the corresponding total amounts of Zn and Cu were 48,369 kg and 7,980 kg, respectively. These figures are based on turnover of the various categories of compound feed and their concentrations of Zn and Cu reported from the industry organizations. The estimated amount of Zn in medical remedies in 2012 was 4,130 kg (data for Normin Sink, only). This amount of Zn represents an addition of 67 % to the Zn in complete feed for piglets. No medical remedies based on Cu are registered in Norway. Most Zn and Cu used in animal feed will be excreted in the manure, which could be used as fertiliser for agricultural fields.

Although data are limited, it is clear that manure represents a major source of Zn and Cu in soils in Norway. The levels of PTM in different agriculture areas depend on the soil types, the quantities of manure added, and the concentrations of Cu and Zn in the manure. Feed for pigs and poultry has Cu and Zn added due to the growth-promoting effects of these elements. However, sources also indicate a specific need for Zn in the diets of weaning pigs (VKM 2014). There is clearly a need for more data on this topic, if our aim is to reduce the levels of Zn and Cu added to feed for monogastric animals.

5 Potentially toxic metals and ecological systems

Effects of toxic metals on the soil microbiome

Whereas "microbiota" is defined as the microbial taxa within a given environment, the term "microbiome" is defined as the genes and genomes of the microbiota, as well as the products of the microbiota and the host environment. Industrial inputs and the agronomic application of feed supplements, fertilisers, pesticides, and metal-contaminated sewage contribute to metal accumulation in the soil (Herland et al., 2000). Toxic metals affect the growth, morphology, and metabolism of soil microorganisms, through functional disturbance, protein denaturation, and/or the destruction of the cell membrane (See Figure 3 and Table 2). Soil microorganisms are essential for the decomposition of soil organic matter; any decrease in the microbial diversity or abundance may adversely affect nutrient absorption from the soil by plants (Giller et al., 1998).

Elevated levels of toxic metals in soils have significant impacts on the population size and overall activity of the microbial communities of soil in contaminated areas. Studies performed in Canada and China have described toxic metal contamination giving rise to shifts in microbial populations (Roane and Kellogg, 1996; Xie et al., 2016). The bioavailability of metals generally decreases with increasing pH, organic matter content, and clay content of soil (Nies et al., 1999).

The presence of metal resistance genes in bacteria not only reflects the anthropocentric view of microbiology, with its embedded history of human antimicrobial use in infectious disease, but also reflects microbial exposure to the metals used in aquacultural and agricultural practices. Pre-dating all human uses, there is also the exposure of microorganisms to localized high levels of toxic metals from natural environmental releases over millennia (Hobman and Crossman, 2015). The authors of this study suggest that the persistence of heavy metal resistance genes indicates a possible future for AMR genes.

However, most antimicrobial drugs are biologically produced and hence will be degraded relatively rapidly in most environments, with the exception of quinolones and tetracyclines, which are related to their chemical properties. Thus, a future reduction in the use of antimicrobials as drugs for treating diseases in animals and humans, and as growth promoters in animal husbandry, will, over time, decrease the selective pressure from these substances.

More research is needed to assist in our understanding of how increased levels of toxic metals influence, through co- and cross-selection, the complex global processes of resistance gene dynamics.

It has been claimed that the long-term accumulation of toxic metals in agricultural soils has the potential to reduce soil productivity by inhibiting soil microbial and fauna populations, and may pose a risk to soil organisms, plants, animals, humans, and our ecosystems (Kochare and Tamir, 2015).

Figure 5 is an illustration of the flux of biological materials in the ecosystem. Toxic metals, resistant bacteria, and genes follow more or less the same channels, and the figure can be used as a general illustration of important processes.

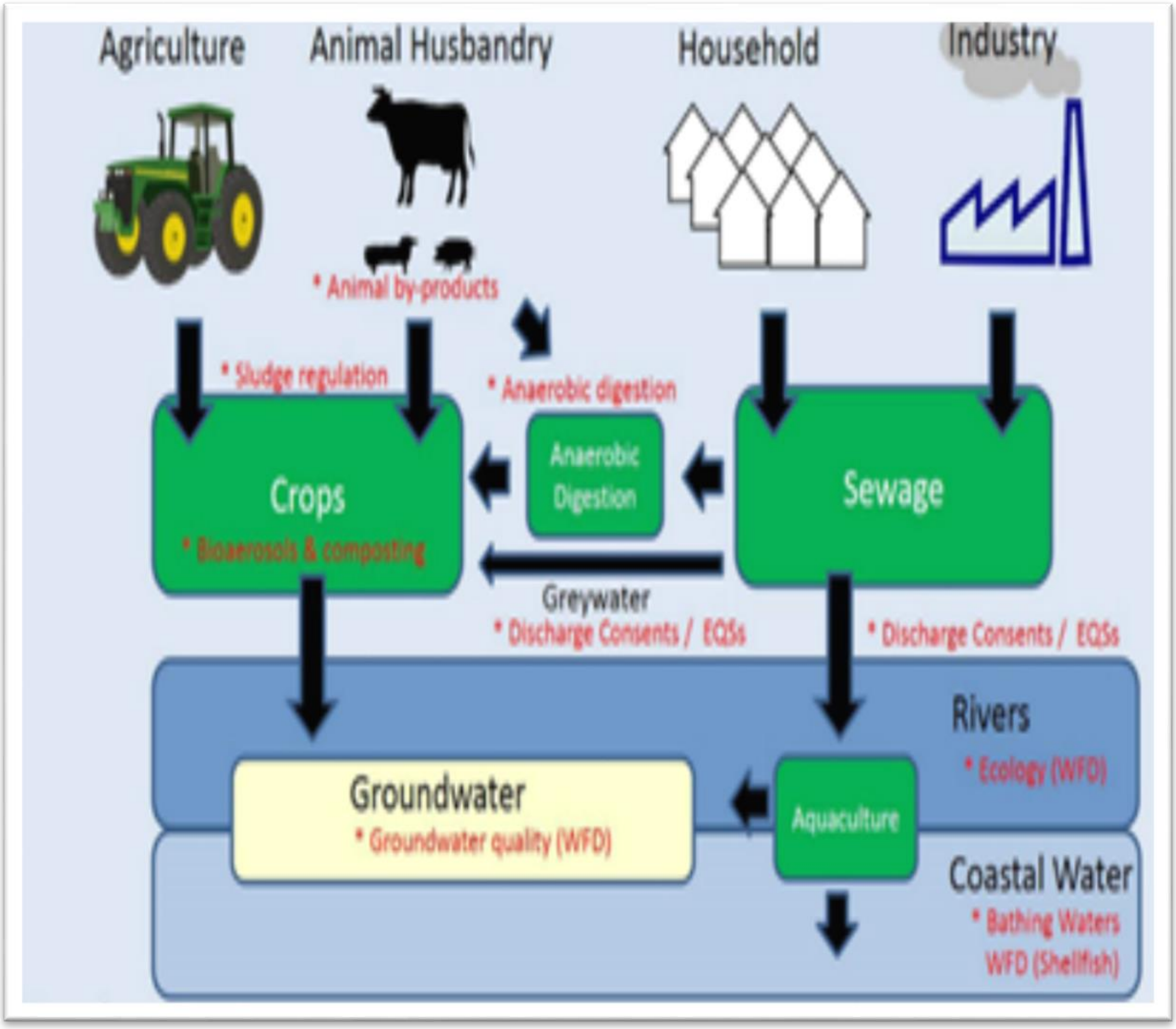

Figure 5. Review of antimicrobial resistance in the environment and its relevance to environmental regulators (Singer et al., 2016).

Toxic metals or antimicrobial compounds can disperse through the environment via multiple and potentially complex pathways, and will remain in the environment unless physically removed, or through uptake by plants used for foods or eaten by animals. Transfer of PTM from crop soils into groundwater, rivers, and eventually marine waters depends on soil pH, cropping strategies, floods, other run-offs, etc. In practice we move from a relatively short time-scale for pharmaceutically produced antimicrobials, bacteria, and genes (months and years) to a more geological time-scale (decades, centuries) for metals. As long as biological and chemical fertilisers contain toxic metals, we must expect that levels in our agricultural soils will build up. This level is generally scant in Norway.

Any ecologically sustainable future for our societies will depend upon intensive recycling of biological and non-biological materials. As toxic metals are found in so many products, a considerable part of this pollution will end up in our rivers, lakes, groundwater, and soils. The understanding of the effects linked to increased efforts to recycle waste materials is in its infancy.

Information about the levels of PTM, like Zn and Cu, in our soils is limited. We also need more data regarding the concentration of other PTM in soils, sewage, by-products, and fertilisers. Althoug present levels in agricultural soils may still be low, the long-term horizon of toxic metals in the environment indicates the importance of applying the precautionary principle in these issues. Thus, discussion of the levels of toxic metals, such as Cu and Zn, added to animal feeds and used in fertiliser products must be discussed and rationalised in order to minimize environmental enrichment of these metals. Furthermore, there is a need for more information regarding Cd, which is the predominant toxic metal in inorganic fertilising products.

As we move towards a more sustainable future, our concern about long-term enrichment of toxic metals in agricultural soils is an arena for research and should be linked to the political agenda. Without taking these concerns into consideration, our efforts towards recycling and less use of new raw materials may have the potential for negative consequences linked to the toxicity of the metals. One of the more likely outcomes could be a link to an increasing problem of metal-resistance in bacteria that undermines our efforts to minimize the spread of AMR in bacteria from different niches.

6 Examples of links between resistance towards potentially toxic metals and other antimicrobial agents

Forty-one articles fulfilled the criteria for evaluation and have been be included in this assessment (**Appendix II**). Most publications are on sewage (n=35), and most focus on the occurrence of toxic metal resistant bacteria, rather than on the ability of metals to induce resistance in bacteria in the environment. No studies were identified that addressed the potential release of toxic metal resistance genes to the environment, via fertilising products.

Most of the articles assessed (**Appendix II**) describe the detection and identification of toxic metal resistance and antimicrobial resistance in different bacterial taxa in different locations and various environmental compartments/niches. Contamination of the environment with toxic metals differs from environmental contamination with antimicrobial compounds as toxic metals are much more stable in the environment than most antimicrobial compounds (quinolones and tetracyclines are exceptions), and thus may hence exert a selective pressure over longer periods.

The majority of the studies included here are observational/descriptive studies that report on the co-existence of antimicrobial resistance determinants and toxic metal resistance determinants in bacteria. The bacterial species vary, and have been isolated from animals, humans, and environments in different countries and regions. No studies were identified that studied environments in Norway. Several variants of combinations of toxic metal resistance and antimicrobial resistance are described; a common feature is that the resistance-encoding genes are associated with mobile genetic elements. Some studies show direct genetic linkage, some show co-existence of resistance in the same isolate, some show co-existence in the same environment, but with unclear causal explanation.

Based on studies presented in **appendix II**, some explanations are provided on how toxic metals in waste, run-off, and manure/sewage from livestock, aquaculture and humans/human activities may contribute to the development of resistant bacteria in the environment (**Table 4**).

Table 4. Probability for development and dissemination of PTM resistance in bacteria in sewage/manure and soil.

Source of resistance	Comment
In organic fertilising products	
Toxic metal resistant bacteria in fertilising products	The probability of the simultaneous presence of AMR bacteria in sewage/manure is high (original articles reviewed in Appendix II).
Toxic metal resistance genes in fertilising products and their mobility	The probability of the presence of toxic metal resistance genes in fertilising products is high and transfer of such genes to bacteria in fertilising products is possible.
Toxic metal residues and development of toxic metal resistant bacteria in fertilising products	The probability of development of toxic metal resistance in susceptible bacteria due to toxic metals in fertilising products is high.

Source of resistance	Comment
In soil/environment	
Spread of toxic metal resistant bacteria from fertilising products to soil/environment	The probability of spread of toxic metal resistant bacteria from sewage/manure to the soil/environment is high.
Spread of toxic metal resistance genes from fertilising products to environmental bacteria	The presence and transfer of toxic metal resistance genes from fertilising products to bacteria in soil is possible.
Development of toxic metal resistance in bacteria in soil/ environment due to toxic metals in fertilising products	Development of resistance in susceptible bacteria in soil due to the presence of toxic metals in fertilising products is possible.

In the articles evaluated in **appendix II**, development of resistance against toxic metals was demonstrated in both sewage and manure. In several studies, several bacterial species were shown to have reduced susceptibility towards several potentially toxic metals, simultaneously. However, neither the source of heavy metal resistant bacteria nor the minimum co-selective concentration (MCC) for resistance induction is known. Co- or cross-resistance against highly important antimicrobials (streptomycin, tetracycline, neomycin) and critically important antimicrobials (e.g., amoxicillin, vancomycin, oxacillin, sulphonamides + trimethoprim, benzylpenicillin/phenoxymethylpenicillin, gentamicin) were identified in many of the bacterial isolates studied in these articles. These suggest that there is genetic linkage or direct genetic causality between genetic determinants to these widely divergent antimicrobials, and metal resistance. The Word Health Organization's criteria used to categorize antimicrobials important to human health has been discussed elsewhere (Collignon et al., 2016).

The data presented in these studies concur with the information presented in **Table 3** regarding mechanisms of resistance against different toxic metals.

Copper resistance

Several resistance mechanisms towards Cu have been described, including efflux pumps and cellular detoxification. These are both intrinsic and acquired characteristics of bacteria, and may occur in combination with AMR determinants in the same bacterial cells. One example of genetic linkage and co-occurrence on the same replicon is the pA17sv1 plasmid in enterococci that harbours resistance determinants towards Cu, macrolides, and glycopeptides (Silveira et al., 2014). This study (Silveira et al., 2014) concluded, "Cu tolerance might contribute to the selection/maintenance of multi-drug resistant *Enterococcus* (including resistance to first-line antibiotics used to treat enterococcal infections) due to the use of Cu compounds (e.g. antiseptics/animal feed supplements)". A proportion of the Cu-compounds used as antiseptics and animal feed supplements will eventually end up in organic fertilisers as manure and sewage, and exert induction of Cu tolerance and multidrug resistant enterococci in these environments due to positive selection of the plasmid host by Cu usage. However, the MCC for such induction is not known. Silveira et al. (2014) also found that enterococci containing Cu-resistance genes were more prevalent in samples from piggeries than from other animal production settings where Cu was used as feed supplement at lower concentrations than in piggeries, indicating positive selection for Cu-resistance determinants in the piggery setting.

In Denmark, glycopeptides were banned as growth promoters in animal production in 1995, and macrolides were banned in 1998. As the glycopeptide and macrolide resistance determinants (*vanA* and *erm*(B)) were shown to be located on the same plasmid in all Danish glycopeptide-resistant *E. faecium*, these bacteria did not decrease significantly until after 1998. However, although the occurrence of the glycopeptide-resistant *E. faecium* decreased, they did not disappear completely. Danish researchers have shown that a *tcrB* gene, which confers resistance to Cu in enterococci, is often located on the same transferable plasmid as the *vanA* and *erm*(B) determinants. Furthermore, the use of copper sulphate as a feed supplement for pigs has been shown to select for Cu resistance mediated by the *tcrB* gene in *E. faecium*, but the continued use of this feed supplement has not been able to maintain high levels of macrolide and glycopeptide resistance (Hasman, 2005; Hasman and Aarestrup, 2005). However, the selective pressure exerted by Cu-containing feed supplements may contribute towards maintaining low levels of these resistant bacteria in the gut microbiota. At re-exposure to glycopeptides or macrolides, the resistant bacteria may rapidly proliferate and become a dominant part of the enterococci population.

In Norway, the glycopeptide avoparcin was never approved for use in swine production, but was used as a feed additive in broiler and turkey production between 1986 and 1995, until implementation of a similar ban in Norway and Denmark. Several studies documented a continuing high prevalence of *vanA*-type vancomycin-resistant enterococci in the Norwegian poultry production several years after the ban (Borgen et al., 2000a; Borgen et al., 2000b; Borgen et al., 2001; Sorum et al., 2004). The occurrence of vancomycin-resistant enterococci in poultry has been investigated as part of the NORM-VET programme. In 2011, 16 % of broiler flocks were positive for *vanA*-type vancomycin-resistant *E. faecium*, and in 2013, 12 % of samples from turkeys were positive. All samples were analysed by a selective method for vancomycin-resistant enterococci (NORM/NORM-VET, 2011; NORM/NORM-VET, 2013). These data show that there is a minor reservoir of vancomycin-resistant enterococci in Norwegian poultry production, but it is not known whether this reservoir is maintained due to the use of feed supplements containing copper sulphate.

The studies mentioned above are examples of genetic linkage to the same replicon, hence co-transferred between antimicrobial and toxic metals, and their respective microbial resistance determinants in the gut flora. This interplay is relevant for similar interactions that may occur in soil microbiota, as organic fertilisers containing toxic metals may have the same function as feed supplements in this context.

Zinc

In Norway, feed supplements containing Zn are approved for use in pigs and poultry, and Zn is used for prevention of piglet diarrhoea. A VKM-report from 2014 concluded that Norwegian pigs are exposed to twice as much Zn as required to fulfil their physiological needs (VKM, 2014). This excess Zn will end up in organic fertilisers when pig and poultry manure is used as such.

Jensen and co-workers concluded from a recent study in Denmark that "amendment of soils with pig slurry has led to a significant increase in soil concentrations of copper and zinc, especially in the latest monitoring period from 1998 to 2014" (Jensen et al., 2016). Another recently published study from Denmark (Song et al., 2017), demonstrated the ability of Zn, in addition to Cu, to co-select for AMR in bacteria in soil. In an experimental model, environmentally relevant levels of Cu and Zn co-selected for tetracycline resistance, while soil spiked with unrealistically high levels of tetracycline did not. The authors concluded that in some cases toxic metals may exert a stronger selection pressure for resistance to an antibiotic than the specific antibiotic itself. It has also been shown that Zn resistance of *S. aureus* of animal origin is strongly associated with resistance against methicillin, and it is suggested that the use of Zn in feed may have contributed to the emergence of livestock associated-MRSA in pigs (Cavaco et al., 2011).

In the natural water environment (water and sediments) Cd, Cu, Ni, Hg, Co, Pb, and Zn concentrations frequently reach levels that exceed their respective MCC values for several bacterial species and, therefore, may drive co-selection. Although several studies have investigated co-selection in the aquatic environment, only a few publications consider soil environments; reviewed by Seiler and Berendonk (2012). In soil, Cu levels reach concentrations that are reported as potentially co-selective for antibiotic resistance genes (Knapp et al., 2011). In contrast, a Zn MCC for soil samples could not be determined because Knapp et al. (2011) did not detect an increasing abundance of antibiotic resistance genes in correlation with elevated Zn concentrations. However, the Zn concentrations of soil samples investigated by Knapp et al. (2011) were relatively low compared with those reported from other soils and maybe within the no-effect range.

Although these studies are concrete examples of apparent genetic linkeage (e.g. co-selection) of antimicrobial and metal resistance, systematic data on high-risk hot spots (such as from Cu and Zn in manure) for human-animal-environmental bacterial interactions, are scarce.

No studies were identified that investigated the abundance of AMR genes (resistome) in the different environmental samples. Therefore, in general we cannot distinguish between the natural resistome and elevated abundance of antibiotic resistance genes in environmental samples; detecting an increase of antibiotic resistance genes in environmental samples is not easy.

7 Uncertainties

The degree of confidence in a final estimation of risk depends on the variability, uncertainty, and assumptions identified in all the previous steps. Discrimination between uncertainty and variability is important in the subsequent selection of risk management options. Biological variation includes the differences in virulence that exist in microbiological populations and variability in susceptibility within the human population and particular sub-populations (http://www.fao.org/docrep/005/y1579e/y1579e05.htm).

In this assessment, a number of uncertainties have been identified related to the probability of formation of, and dissemination of, AMR due to the release of toxic metals to the environment. Many of these uncertainties are due to our limited understanding of the complex processes occurring at spatiotemporal scales not fully amendable to experimental investigation. A quantitative framework remains to be developed. There are many data gaps and detailed data on the current and future use of toxic metals, along with their environmental levels, are not readily available. Without these data, estimating the selective levels and MCC that could potentially induce increased AMR is challenging. The present methods for determining AMR in environmental samples are primarily culture-based laboratory studies (± antimicrobials) or using culture-independent methods and examining for the presence of antimicrobial resistance genes (ARGs), using PCR or sequencing. The latter methods do not fully capture the potential for co-selection with toxic metals. There are also uncertainties regarding the ability of, toxic metal-resistant bacterial strains to colonize humans or animals, the extent of such colonization, and the ability of their resistance genes to be transferred to resident bacterial species in the environment. Some sources of the uncertainties identified are as follows:

- Knowledge of antibiotic resistance genes (resistome) abundance in the different environmental samples is not readily available or systematically collected. Therefore, in general, we often cannot distinguish between the natural resistome and elevated abundance of antibiotic resistance genes in the different environmental samples.
- Baseline data regarding toxic metal concentration in the environment are limited.
- Baseline data regarding toxic metal resistant bacteria in the environment are limited.
- Our ability to predict the long-terms effects of toxic metals in fertilising products on the environment, regarding the development and persistence of AMR, is limited.

• Our understanding of the genetic interactions and spread that occur in bacteria of environmental origin and the development of AMR due to exposure to PTM needs to be improved.

- AMR is an evolving situation; those factors that may promote/reduce the transmission of bacteria resistant to antimicrobials and their corresponding gene determinants are not fully understood. The problem is a complex system that is challenging to address with linear model type approaches.

- Results from controlled laboratory experiments that investigate co-selection of resistance to a single toxic metal and antimicrobial compound may be difficult to extrapolate to the complex chemistry of environmental toxic metals.

- The specific conditions and circumstances that give rise horizontal gene transfer events can rarely be identified.

- For uncertainties regarding the technical aspects of laboratory methods, see data gaps.

8 Conclusions (with answers to the terms of reference)

The Norwegian Food Safety Authority would like VKM to give an opinion on the following questions related to the influence of potentially toxic metals on antimicrobial resistance:

Can the content of arsenic (As), cadmium (Cd), chromium (CrIII + CrVI), copper (Cu), lead (Pb), mercury (Hg), nickel (Ni), and zinc (Zn) levels in soil and fertilising products that are relevant for Norway play a role in the development, spreading, and persistence of bacterial resistance to these elements, as well as cross or co-resistance to antimicrobial agents?

8.1 Toxic metals in soil relevant for Norway

There is no systematic monitoring of toxic metals in Norwegian environments, and, based on available data, it appears that the levels are highly variable, depending on geology and characteristics of the soil. Human activities over centuries, such as industrial, urban, agricultural, and aquacultural activities, have resulted in increased levels of toxic metals in the environment, especially in areas where such activities have been intensive or combined. It is currently not possible to relate these partly unknown and variable levels to their impact on development, dissemination, and persistence of AMR.

8.2 Toxic metals in fertilising products relevant for Norway

As for soil, data on toxic metals in fertilising materials are fragmented and limited. Fertilising materials, in the form of sewage sludge or livestock manure, will either add to or ”dilute” already existing levels of these toxic metals in soil, especially in areas of intensive agriculture. The additive effect of toxic metals in fertilising materials must be regarded from a long-term perspective, as these metals accumulate in the environment.
It is not possible to relate these partly unknown and variable levels with their impact on development, dissemination, and persistence of AMR. However, areas of intensive agriculture may be regarded as “hot spots” for interactions between bacteria of environmental, animal, and human origin, and the toxic metals.
In this report, we focus in particular on the metals that are actively added to the environmental cycles through animal feed (Zn, Cu). Although other PTM may also be relevant, we consider the levels of these to be low in Norway.

In sewage sludge, Cd is considered the most important toxic metal with respect to environmental contamination. As Zn and Cu are added to swine and poultry feed in levels exceeding these animals' physiological needs, these metals are the most relevant to consider in livestock manure.

8.3 Development of antimicrobial resistance

Development of AMR can be partly regarded as a dose- and time dependent response to exposure to different drivers for resistance. There is a strong indication that the PTM evaluated in this opinion are driving forces for the development of AMR in exposed bacteria, but the dose- and time exposures most likely to have greatest impact are not known. The naturally occurring background resistance in environmental bacteria complicates our estimation of the effect of toxic metal exposure on the development of AMR, and we are currently unable to distinguish readily between the natural resistome and an elevated abundance of AMR in different environmental samples due to metal contamination. Heavy metal driven co-selection of AMR in environments impacted by agriculture and aquaculture should especially focus on Cd, Cu, and Zn as co-selecting factors for the development of AMR.

The persistence and dissemination of AMR can occur when metal resistance within bacteria confers cross- and/or co-resistance to particular antibiotics. The term minimum co-selective concentration (MCC) of metals was recently introduced as a term that specifies the minimum toxic metal concentration that correlates with detection of increased AMR. The use of the MCC term is an interesting approach, and, in the future, may prove to be a useful method for assessment of the co-selective effect of a toxic metal. However, considerable field- and laboratory-based research is needed to establish the MCC-approach as an acknowledged and unifying method comparable to the MIC methods used by EUCAST and CLSI.
More research is needed to explain the relationship between development of resistance against potential toxic metals and resistance toward antimicrobial agents resistance in bacteria.

8.4 Spread of antimicrobial resistance

Many examples of cross- and co-resistance between toxic metals and antibiotic resistance are described in the literature. Most important are those cases where toxic metal resistance determinants are genetically linked to resistance determinants towards *highly important* and *critically important* antibiotics. Emergence of livestock-associated methicillin-resistant *S. aureus* in pigs is one of the most alarming examples of AMR. The association between resistance to Zn and methicillin-resistance in *S. aureus* of animal origin suggests that the use of Zn as a feed supplement could have contributed to contribute to the persistence, amplification, and dissemination of MRSA in pigs, rather than initial development.

8.5 Persistence of antimicrobial resistance

Traits conferring resistance to antimicrobial compounds have been present in some bacteria since times pre-dating human society, probably as defence mechanisms to antibiotics produced by bacterial communities.

The various mechanisms for current resistance are most often known and understood within an evolutionary perspective. However, we do not fully understand the mechanisms behind the persistence of AMR. Removal of selective conditions for development and spread of resistance may result in the levels of resistance decreasing, but not necessarily lead to a full disappearance. The presence of a minor proportion of a bacterial population that is resistant means that it has the potential to outcompete the remaining population should that population again be exposed to a corresponding antibiotic or toxic metal.
The interaction between antibiotics / toxic metals / disinfectant agents and bacteria may be a major cause for development of AMR in bacteria.
Through the use of fertilising materials, the bacterial influx to the environment belongs to the large group of gut microbiota. These bacteria are adapted to the intestinal environment, and their environmental survival abilities are variable. Much of this microbiota will die out and not influence the environmental microbiota over time. Composting of livestock manure and the production process of sewage sludge reduces the number of microbes added to the soil and environment. However, data on the long-term fate of ARB and AMR genes originating from an intestinal environment are fragmented and limited.

9 Data gaps

We have limited data on the following relevant topics:

- The concentrations of potentially toxic metals in sewage, animal manure, and soil in different geographical areas in Norway.
- Toxic metal resistant bacteria in sewage.
- Toxic metal resistant bacteria in agriculture areas where fertilising products have been used.
- Toxic metal resistant bacteria in soil in Norway ("background resistance").
- Activity of toxic metals against different bacterial species in fertilising products (manure, sewage, etc.) and in soil.
- Comprehensive longitudinal and quantitative data from studies that have examined the dissemination of AMR pathogens from livestock manure and sewage application in the environment.
- Updated data on the concentration of toxic metals in fertilising products, organic (sludge and livestock manure) as well as inorganic, and soils in Norway.
- Development of toxic metal resistance in environmental bacteria.
- Development of toxic metal resistance in "un-culturable" bacteria.
- Dissemination of toxic metal resistance genes via fertilising products.
- Links between the leve and concentartion of PTM in fertilising products and soil and development of resistance in bacteria.
- Sub-inhibitory concentrations of metals that induce resistance in different bacterial species at laboratory level, in sewage, manure, soil, and environment.
- Estimation of MCC for toxic/heavy metals in Norway.
- Data from evidence-based studies regarding the impact of heavy metal resistant bacteria in the environment on animals and humans.
- Relationship between development of resistance against potential toxic metals and resistance aganist antimicrobial agents in bacteria.

10 References

Aarestrup F.M., Hasman H., Jensen L.B., Moreno M., Herrero I.A., Dominguez L., Finn M., Franklin A. (2002) Antimicrobial resistance among enterococci from pigs in three European countries. Appl Environ Microbiol 68:4127-9.

Ackerley D.F., Barak Y., Lynch S.V., Curtin J., Matin A. (2006) Effect of chromate stress on *Escherichia coli* K-12. J Bacteriol 188:3371-81. DOI: 10.1128/JB.188.9.3371-3381.2006.

Alam M.Z., Ahmad S., Malik A. (2011) Prevalence of heavy metal resistance in bacteria isolated from tannery effluents and affected soil. Environ Monit Assess 178:281-91. DOI: 10.1007/s10661-010-1689-8.

Alonso A., Sanchez P., Martinez J.L. (2001) Environmental selection of antibiotic resistance genes. Environ Microbiol 3:1-9.

Amachawadi R.G., Shelton N.W., Shi X., Vinasco J., Dritz S.S., Tokach M.D., Nelssen J.L., Scott H.M., Nagaraja T.G. (2011) Selection of fecal enterococci exhibiting tcrB-mediated copper resistance in pigs fed diets supplemented with copper. Appl Environ Microbiol 77:5597-603. DOI: 10.1128/AEM.00364-11.

Amundsen C.E., Paulsrud B., Nedland K.T., Høgåsen H., Gjerde B., Mohn H. (2001) Miljøgifter og smittestoffer i organisk avfall. Status og veien videre. Jordforsk.

Anssour L., Messai Y., Estepa V., Torres C., Bakour R. (2016) Characteristics of ciprofloxacin-resistant Enterobacteriaceae isolates recovered from wastewater of an Algerian hospital. J Infect Dev Ctries 10:728-34. DOI: 10.3855/jidc.6727.

Baker-Austin C., Wright M.S., Stepanauskas R., McArthur J.V. (2006) Co-selection of antibiotic and metal resistance. Trends Microbiol 14:176-82. DOI: 10.1016/j.tim.2006.02.006.

Bednorz C., Oelgeschlager K., Kinnemann B., Hartmann S., Neumann K., Pieper R., Bethe A., Semmler T., Tedin K., Schierack P., Wieler L.H., Guenther S. (2013) The broader context of antibiotic resistance: zinc feed supplementation of piglets increases the proportion of multi-resistant *Escherichia coli in vivo*. Int J Med Microbiol 303:396-403. DOI: 10.1016/j.ijmm.2013.06.004.

Borgen K., Simonsen G.S., Sundsfjord A., Wasteson Y., Olsvik O., Kruse H. (2000a) Continuing high prevalence of VanA-type vancomycin-resistant enterococci on Norwegian poultry farms three years after avoparcin was banned. J Appl Microbiol 89:478-85.

Borgen K., Sorum M., Kruse H., Wasteson Y. (2000b) Persistence of vancomycin-resistant enterococci (VRE) on Norwegian broiler farms. FEMS Microbiol Lett 191:255-8.

Borgen K., Sorum M., Wasteson Y., Kruse H. (2001) VanA-type vancomycin-resistant enterococci (VRE) remain prevalent in poultry carcasses 3 years after avoparcin was banned. Int J Food Microbiol 64:89-94.

Cabral L., Junior G.V., Pereira de Sousa S.T., Dias A.C., Lira Cadete L., Andreote F.D., Hess M., de Oliveira V.M. (2016) Anthropogenic impact on mangrove sediments triggers differential responses in the heavy metals and antibiotic resistomes of microbial communities. Environ Pollut 216:460-9. DOI: 10.1016/j.envpol.2016.05.078.

Caldini G., Cenci G., Morozzi G. (1987) Resistotype heterogeneity of *Escherichia coli* strains isolated from humans, animals and sewage waters. Zentralbl Bakteriol Mikrobiol Hyg B 184:206-13.

Carlin A., Shi W., Dey S., Rosen B.P. (1995) The ars operon of *Escherichia coli* confers arsenical and antimonial resistance. J Bacteriol 177:981-6.

Cavaco L.M., Hasman H., Aarestrup F.M. (2011) Zinc resistance of *Staphylococcus aureus* of animal origin is strongly associated with methicillin resistance. Vet Microbiol 150:344-8. DOI: 10.1016/j.vetmic.2011.02.014.

Clarkson T.W., Magos L. (2006) The toxicology of mercury and its chemical compounds. Crit Rev Toxicol 36:609-62. DOI: 10.1080/10408440600845619.

Collignon P.C., Conly J.M., Andremont A., McEwen S.A., Aidara-Kane A., World Health Organization Advisory Group B.M.o.I.S.o.A.R., Agerso Y., Andremont A., Collignon P., Conly J., Dang Ninh T., Donado-Godoy P., Fedorka-Cray P., Fernandez H., Galas M., Irwin R., Karp B., Matar G., McDermott P., McEwen S., Mitema E., Reid-Smith R., Scott H.M., Singh R., DeWaal C.S., Stelling J., Toleman M., Watanabe H., Woo G.J. (2016) World Health Organization Ranking of Antimicrobials According to Their Importance in Human Medicine: A Critical Step for Developing Risk Management Strategies to Control Antimicrobial Resistance From Food Animal Production. Clin Infect Dis 63:1087-1093. DOI: 10.1093/cid/ciw475.

da Costa P.M., Loureiro L., Matos A.J. (2013) Transfer of multidrug-resistant bacteria between intermingled ecological niches: the interface between humans, animals and the environment. nt J Environ Res Public Health 14;10(1):278-294.

de Vicente A., Aviles M., Codina J.C., Borrego J.J., Romero P. (1990) Resistance to antibiotics and heavy metals of *Pseudomonas aeruginosa* isolated from natural waters. J Appl Bacteriol 68:625-32.

Dhakephalkar P.K., Chopade B.A. (1994) High levels of multiple metal resistance and its correlation to antibiotic resistance in environmental isolates of Acinetobacter. Biometals 7:67-74.

Esser K. (1996) Reference concentrations for heavy metals in mineral soils, oat, and orchard grass (Dáctylis glomeratá) from three agricultural regions in Norway. Water, Air, and Soil Pollution 89:375-397.

Ferreira da Silva M., Vaz-Moreira I., Gonzalez-Pajuelo M., Nunes O.C., Manaia C.M. (2007) Antimicrobial resistance patterns in *Enterobacteriaceae* isolated from an urban wastewater treatment plant. FEMS Microbiol Ecol 60:166-76. DOI: 10.1111/j.1574-6941.2006.00268.x.

Filali B.K., Taoufik J., Zeroual Y., Dzairi F.Z., Talbi M., Blaghen M. (2000) Waste water bacterial isolates resistant to heavy metals and antibiotics. Curr Microbiol 41:151-6.

Foster T.J. (1987) The genetics and biochemistry of mercury resistance. Crit Rev Microbiol 15:117-40. DOI: 10.3109/10408418709104455.

Fraise A.P., Maillard J.-Y., Sattar S. (2012) Principles and Practice of Disinfection, Preservation and Sterilization. John Wiley & Sons.

Freitas A.R., Coque T.M., Novais C., Hammerum A.M., Lester C.H., Zervos M.J., Donabedian S., Jensen L.B., Francia M.V., Baquero F., Peixe L. (2011) Human and swine hosts share vancomycin-resistant Enterococcus faecium CC17 and CC5 and Enterococcus faecalis CC2 clonal clusters harboring Tn1546 on indistinguishable plasmids. J Clin Microbiol 49:925-31. DOI: 10.1128/JCM.01750-10.

Gao P., He S., Huang S., Li K., Liu Z., Xue G., Sun W. (2015) Impacts of coexisting antibiotics, antibacterial residues, and heavy metals on the occurrence of erythromycin resistance genes in urban wastewater. Appl Microbiol Biotechnol 99:3971-80. DOI: 10.1007/s00253-015-6404-9.

Giller K.E., Witter E., Mcgrath S.P. (1998) Toxicity of heavy metals to microorganisms and microbial processes in agricultural soils: a review. Soil Biol. Biochem. 30:1389-1414.

Habi S., Daba H. (2009) Plasmid incidence, antibiotic and metal resistance among enterobacteriaceae isolated from Algerian streams. Pak J Biol Sci 12:1474-82.

Harrison J.J., Ceri H., Turner R.J. (2007) Multimetal resistance and tolerance in microbial biofilms. Nat Rev Microbiol 5:928-38. DOI: 10.1038/nrmicro1774.

Hasman H. (2005) The tcrB gene is part of the tcrYAZB operon conferring copper resistance in *Enterococcus faecium* and *Enterococcus faecalis*. Microbiology 151:3019-25. DOI: 10.1099/mic.0.28109-0.

Hasman H., Aarestrup F.M. (2005) Relationship between copper, glycopeptide, and macrolide resistance among *Enterococcus faecium* strains isolated from pigs in Denmark between 1997 and 2003. Antimicrob Agents Chemother 49:454-6. DOI: 10.1128/AAC.49.1.454-456.2005.

Heck K., De Marco E.G., Duarte M.W., Salamoni S.P., Van Der Sand S. (2015) Pattern of multiresistant to antimicrobials and heavy metal tolerance in bacteria isolated from sewage sludge samples from a composting process at a recycling plant in southern Brazil. Environ Monit Assess 187:328. DOI: 10.1007/s10661-015-4575-6.

Henriette C., Petitdemange E., Raval G., Gay R. (1991) Mercuric reductase activity in the adaptation to cationic mercury, phenyl mercuric acetate and multiple antibiotics of a gram-negative population isolated from an aerobic fixed-bed reactor. J Appl Bacteriol 71:439-44.

Herland B.J., Taylor D., Wither K. (2000) The distribution of mercury and other trace metals in the sediments of the Mersey Estuary over 25 years 1974- 1998. Sci Total Environ 253:45-62.

Hobman J.L., Crossman L.C. (2015) Bacterial antimicrobial metal ion resistance. J Med Microbiol 64:471-97. DOI: 10.1099/jmm.0.023036-0.

Holzel C.S., Muller C., Harms K.S., Mikolajewski S., Schafer S., Schwaiger K., Bauer J. (2012) Heavy metals in liquid pig manure in light of bacterial antimicrobial resistance. Environ Res 113:21-7. DOI: 10.1016/j.envres.2012.01.002.

Hu N., Zhao B. (2007) Key genes involved in heavy-metal resistance in Pseudomonas putida CD2. FEMS Microbiol Lett 267:17-22. DOI: 10.1111/j.1574-6968.2006.00505.x.

Huddleston J.R. (2014) Horizontal gene transfer in the human gastrointestinal tract: potential spread of antibiotic resistance genes. Infect Drug Resist 7:167-76. DOI: 10.2147/IDR.S48820.

IFT. (2006) Antimicrobial resistance: Implications for the food system, Institute of Food Technologists.

Imlay J.A. (2003) Pathways of oxidative damage. Annu Rev Microbiol 57:395-418. DOI: 10.1146/annurev.micro.57.030502.090938.

Jacob M.E., Fox J.T., Nagaraja T.G., Drouillard J.S., Amachawadi R.G., Narayanan S.K. (2010) Effects of feeding elevated concentrations of copper and zinc on the antimicrobial susceptibilities of fecal bacteria in feedlot cattle. Foodborne Pathog Dis 7:643-8. DOI: 10.1089/fpd.2009.0401.

Jaroslawiecka A., Piotrowska-Seget Z. (2014) Lead resistance in micro-organisms. Microbiology 160:12-25. DOI: 10.1099/mic.0.070284-0.

Jensen J., Larsen M.M., Bak J. (2016) National monitoring study in Denmark finds increased and critical levels of copper and zinc in arable soils fertilized with pig slurry. Environ Pollut 214:334-340. DOI: 10.1016/j.envpol.2016.03.034.

Ji X., Shen Q., Liu F., Ma J., Xu G., Wang Y., Wu M. (2012) Antibiotic resistance gene abundances associated with antibiotics and heavy metals in animal manures and agricultural soils adjacent to feedlots in Shanghai; China. J Hazard Mater 235-236:178-85. DOI: 10.1016/j.jhazmat.2012.07.040.

Kahlmeter G. (2015) The 2014 Garrod Lecture: EUCAST - are we heading towards international agreement? J Antimicrob Chemother 70:2427-39. DOI: 10.1093/jac/dkv145.

Kaur S., Kamli M.R., Ali A. (2011) Role of arsenic and its resistance in nature. Can J Microbiol 57(10):769-774.

Khan Z., Nisar M.A., Hussain S.Z., Arshad M.N., Rehman A. (2015) Cadmium resistance mechanism in *Escherichia coli* P4 and its potential use to bioremediate environmental cadmium. Appl Microbiol Biotechnol 99:10745-57. DOI: 10.1007/s00253-015-6901-x.

Khesin R.B., Karasyova E.V. (1984) Mercury-resistant plasmids in bacteria from a mercury and antimony deposit area. Mol Gen Genet 197:280-5.

Knapp C.W., McCluskey S.M., Singh B.K., Campbell C.D., Hudson G., Graham D.W. (2011) Antibiotic resistance gene abundances correlate with metal and geochemical conditions in archived Scottish soils. PLoS One 6:e27300. DOI: 10.1371/journal.pone.0027300.

Kochare T., Tamir B. (2015) Assessment of Dairy Feeds for Heavy Metals. American Scientific Research Journal for Engineering. Technology and Sciences 11:20-31.

Kotas J., Stasicka Z. (2000) Chromium occurrence in the environment and methods of its speciation. Environ Pollut 107:263-83.

Kruger M.C., Bertin P.N., Heipieper H.J., Arsene-Ploetze F. (2013) Bacterial metabolism of environmental arsenic--mechanisms and biotechnological applications. Appl Microbiol Biotechnol 97:3827-41. DOI: 10.1007/s00253-013-4838-5.

Lemire J.A., Harrison J.J., Turner R.J. (2013) Antimicrobial activity of metals: mechanisms, molecular targets and applications. Nat Rev Microbiol 11:371-84. DOI: 10.1038/nrmicro3028.

Li Y., Liu B., Zhang X., Gao M., Wang J. (2015) Effects of Cu exposure on enzyme activities and selection for microbial tolerances during swine-manure composting. J Hazard Mater 283:512-8. DOI: 10.1016/j.jhazmat.2014.09.061.

Lin H., Sun W., Zhang Z., Chapman S.J., Freitag T.E., Fu J., Zhang X., Ma J. (2016) Effects of manure and mineral fertilization strategies on soil antibiotic resistance gene levels and microbial community in a paddy-upland rotation system. Environ Pollut 211:332-7. DOI: 10.1016/j.envpol.2016.01.007.

Macomber L., Hausinger R.P. (2011) Mechanisms of nickel toxicity in microorganisms. Metallomics 3:1153-62. DOI: 10.1039/c1mt00063b.

Martins V.V., Zanetti M.O., Pitondo-Silva A., Stehling E.G. (2014) Aquatic environments polluted with antibiotics and heavy metals: a human health hazard. Environ Sci Pollut Res Int 21:5873-8. DOI: 10.1007/s11356-014-2509-4.

Matuschek E., Brown D.F., Kahlmeter G. (2014) Development of the EUCAST disk diffusion antimicrobial susceptibility testing method and its implementation in routine microbiology laboratories. Clin Microbiol Infect 20:O255-66. DOI: 10.1111/1469-0691.12373.

Miranda C.D., Castillo G. (1998) Resistance to antibiotic and heavy metals of motile aeromonads from Chilean freshwater. Sci Total Environ 224:167-76.

Mortvedt J.J. (1996) Heavy metal contaminants in inorganic and organic fertilizers. Fertilizer Research 43:55-61.

Naik M.M., Dubey S.K. (2013) Lead resistant bacteria: lead resistance mechanisms, their applications in lead bioremediation and biomonitoring. Ecotoxicol Environ Saf 98:1-7. DOI: 10.1016/j.ecoenv.2013.09.039.

Narayani M., Vidya Shetty K. (2014) Reduction of hexavalent chromium by a novel Ochrobactrum sp. - microbial characteristics and reduction kinetics. J Basic Microbiol 54:296-305. DOI: 10.1002/jobm.201200183.

Naureen A., Rehman A. (2016) Arsenite oxidizing multiple metal resistant bacteria isolated from industrial effluent: their potential use in wastewater treatment. World J Microbiol Biotechnol 32:133. DOI: 10.1007/s11274-016-2079-3.

Nielsen K.M., Bøhn T., Townsend J.P. (2014) Detecting rare gene transfer events in bacterial populations Front. Microbiol. 4:415.

Nies D.H. (1999) Microbial heavy-metal resistance. Appl Microbiol Biotechnol 51:730-50.

Nies D.H., Silver S. (1995) Ion efflux systems involved in bacterial metal resistances. J Ind Microbiol 14:186-99.

Nofima (2016). Karakterisering av slam fra tre kommersielle settefiskanlegg gjennom ett produksjonsår.Hovednæringsstoff, mineraler og tungmetaller. Rapport 33/2016.

NORM/NORM-VET. (2011) Usage of Antimicrobial Agents and Occurrence of Antimicrobial Resistance in Norway, Tromsø / Oslo 2012. ISSN:1502-2307 (print) / 1890-9965 (electronic).

NORM/NORM-VET. (2013) Usage of Antimicrobial Agents and Occurrence of Antimicrobial Resistance in Norway, Tromsø / Oslo 2014. ISSN:1502-2307 (print) / 1890-9965 (electronic).

Novo A., Andre S., Viana P., Nunes O.C., Manaia C.M. (2013) Antibiotic resistance, antimicrobial residues and bacterial community composition in urban wastewater. Water Res 47:1875-87. DOI: 10.1016/j.watres.2013.01.010.

Pal C., Bengtsson-Palme J., Rensing C., Kristiansson E., Larsson D.G. (2014) BacMet: antibacterial biocide and metal resistance genes database. Nucleic Acids Res 42:D737-43. DOI: 10.1093/nar/gkt1252.

Patra S., Das T.K., Ghosh S., Sarkar D., Jana B.B. (2010) Cadmium tolerance and antibiotic resistance of *Pseudomonas* sp. isolated from water, sludge and fish raised in wastewater-fed tropical ponds. Indian J Exp Biol 48:383-93.

Phan T.N., Buckner T., Sheng J., Baldeck J.D., Marquis R.E. (2004) Physiologic actions of zinc related to inhibition of acid and alkali production by oral streptococci in suspensions and biofilms. Oral Microbiol Immunol 19:31-8.

Qian M., Wu H., Wang J., Zhang H., Zhang Z., Zhang Y., Lin H., Ma J. (2016) Occurrence of trace elements and antibiotics in manure-based fertilizers from the Zhejiang Province of China. Sci Total Environ 559:174-81. DOI: 10.1016/j.scitotenv.2016.03.123.

Rahube T.O., Yost C.K. (2012) Characterization of a mobile and multiple resistance plasmid isolated from swine manure and its detection in soil after manure application. J Appl Microbiol 112:1123-33. DOI: 10.1111/j.1365-2672.2012.05301.x.

Rajkumar B., Sharma G.D., Paul A.K. (2012) Isolation and characterization of heavy metal resistant bacteria from Barak River contaminated with pulp paper mill effluent, South Assam. Bull Environ Contam Toxicol 89:263-8. DOI: 10.1007/s00128-012-0675-y.

Rathnayake I.V., Megharaj M., Krishnamurti G.S., Bolan N.S., Naidu R. (2013) Heavy metal toxicity to bacteria - are the existing growth media accurate enough to determine heavy metal toxicity? Chemosphere 90:1195-200. DOI: 10.1016/j.chemosphere.2012.09.036.

Riber L., Poulsen P.H., Al-Soud W.A., Skov Hansen L.B., Bergmark L., Brejnrod A., Norman A., Hansen L.H., Magid J., Sorensen S.J. (2014) Exploring the immediate and long-term impact on bacterial communities in soil amended with animal and urban organic waste fertilizers using pyrosequencing and screening for horizontal transfer of antibiotic resistance. FEMS Microbiol Ecol 90:206-24. DOI: 10.1111/1574-6941.12403.

Roane T.M., Kellogg S.T. (1996) Characterization of bacterial communities in heavy metal contaminated soils. Can J Microbiol 42:593-603.

Roca I., Akova M., Baquero F., Carlet J., Cavaleri M., Coenen S., Cohen J., Findlay D., Gyssens I., Heuer O.E., Kahlmeter G., Kruse H., Laxminarayan R., Liebana E., Lopez-Cerero L., MacGowan A., Martins M., Rodriguez-Bano J., Rolain J.M., Segovia C., Sigauque B., Tacconelli E., Wellington E., Vila J. (2015) The global threat of antimicrobial resistance: science for intervention. New Microbes New Infect 6:22-9. DOI: 10.1016/j.nmni.2015.02.007.

Rosen B.P. (2002) Biochemistry of arsenic detoxification. FEBS Lett 529:86-92.

Rosewarne C.P., Pettigrove V., Stokes H.W., Parsons Y.M. (2010) Class 1 integrons in benthic bacterial communities: abundance, association with Tn402-like transposition modules and evidence for coselection with heavy-metal resistance. FEMS Microbiol Ecol 72:35-46. DOI: 10.1111/j.1574-6941.2009.00823.x.

Rudrik J.T., Bawdon R.E., Guss S.P. (1985) Determination of mercury and organomercurial resistance in obligate anaerobic bacteria. Can J Microbiol 31:276-81.

Salmore A.K., Hollis E.J., McLellan S.L. (2006) Delineation of a chemical and biological signature for stormwater pollution in an urban river. J Water Health 4:247-62.

Saltikov C.W., Olson B.H. (2002) Homology of *Escherichia coli* R773 arsA, arsB, and arsC genes in arsenic-resistant bacteria isolated from raw sewage and arsenic-enriched creek waters. Appl Environ Microbiol 68:280-8.

Schiering N., Kabsch W., Moore M.J., Distefano M.D., Walsh C.T., Pai E.F. (1991) Structure of the detoxification catalyst mercuric ion reductase from *Bacillus* sp. strain RC607. Nature 352:168-72. DOI: 10.1038/352168a0.

Seiler C., Berendonk T.U. (2012) Heavy metal driven co-selection of antibiotic resistance in soil and water bodies impacted by agriculture and aquaculture. Front Microbiol 3:399. DOI: 10.3389/fmicb.2012.00399.

Serikstad G.L., McKinnon K., Eggen T. (2012) Uønskete stoffer i husdyrgjødsel-Konvensjonell husdyrgjødsel brukt i økologisk drift - er det problematisk?, Bioforsk Rapport Bioforsk Report

Silveira E., Freitas A.R., Antunes P., Barros M., Campos J., Coque T.M., Peixe L., Novais C. (2014) Co-transfer of resistance to high concentrations of copper and first-line antibiotics among *Enterococcus* from different origins (humans, animals, the environment and foods) and clonal lineages. J Antimicrob Chemother 69:899-906. DOI: 10.1093/jac/dkt479.

Silver S. (1998) Genes for all metals--a bacterial view of the periodic table. The 1996 Thom Award Lecture. J Ind Microbiol Biotechnol 20:1-12.

Silver S., Phung L T. (2005) A bacterial view of the periodic table: genes and proteins for toxic inorganic ions. J Ind Microbiol Biotechnol 32:587-605. DOI: 10.1007/s10295-005-0019-6.

Silver S., Phung L.T. (1996) Bacterial heavy metal resistance: new surprises. Annu Rev Microbiol 50:753-89. DOI: 10.1146/annurev.micro.50.1.753.

Singer A.C., Shaw H., Rhodes V., Hart A. (2016) Review of Antimicrobial Resistance in the Environment and Its Relevance to Environmental Regulators. Front Microbiol 7:1728. DOI: 10.3389/fmicb.2016.01728.

Smith H.W., Parsell Z., Green P. (1978) Thermosensitive antibiotic resistance plasmids in enterobacteria. J Gen Microbiol 109:37-47. DOI: 10.1099/00221287-109-1-37.

Song J., Rensing C., Holm P.E., Virta M., Brandt K.K. (2017) Comparison of Metals and Tetracycline as Selective Agents for Development of Tetracycline Resistant Bacterial Communities in Agricultural Soil. Environ Sci Technol. 7;51(5):3040-3047.

Sørum M., Holstad G., Lillehaug A., Kruse H. (2004) Prevalence of vancomycin resistant enterococci on poultry farms established after the ban of avoparcin. Avian Dis 48:823-8. DOI: 10.1637/7197-042004R.

SSB. (2007) Kommunale avløp. Ressursinnsats, utslipp, rensing og slamdisponering 2006. ISBN 978-82-537-7297-4 ISBN 978-82-537-7298-1 Oslo.

Stepanauskas R., Glenn T.C., Jagoe C.H., Tuckfield R.C., Lindell A.H., McArthur J.V. (2005) Elevated microbial tolerance to metals and antibiotics in metal-contaminated industrial environments. Environ Sci Technol 39:3671-8.

Summers A.O., Wireman J., Vimy M.J., Lorscheider F.L., Marshall B., Levy S.B., Bennett S., Billard L. (1993) Mercury released from dental "silver" fillings provokes an increase in mercury- and antibiotic-resistant bacteria in oral and intestinal floras of primates. Antimicrob Agents Chemother 37:825-34.

Szczepanowski R., Krahn I., Puhler A., Schluter A. (2004) Different molecular rearrangements in the integron of the IncP-1 beta resistance plasmid pB10 isolated from a wastewater treatment plant result in elevated beta-lactam resistance levels. Arch Microbiol 182:429-35. DOI: 10.1007/s00203-004-0726-9.

Tchounwou P.B., Yedjou C.G., Patlolla A.K., Sutton D.J. (2012) Heavy metal toxicity and the environment. EXS 101:133-64. DOI: 10.1007/978-3-7643-8340-4_6.

Teitzel G.M., Parsek M.R. (2003) Heavy metal resistance of biofilm and planktonic *Pseudomonas aeruginosa*. Appl Environ Microbiol 69:2313-20.

Timoney J.F., Port J., Giles J., Spanier J. (1978) Heavy-metal and antibiotic resistance in the bacterial flora of sediments of New York Bight. Appl Environ Microbiol 36:465-72.

Varela A.R., Andre´ S., Nunes O.C., Manaia C.M. (2014) Insights into the relationship between antimicrobial residues and bacterial populations in a hospital-urban wastewater treatment plant system. Water Research 54:327-336.

Verma T., Garg S.K., Ramteke P.W. (2009) Genetic correlation between chromium resistance and reduction in Bacillus brevis isolated from tannery effluent. J Appl Microbiol 107:1425-32. DOI: 10.1111/j.1365-2672.2009.04326.x.

Viti C., Marchi E., Decorosi F., Giovannetti L. (2014) Molecular mechanisms of Cr(VI) resistance in bacteria and fungi. FEMS Microbiol Rev 38:633-59. DOI: 10.1111/1574-6976.12051.

VKM. (2009) Risk assessment of contaminants in sewage sludge applied on Norwegian soils.

VKM. (2014) Zinc and copper in pig and poultry production – fate and effects in the food chain and the environment, Norwegian Scientific Committee for Food Safety, ISBN 978-82-8259-093-8.

Wales A.D., Davies R.H. (2015) Co-Selection of Resistance to Antibiotics, Biocides and Heavy Metals, and Its Relevance to Foodborne Pathogens. Antibiotics (Basel) 4:567-604. DOI: 10.3390/antibiotics4040567.

Wise S.S., Holmes A.L., Ketterer M.E., Hartsock W.J., Fomchenko E., Katsifis S., Thompson W.D., Wise J.P., Sr. (2004) Chromium is the proximate clastogenic species for lead chromate-induced clastogenicity in human bronchial cells. Mutat Res 560:79-89. DOI: 10.1016/j.mrgentox.2004.02.009.

Xie Y., Fan J., Zhu W., Amombo E., Lou Y., Chen L., Fu J. (2016) Effect of Heavy Metals Pollution on Soil Microbial Diversity and Bermudagrass Genetic Variation. Front Plant Sci 7:755. DOI: 10.3389/fpls.2016.00755.

Yamina B., Tahar B., Marie Laure F. (2012) Isolation and screening of heavy metal resistant bacteria from wastewater: a study of heavy metal co-resistance and antibiotics resistance. Water Sci Technol 66:2041-8. DOI: 10.2166/wst.2012.355.

Yazdankhah S., Rudi K., Bernhoft A. (2014) Zinc and copper in animal feed - development of resistance and co-resistance to antimicrobial agents in bacteria of animal origin. Microb Ecol Health Dis 25. DOI: 10.3402/mehd.v25.25862.

Yazdankhah S.P., Scheie A.A., Hoiby E.A., Lunestad B.T., Heir E., Fotland T.O., Naterstad K., Kruse H. (2006) Triclosan and antimicrobial resistance in bacteria: an overview. Microb Drug Resist 12:83-90. DOI: 10.1089/mdr.2006.12.83.

Yu Z., Gunn L., Wall P., Fanning S. (2017) Antimicrobial resistance and its association with tolerance to heavy metals in agriculture production. Food Microbiol 64:23-32. DOI: 10.1016/j.fm.2016.12.009.

Zscheck K.K., Murray B.E. (1993) Genes involved in the regulation of beta-lactamase production in enterococci and staphylococci. Antimicrob Agents Chemother 37:1966-70.

Appendix I

According to the Norwegian regulations, organic fertilising products are divided into four categories based on their content of PTM. The different quality classes defined for organic fertilising products are shown in the Table below.

Table 1. Quality demands for organic fertilising products. Maximum levels for potentially toxic metals (mg/kg DM) in different quality classes of sewage sludge.

Class:	**0** **mg/kg DM**	**I** **mg/kg DM**	**II** **mg/kg DM**	**III** **mg/kg DM**
Cadmium (Cd)	0.4	0.8	2	5
Lead (Pb)	40	60	80	200
Mercury (Hg)	0.2	0.6	3	5
Nickel (Ni)	20	30	50	80
Zinc (Zn)	150	400	800	1500
Copper (Cu)	50	150	650	1000
Chromium (Cr)	50	60	100	150
Application	No limitation on agriculture areas, and green areas (e.g. private gardens, parks)	40 tons dry matter per hectare per 10 year on agriculture areas, private gardens and parks.	20 tons dry matter per hectare per 10 year on agriculture areas, private gardens and parks.	Maximum 5 cm on green areas

Products within <u>class 0</u> can be used on agriculture areas and green areas with no limitation. The amount used is adjusted to the plants need for nutrients.

Products satisfying <u>class I</u> can be applied on agriculture areas, private gardens and parks at no more than 40 tonnes dry matter per hectare per 10 years. The products can also be used on green areas where foods or forage crops are not to be grown. The product shall be applied at maximum 5 cm layers and mixed into the soil at the site.

Products complying with <u>class II</u> can be applied on agriculture areas, private gardens and parks at no more than 20 tonness dry matter per hectare per 10 years. The products can also be used on green areas and similar areas where food or forage crops are not to be grown. The product shall be applied at maximum 5 cm layers and mixed into the soil at the site.

Products within <u>class III</u> may be used on green areas where food or forage crops are not to be grown. The product shall be applied at maximum 5 cm layers every 10 year and mixed into the soil at the site. When used as cover on landfills the layer shall not exceed 15 cm.

Raw materials included in products of class I and II must comply with the requirements for heavy metal contents of class II. Similarly, raw materials included in class III products shall comply with the heavy metal requirements for class III. Products higher then class 0 can only be used on agricultural land if the heavy metal content in the soil is below the maximum levels given in Table 2.

Table 2. Quality demands for soil. Maximum levels for potentially toxic metals (mg/kg DM) in soil where sewage sludge can be applied.

Potentially toxic metals	**Maximum levels in soil** **mg/kg DM**
Cadmium (Cd)	1
Lead (Pb)	50
Mercury (Hg)	1
Nickel (Ni)	30
Zinc (Zn)	150
Copper (Cu)	50
Chromium (Cr)	100

Appendix II

Table 1. Evaluations of the published articles regarding development of AMR due to exposure to As, Cd, Cu, Hg, Ni, Pb, Zn in sewage.

Reference	Country	Sample Number (if stated or applicable)	Bacterial species	Toxic metals and antimicrobial agents investigated	Conclusion
(Anssour et al., 2016)	Spain	Hospital effluent (sewage) N = not stated	*E. coli, Escherichia vulneris, Klebsiella pneumoniae, Klebsiella oxytoca, Citrobacter freundii, and Citrobacter koseri/farmeri.*	Cd, Zn, Hg Nalidixic acid, ciprofloxacin, ofloxacin, and pefloxacin.	This study highlighted bacterial multiple-antibiotic and toxic metal resistance in hospital effluents, which is linked to ciprofloxacin resistance through selective pressure, co-resistance, and cross-resistance. This should draw attention to the consequences of exposure of bacteria to fluoroquinolones in general, and ciprofloxacin in particular, through their heavy and/or inappropriate use and their release in hospital effluents.
(Cabral et al., 2016)	Brazil	Sediment sampling of different mangrove sites N: not indicated	*Gammaproteobacteria, Deltaproteobacteria, Alphaproteobacteria, Betaproteobacteria Flavobacteriia Bacteroidia, Clostridia, Bacilli, Chlorobia, Epsilonproteobacteria, Planctomycetia, Actinobacteria, Solibacteres, Methanomicrobia, Spirochaetia*	Cu, Zn, Cr, Ni, Cd, Pb, Hg, and antibiotic resistome	The concentration Zn, Cr, Pb, Cu, Ni, Cd, and Hg and abundance of genes and transcripts involved in resistance to toxic compounds have been closely associated with sites impacted with **petroleum, sludge and other urban waste**. The most abundant active microorganism in the Oil Mgv sediment was related to a species of sulphate-reducing bacteria (*Desulfobacterium autotrophicum*), suggesting that this microorganism is involved in heavy metal transformation in this site.

Reference	Country	Sample Number	Bacterial species	Susceptibility to toxic metals and antimicrobial agents	Conclusion
(Naureen and Rehman, 2016)	Sweden	Industrial wastewater N = not stated	Arsenite-oxidizing bacteria, isolated from industrial wastewater *Bacillus cereus, Acinetobacter junii*	As, Pd, Cd, Cr, Cu	Both *B. cereus* and A. junii could tolerate As^{3+} up to 40 mM. Both arsenite-oxidizing strains can transform more than 85 % of As^{3+} into As^{5+} from the industrial wastewater and bacterial treated wastewater can be used for irrigation purposes, at least.
(Lin et al., 2016)	China	Paddy upland rotation system to mineral fertilizer (NPK) and different application dosages of manure combined with NPK. N = not applicable	Detection of antimicrobial resistance gene (ARG) from microbial community	Cu, Zn Tetracycline ARGs (tetA, tetB, tetC, tetG and tetW), two sulphonamide ARGs (sul1 and sul2) and one genetic element (IntI1)	Manure application could change the soil resistome without changing the microbial community at large, but excessive use of manure would induce The accumulation of Cu and Zn could act as one important force, driving the observed bloom of soil ARGs. Determination of antibiotics and heavy metals in soils suggested that the observed bloom of soil ARGs might be closely associated with the accumulation of Cu and Zn in soil.

Reference	Country	Sample Number	Bacterial species	Susceptibility to toxic metals and antimicrobial agents	Conclusion
(Gao et al., 2015)	China	Urban wastewater N = Six batches of wastewater samples were taken in the sterile polypropylene bottles. Each sample was composite by wellmixing equal volumes (about 1 L) per hour for 4 h.	Antimicrobial resistance genes (against heavy metals, antibiotics and other antibacterial agents)	Cd, Cr, Cu Zn, Pb, NiMacrolides, tetracyclines, sulphonamides, quinolones, antibacterial residues (triclosan)	A relationship between certain antibiotics, antibacterial residues, and heavy metals and ERY-ARGs was demonstrated. ERY presented significant correlations ($0.883<r<0.929$, $P<0.05$) with ere(A), ere(B), and mef(A)/mef(E) genes, while tetracycline exhibited a significant correlation ($r=0.829$, $P<0.05$) with erm(B) genes. It is noteworthy that triclosan correlated significantly ($0.859<r<0.956$, $P<0.05$) with ere(A), ere(B), mef(A)/mef(E), and erm(B) genes. In addition, significantly positive correlations ($0.823<r<0.871$, $P<0.05$) were observed between Zn and Pb and certain ERY-ARGs (i.e., ere(B), mef(A)/mef(E), erm(B), etc.).

Reference	Country	Sample Number	Bacterial species	Susceptibility to toxic metals and antimicrobial agents	Conclusion
(Khan et al., 2015)	Pakistan	Industrial wastewater containing heavy metals in the sewerage system. N = not stated	*E. coli* N=2	Cd	Heavy metal resistant *E. coli* P4 were isolated from industrial wastewater showing optimum growth at 30 °C and pH 7. The bacteria were able to resist Cd^{2+} (10.6 mM), Zn^{2+} (4.4 mM), Pb^{2+} (17 mM), Cu^{2+} (3.5 mM), Cr^{6+} (4.4 mM), As^{2+} (10.6 mM), and Hg^{2+} (0.53 mM). *E. coli* P4 could remove 18.8, 37, and 56 % Cd^{2+} from the aqueous medium after 48, 96, and 144 h, respectively. Amplification of czcB gene, a component of czcCBA operon, from genomic DNA suggested that the Cd^{2+} resistance in *E. coli* P4 is chromosomal. *E. coli* P4 also harbours smtAB gene that plays a significant role in Cd^{2+} resistance, and the bacteria can be used to ameliorate the toxic metal ions from the wastewater.
(Heck et al., 2015)	Brazil	Organic waste and sewage sludge generated during wastewater treatment N = Not stated	*Pseudomonas* spp. *Enterobacter* spp. *Ochrobactrum* spp. N= 344 isolates	Cr, Zn, Pb, Cu Ampicillin, amoxicillin + clavulanic acid, cephalosporins – cephalothin, cefoxitin, ceftriaxone, chloramphenicol; aminoglycosides – streptomycin, and gentamicin (10 µg); fluoroquinolones – ciprofloxacin, norfloxacin; carbapenems – imipenem; nitrofurantoin; sulphamethoxazole – trimethoprim, and tetracycline.	The composting process associated with sewage sludge monitored at a recycling plant in southern Brazil exhibited a high index of bacteria multiresistant to antimicrobials, with the highest resistance percentage being to nitrofurantoin and β-lactams. Biocidal concentration standards for Cr and Cu showed Cu to be the metal that had the highest biocidal concentration to the strains isolated at the beginning and end of the process. *Pseudomonas* and *Ochrobactrum* were the genera identified that had the highest AMR and heavy metal tolerance profile, simultaneously.

Reference	Country	Sample Number	Bacterial species	Susceptibility to PTM and antimicrobial agents	Conclusion
(Varela et al., 2014)	Portugal	Hospital effluent and in the receiving wastewater treatment plant. Seven samples were collected from the hospital effluent (one per month in October 2010, January-April 2011, June-July 2011), 21 from the raw inflow, and 21 from the treated effluent of the wastewater treatment plant (three consecutive days in the months of October 2010, March 2011, May 2011).	*Bacteroidetes*, related with the genus *Prevotella*, *Bacteroidetes*, related with the genus *Alistipes*, *Proteobacteria*, related with the genus Acinetobacter *Firmicutes*, related with the genus *Faecalibacterium* *Proteobacteria*, related with the genus *Aeromonas* *Firmicutes*, related with the class *Clostridia* Firmicutes, related with the class *Clostridia*	As, Cd, Cr, Hg, Pb, Penicillin G, Penicillin V Ciprofloxacin, Ofloxacin, Sulphamethoxazole, Sulphathiazole, Sulphamethazine, Tetracycline, Oxytetracycline, Doxycycline, Chlortetracycline	The concentrations of fluoroquinolones, As and Hg were, in general, higher in hospital effluent than in raw inflow, while the opposite was observed for tetracyclines, sulphonamides and penicillin G. The prevalence of ciprofloxacin resistance was significantly higher in hospital effluent than in raw inflow. The concentration of antimicrobial residues was significantly correlated with the prevalence of antibiotic resistant bacteria and with variations in the bacterial community. Hospital effluent was confirmed as a relevant, although not unique, source of antimicrobial residues and antibiotic resistant bacteria to the wastewater treatment plant. Moreover, given the high loads of antibiotic residues and antibiotic resistant bacteria that may occur in hospital effluents, these wastewater habitats may represent useful models to study and predict the impact of antibiotic residues on bacterial communities.

(Martins et al., 2014)	Brazil	Water samples were collected in sterile flasks from the Pardo River in São Paulo State in Brazil and from a lake in São Paulo University. N = not stated	*P. aeruginosa*	Cu, Zn, Hg Amikacin, amoxicillin, aztreonam, cefepime, ceftazidime, ceftriaxone, cephalothin, ciprofloxacin, chloramphenicol, gentamicin, meropenen, streptomycin, tetracycline, imipenem, piperacillin–tazobactam, polymyxin B, rifampicin, ticarcillin–clavulanic acid, tobramycin, and trimethoprim	This study demonstrated that a conjugative plasmid present in *P. aeruginosa* isolated from river water presented coresistance to tetracycline and Cu, reinforcing the concern that antibiotic resistance by the acquisition of plasmids can be induced by the selective pressure of heavy metals in the environment. Therefore, not only the indiscriminate use of antibiotics, but also the contamination of the environment by heavy metals, can lead to serious problems, including risk and damage to human health.

Reference	Country	Sample Number	Bacterial species	Susceptibility to PTM and antimicrobial agents	Conclusion
(Novo et al., 2013)	Portugal	Raw and treated wastewater composite samples were collected from an urban treatment plant over 14 sampling dates. N=28	*Epsilonproteobacteria,* related with *Arcobacter, Gammaproteobacteria,* related with *Acinetobacter, Gammaproteobacteria* related with *Pseudomonadales, Fusobacteria* related with *Streptobacillus, Betaproteobacteria* related with *Comamonas Epsilonproteobacteria related with Sulfurimonas, Bacteroidetes* *Betaproteobacteria,* related with *Acidovorax, Firmicutes* related with *Clostridium, Gammaproteobacteria* related with *Rheinheimera, Betaproteobacteria,* related with *Simplicispira, Bacteroidetes,* related with *Leadbetterella, Betaproteobacteria,* related with *Comamonas, Firmicutes* related with the genus, *Phascolarctobacterium, Betaproteobacteria,* related with *Comamonas Betaproteobacteria, related with Curvibacter*	As, Cd, Pb, Cr and Hg Tetracyclines, penicillins, sulphonamides, quinolones, triclosan,	The concentration of tetracyclines, penicillins, sulphamides and quinolones and the abundance of antibiotic resistant cultivable bacteria in the raw wastewater were positively correlated with the abundance of *Epsilonproteobacteria* in treated wastewater and negatively with *Gamma*, *Betaproteobacteria* and *Firmicutes.*

(Rajkumar et al., 2012)	India	Wastewater from river contaminated with paper and pulp effluents N = not stated	A group of 15 heavy metal resistant bacteria (different degrees of chromium tolerance): Faecal coliform, *B. cereus, E. coli*	Cr, Ni, Cu, Co, Cd, Tetracycline, Streptomycin, Polymyxin, Vancomycin, Penicillin G, Ampicillin, Chloramphenicol, Gentamycin, Erythromycin, Rifampicin	Four isolates showed 34 %–49 % growth at a concentration of 4.0 mM of Cr^{6+} and subjected to chromium reduction assay under aerobic conditions. These 4 isolates also showed different degrees of resistance to other heavy metals like Ni, Cu, Co and Cd. Antibiotic sensitivity profile of these selected bacterial strains was determined against 10 different antibiotics. Isolate E (4) appeared to be most susceptible, being inhibited by eight antibiotics and resistant to penicillin G and ampicillin. The isolate E (3) was resistant to as many as five antibiotics and showed susceptible responses to the other antibiotics. Both the isolates K(6)PA6 and D (2) were resistant to four antibiotics and showed intermediate to susceptible responses to the other antibiotics.

Reference	**Country**	**Sample** **Number**	**Bacterial species**	**Susceptibility to PTM and antimicrobial agents**	**Conclusion**
(Yamina et al., 2012)	Algeria	Wastewater N = not stated	*Aeromonas* sp., *Alcaligens* sp., *E. coli, P. aeruginosa, Bacillus* sp., *Pasteurella* sp., *Enterobacter* sp., *Klebsiella* sp. *Micrococcus luteus, Staphylococcus*	Zn, Pb, Cr, Cd Ampicillin, cephalothin, gentamicin, kanamycin, amikacin, doxycycline, nalidixic acid	77% and 15% of bacteria resistant against heavy metals were, Gram-negative, and Gram-positive, respectively. 15% of the isolates were resistant towards one antibiotic and 85% resistant against multi and two antibiotics. Among 13 metal-resistant isolates, 11 (85%) were MDR-resistant. *M. luteus* was considered as the more heavy metal resistant.

(Ferreira da Silva et al., 2007)	Brazil	Wastewater N = ?	*Enterobacteriaceae* *Pseudomonas* spp. *Bacillus s*pp.	Cr, Hg, Ag amikacin, chloramphenicol, gentamicin, sulphamethoxazole-trimethoprim, tetracycline, norfloxacin, ciprofloxacin, tobramycin and cefotaxime	Tolerance to Cr was observed mainly in Gram-positive bacteria, but for Ag and Hg the tolerant bacteria were predominately Gram negative. The authors hreported a correlation between Hg / Ag and other antimicrobial agents. This correlation has not been reported for Cr.

Reference	Country	Sample Number	Bacterial species	Susceptibility to PTM and antimicrobial agents	Conclusion
(Narayani and Vidya Shetty, 2014)	India	Aerator water of an activated sludge process of a wastewater treatment facility of a dye and pigment based specialty chemical industry N=1	A Gram negative hexavalent chromium (Cr(VI)) reducing bacteria, *Ochrobactrum* sp. Cr-B4	Cr, Fe, Cu Zn, Ni, Cu, Pb Chloramphenicol Nalidixic acid Kanamycin Streptomycin Novobiocin Gentamycin	It exhibited resistance against all examined heavy metal ions, and novobiocin. The strain, *Ochrobactrum* sp. Cr-B4 isolated in this study had a very high Cr(VI) resistance of 1000 mg L^{1} with an ability of reducing Cr(VI) to Cr(III) very efficiently. This strain, being highly resistant toward multiple metals, can survive in wastewater even in the presence of these metals and be used to reduce Cr(VI). Thus, this study indicates the potential use of this technology at an industrial level.

(Alam et al., 2011)	India	Tannery effluent samples were collected from the outlet of combined effluent treatment plant at Jajmau, Kanpur (India) in a 5-l sterile plastic container. Soil samples were collected from the agricultural fields irrigated with the tannery effluents. Each time five samples were collected from five different fields and a composite sample was made for analysis. The samples were collected in sterilized polythene bags with help of sterilized spatula. A total of five samplings were carried out and samples were collected at every 3 months. N = 5	Group I (n = 84). This group include Gram-negative bacilli (*Pseudomonas, Enterobacter, Pantoea, Stenotrophomonas, Aeromonas, Alcaligenes*) Group II (n = 52). It include Gram-positive spore forming bacilli (*Bacillus*) Group III (n = 37). Consist of Gram-positive non-spore forming bacilli (*Exiguobacterium, Brochothrix, Aureobacterium*) Group IV (n = 25). This division include Gram-positive cocci (*Staphylococcus, Micrococcus*)	Cr^{6+}, Cr^{3+}, Ni^{2+}, Zn^{2+}, Cu^{2+}, Cd^{2+}, and Hg^{2+} Ampicillin Amoxicillin Gentamycin Kanamycin Tetracycline Streptomycin Methicillin Doxycycline Chloramphenicol Co-trimoxazole	The majority of these metal-resistant isolates were sensitive to the ten commonly used antibiotics. Out of 198 isolates, 114 were fsensitive to all antibiotics whereas only two isolates were resistant to maximum eight antibiotics. 41 and 40 isolates (20.7% and 20.2%) were resistant to methicillin and amoxicillin, respectively.

Reference	Country	Sample Number	Bacterial species	Susceptibility to PTM and antimicrobial agents	Conclusion
(Patra et al., 2010)	India	From water, sludge, and intestines of fish raised in different wastewater ponds along an effluent gradient in sewage treatment plant. N: six replicate samples of water and sludge during May 2005-March 2006, at monthly intervals. Six rohu (*Labeo rohia*) from two maturation ponds and 6 tilapia from all the three ponds were obtained every month.	*Pseudomonas* sp	Cd Netilmicin, colistin, tobramycin, polymyxin, gentamicin, ticracillin, imipenem, ciprofloxacin	*Pseudomonas* sp collected from the samples water, sludge, or fish exhibited growth response in presence of low concentrations of Cd (0.05 mM) in the culture medium. However, *Pseudomonas* sp failed to grow when the Cd level in the culture medium was 3 mM. The *Pseudomonas* sp. isolated from the intestines of tilapia did not have resistance to any of the ten antimicrobial agents. However, the bacteria from raw sewage, water and sediment of the anaerobic pond were resistant to seven of ten antibiotics tested.
(Verma et al., 2009)	India	Treated tannery effluent was collected from a common effluent treatment plant. N = not stated	*Bacillus* spp.	Cr, Mn, As, Zn, Co, Cd, Hg, Polymyxin B, chloramphenicol, kanamycin, tetracycline, bacitracin, streptomycin, ampicillin, carbenicillin, co-trimazole, nalidixic acid, cephaloridin	All chromate resistant *Bacillus* spp. were found tolerant to multiple metals, suggesting that they can withstand the presence of other metallic ions and perform the desired activity. *Bacillus brevis* was resistant to elevated Cr(VI) levels and may potentially reduce it in short time from an environment where other metal ions are also present in addition to Cr ions. The strain tested shows a positive correlation between genetic basis of Cr(VI) resistance and reduction. Such strains may potentially be useful in biotechnological applications and *in situ* Cr(VI) bioremediation.

Reference	Country	Sample Number	Bacterial species	Susceptibility to PTM and antimicrobial agents	Conclusion
(Hu and Zhao, 2007)	China	Sewage sludge Genetic characterization of Cd-resistance. N = not applicable	*Pseudomonas putida* CD2	Cd, Co, Cu, Zn, Ni, Pb, Mn	Strain CD2 exhibited high maximal tolerant concentrations (MTC) for a large spectrum of divalent metals. The DNA sequences of the contiguous region from the transposon Tn5 insertion sites were determined by inverse PCR. Six genes involved in cadmium resistance were identified. These genes were from three gene clusters: *czcCBA1, cadA2R* and *colRS*. The homologues of the first two gene clusters were predicted to be metal efflux systems, whereas the products of *colRS*, ColR and ColS, were thought to be a two-component signal transduction system. ColRS also has a function in regulating multi-metal resistance.
(Habi and Daba, 2009)	Algeria	Surface water to assess impact of faecal and/or metal pollution, heavy metals, antibiotic resistance, and plasmid incidence N = not stated	*Enterobacteriaceae* N=208	Pb, Cd, Hg, Streptomycin, kanamycin, tetracycline, chloramphenicol, doxycycline, furan	No difference was found between stream water subjected or not to contamination from metallic or poultry waste. The frequency of strains carrying plasmids was higher in urban wastewater than metal and/or low faecal polluted stream water. No correlation was observed between plasmid and metal resistance. Resistance against heavy metals and antibiotics were chromosomally located.

Reference	Country	Sample Number	Bacterial species	Susceptibility to PTM and antimicrobial agents	Conclusion
(Ferreira da Silva et al., 2007)	Portugal	Wastewater of a municipal treatment plant: Over 18 months, enterobacteria were isolated from the raw (189 isolates) and treated (156 isolates) wastewater of a municipal treatment plant. N: not stated	*Escherichia* spp. *Klebsiella* spp. *Shigella* spp.	Ni, Cd, Cr, Hg, Zn Hydrogen peroxide, sodium hypochlorite, quaternary ammonium/formaldehyde and iodine. Amoxicillin gentamicin, ciprofloxacin, tetracycline, sulphamethoxazole/ trimethoprim, sulphamethoxazole cephalothin streptomycin	Compared with the raw influent, the treated wastewater presented higher relative proportions of *Escherichia* spp. isolates resistant to ciprofloxacin and cephalothin (P=0.0001 and P=0.05, respectively). Except for Hg, which showed a positive correlation with tetracycline and sulfamethoxazole/trimethoprim, no significant positive correlations were observed between antibiotic, disinfectant and heavy metal resistance. The variable regions of class 1 integrons, detected in *c.* 10% of the *Escherichia* spp. isolates, contained predominantly the gene cassettes aadA1/dhfrI.
(Rosewarne et al., 2010)	Australia	Sediments were collected from 30 spatially distinct locations N = not stated	*Proteobacteria*	Zn, Cu, Hg, Pb	Characterization of class 1 integrons in bacteria cultured from selected sediment samples identified an association with complete Tn402-like transposition modules, and the potential for co-selection of heavy metal and antibiotic resistance mechanisms in benthic environments.

Reference	Country	Sample Number	Bacterial species	Susceptibility to PTM and antimicrobial agents	Conclusion
(Szczepanowski et al., 2004)	Germany	Bacteria isolated from wastewater treatment plant N = not stated	*E. coli* DH5a (pB10) containing the multiresistance plasmid pB10 or the pB10 derivative (pB10-2)	Hg Ampicillin, streptomycin, sulphonamides, tetracycline	The combined action of an integron and the conjugative DNA-transfer modules of an IncP-1 broad-host-range plasmid are of particular importance for the rapid formation and dissemination of enhanced resistance properties. The molecular study of the genome indicates that different molecular rearrangements in the integron of the IncP-1b resistance plasmid pB10 result in elevated b-lactam resistance levels.
(Salmore et al., 2006)	USA	Storm water: Ten sites were chosen along 24 river kilometres; three of the sites were within the combined sewer area of the Menomonee (sites 6–8) and two additional sites were below the confluence with the Milwaukee River (sites 9–10), also within the combined sewer system. The upper Menomonee River flows through a natural channel with intermittent stone reinforcement walls on one or both sides surrounded by suburban/urban residential land (sites 1–5). N= not stated	*E. coli*	Cr, Pb, Zn Chlorotetracycline, Kanamycin, Nalidixic acid, Neomycin, Penicillin, Tetracycline, Streptomycin, Sulphathiazole	This study demonstrates how storm water delivers heavy metals, chemicals and faecal indicator bacteria into receiving waters and can be a major contributor to degraded water quality in urban rivers. Cr, Zn, Pb and total phosphorus were elevated in stormwater, but *E. coli* levels did not correlate with this chemical signature. The results from antibiotic resistance testing suggested that the elevated *E. coli* levels observed after storm events are a mixture of human and non-human sources. The results of this study demonstrate that there is a considerable *E. coli* load to receiving waters from stormwater and that sanitary sewage inputs may not be limited to recognized sewer overflows.

Reference	Country	Sample Number	Bacterial species	Susceptibility to PTM and antimicrobial agents	Conclusion
(Filali et al., 2000)	Morocco	Sewage samples were collected aseptically from 16 sites in Casablanca city N = not stated	*Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Staphylococcus* sp.	Cd, Hg, Ag, Cu, Zn Ampicillin, Amoxicillin, Ceftriaxone, Cefotaxione, Ciprofloxacin, Rifampicin, Chloramphenicol, Tetracycline, Penicillin G, Spiramycin.	The strains most resistant to all tested products belonged to *P. fluorescens*, *P. aeruginosa*, *Klebsiella pneumoniae*, *Proteus mirabilis*, and *Staphylococcus* sp., which showed a net multiresistance against heavy metals, antibiotics, and aromatic compounds.
(Saltikov and Olson, 2002)	USA	Raw sewage and arsenic enriched creek waters N= not stated	Hot Creek isolates: *Enterobacter cloacae, Klebsiella* sp., *Yersinia intermedia, Acinetobacter calcoaceticus, Xanthomonas oryzae, Janthinobacterium lividium, Pseudomonas corrugata* South Hawee isolates: *Serratia fonticola, Pseudomonas corrugata, Acinetobacter genospecies 15, Pseudomonas corrugata, Pseudomonas vescicularis, Pseudomonas corrugata, Serratia fonticola, Pseudomonas cichorii, Serratia fonticola*	As	PCR results showed that the ars operon is conserved in some enteric bacteria isolated from creek waters and raw sewage. The occurrence of the arsBC genotype was about 50% in raw sewage enteric bacteria, while arsA was detected in only 9.4% of the isolates (n = 32). The arsABC and arsBC genotypes occurred more frequently in enteric bacteria isolated from creek samples: 71.4 and 85.7% (n =7), respectively. Average sequence divergence within arsB for six creek enteric bacteria was 20% compared to that of the *E. coli* R773 ars operon. Only 1 of 11 pseudomonads screened by PCR was positive for arsB. The results from this study suggest that significant divergence has occurred in the ars operon among As-resistant *E. coli* strains and in *Pseudomonas* spp.

Reference	Country	Sample Number	Bacterial species	Susceptibility to PTM and antimicrobial agents	Conclusion
(Dhakephalkar and Chopade, 1994)	India	Bacterial isolates were isolated from environmental sources like soil, river water, sewage, treated industrial effluent and laboratory effluent N = not stated	*Acinetobacter*	13 heavy metals including As, Ag, Cd, Co, Cr, Cu, Hg, Mo, Ni, Pb, Bi, Zn, Mo Penicillin, carbenicillin, cephalotaxime, rifampicin, neomycin, chloramphenicol, nalidixic acid	All environmental isolates of *Acinetobacter* were resistant against multiple metal ions (minimum 13 ions). The maximum number of strains was found to be sensitive to Hg (60%) while all isolates were resistant to Cu, Pb, boron and tungsten. All but 1 of the multiple metal resistant environmental isolates were also resistant to multiple antibiotics. 88.2% of the Cd-resistant isolates were resistant against penicillin and ampicillin. All Hg-resistant strains were resistant against ampicillin, carbenicillin, and chloramphenicol, while 87.5% of these strains were resistant against kanamycin, tetracycline.
(Miranda and Castillo, 1998)	Chile	Water samples were collected during a 1-year period from three different water sources: slightly polluted waters, N = 18+11+15 .	172 motile *Aeromonas* isolates	Cd, Cu, Hg, Cr Carbenicillin erythromycin, chloramphenicol kanamycin, streptomycin, gentamicin, amikacin, cephradine, tetracycline, nalidixic acid, trimethoprim-sulfamethoxazole	Moderately polluted waters showed lower antibiotic multiresistance and metal susceptibility than unpolluted and highly polluted ones. This study suggests that it is not possible to detect a higher prevalence of resistant *Aeromonas* as a function of faecal pollution level at the source of isolation. Polluted and unpolluted waters in Chile may play an important role as reservoirs of antibiotic and heavy metal resistant *Aeromonas* thereby posing a potential public health hazard.

Reference	Country	Sample Number	Bacterial species	Susceptibility to PTM and antimicrobial agents	Conclusion
(de Vicente et al., 1990)	Spain	Fresh and seawater. Samples were collected from 20 stations in 3 different geographical area (river and sea), which are exposed to domestic sewage disposal N = 60	*P. aeruginosa*	As, Ag, Cr, Hg, **Cd**, Pb, Mo Amikacin, carbenicillin, ampicillin, tetracycline, tobramycin, colistin	There was a close relationship between the degree of pollution and the frequency of heavy metal resistant strains of *P. aeruginosa*. The highest frequencies of resistance to **Hg and As**were obtained from **marine environments** with little faecal pollution, where the highest incidence of multi-resistant strains was also observed.
(Henriette et al., 1991)	France	Sewage treated in biofilter included wastewaters with hospital N = not stated	*Acinetobacter, Moraxella, Aeromonas, Vibrio, alcaligenes, Flavobacterium, Pseudomonas, Klebsiella, Serratia* N= 88 isolates	Hg, Penicillin, ampicillin, cefalotin, cefazolin, neomycin, kanamycin, amikacin, gentamicin, netilimicin, chloramphenicol, tetracycline, minocycline, erythromycin, spiramycin, prestinamycin, rifampicin, streptomycin, bacitracin, colistin	The high percentage of MDR and Hg resistance may be due to simultaneous selection that exhibited mucoid colonies and tolerance to these two categories (antibiotic and Hg) of stress agents.

Reference	Country	Sample Number	Bacterial species	Susceptibility to PTM and antimicrobial agents	Conclusion
(Smith et al., 1978)	USA	Many bacterial isolates from human, animal, including 275 bacterial isolates from **sewage and rivers**. Thermosensitive plasmids were found in all the samples of sewage and river water. N = 375	Enterobacteria (*E. coli* K12, *Salmonella typhimurium, Klebsiella pneumoniae*)	As, Ag, Cu, Co, Te. Streptomycin; sulphonamide; chloramphenicol tetracycline; spectinomycin; neomycin; ampicillin.	A high proportion of thermosensitive plasmids mediated resistance to Hg, As and tellurite; the Hg and As resistance, but not the tellurite resistance, was largely confined to plasmids that provided resistance to chloramphenicol in addition to several other antibiotics.

(Rudrik et al., 1985)	USA	Sewage N = not stated	Anaerobic clinical and sewage isolates: *Bacteriodes ruminicula,* *Clostridium perfringens* *Enterobacteriaceae*	Hg Chloramphenicol, tetracycline, gentamicin, cephalotin, erythromycin, kanamycin, ampicillin, carbenicillin, streptomycin	Four strains of anaerobic bacteria were Hg-resistant. Unlike anaerobic organisms involved in Hg-resistance, the anaerobes isolated did not contain plasmids and exhibit inducible Hg-resistance nor demonstrate MDR. The data suggest that Hg-resistance is chromosomally mediated, the presence of mobile genetic elements (plasmids, transposon) could not be ruled out.

Reference	Country	Sample Number	Bacterial species	Susceptibility to PTM and antimicrobial agents	Conclusion
(Timoney et al., 1978)	USA	Sediment; Sewage sludge N= not stated N = ?	***Bacillus*** spp.	Hg, Cd, Zn, Ampicillin, streptomycin, kanamycin, chloramphenicol, tetracycline	***Bacillus*** strains with combined ampicillin and Hg resistances were almost six times as frequent at the sludge dumpsite as in control sediment. Also, Hg resistance was frequently linked with other heavy metal resistance, and in substantial proportion of ***Bacillus*** strains, involved reduction to volatile metallic Hg (Hg˚).
Varma et al., 1978	USA	Raw sewage N = not indicated	***Enterobacteriaceae*** ***Achromobacteriaceae***	As, Pb, Hg, Cd, Cu, Ag, Bi Ampicillin, Carbenicillin, Penicillin, Tetracycline	All Pb resistant bacteria in ***Enterobacteriaceae*** showed simultaneous resistance to As, Hg, Ag and penicillin.

Table 2. Evaluations of the published articles regarding development of AMRr due to exposure to As, Cd, Cu, Hg, Ni, Pb, Zn in manure.

Reference	Country	Sample Number	Bacterial species	PTM and antimicrobial agents investigated	Conclusion
(Qian et al., 2016)	China	From February 2013 to January 2014, 219 manure-based fertiliser samples were collected; 38 samples were classified as being from chicken manure, 38 from cattle manure, 116 from pig manure and 27 from a mixture of pig and cattle manure or chicken and cattle manure. Samples were sealed in a plastic container and stored at −20 °C prior to analysis. All samples were analysed within a week of collection.	_	As, Cr, Hg, Pb, Cd 23 antimicrobial agents belong to 4 classes: sulphonamides, tetracyclines, fluoroquinolones and chloramphenicols	There were significant positive relationships between Zn and Cu. Of the fertilizer samples, 63.9% were positive for at least one antibiotic. Furthermore, approximately 10% of the collected fertilizer samples could pose a high ecological risk due to the presence of one antibiotic compound. As the co-existence of antibiotic and trace element can alter their individual effects on pollution, the potential risk of antibiotics and trace elements for co-selection and stimulation of resistance in bacterial populations.
(Li et al., 2015)	China	Fresh manure from a swine farm in the rural area of Beijing N = not indicated	Microbial community, not specified	Cu Tylosin, Vancomycin	High-Cu exposure to the microbial community during the composting not only selected for Cu resistance but also co-selected for antibiotic resistance (tylosin and vancomycin), which was of significance because the tolerance might be transferred to the soil after the land application of composted manure. These results may also have implications in the ecological risk of animal wastes contaminated by the heavy metal Cu.

Reference	Country	Sample Number	Bacterial species	Susceptibility to PTM and antimicrobial agents	Conclusion
(Riber et al., 2014)	Denmark	Cattle manure, sewage sludge or municipal solid waste compost N = not stated	*Pseudomonas* spp.	Hg Gentamicin, tetracycline, streptomycin, kanamycin, ciprofloxacin	Fertilizer amendment had a transient impact on the resistance profile of the soil community; abundance of resistant isolates decreased with time after fertiliser application, but persistent strains appeared multiresistant, also in unfertilised soil. Finally, the ability of a *P. putida* strain to take up resistance genes from indigenous soil bacteria by HGT was present only in week 0, indicating a temporary increase in prevalence of transferable ARG. It was concluded that the impact of fertilizer amendment is transient and quickly declines towards a basic level at which transfer frequency of resistance seems negligible for several reasons. Interestingly, the authors mention that the abundance of antibiotic-resistant *Pseudomonas* will decrease, but remaining strains are multiresistant, also in the unfertilized soil, indicating that soil may be an underrated reservoir of resistance traits.
(Ji et al., 2012)	China	Samples from manures, and soils collected from multiple feedlots N = not stataed		Zn, Cu Chloramphenicol, Tetracyclines, sulphonamide	The results revealed the presence of chloramphenicol, sulphonamides and tetracyclines. Typical heavy metals, such as Cu, Zn, and As, were detected. All ARGs tested were detected in the collected samples except *tetB(P)*, which was absent in animal manures. Overall, sulphonamide ARGs were more abundant than tetracycline ARGs. Except for *sulII*, only a weak positive correlation was found between ARGs and their corresponding antibiotics. On the contrary, significant positive correlations ($p<0.05$) were found between some ARGs and typical heavy metals. For example, *sulA* and *sulIII* were strongly correlated with levels of Cu, Zn and Hg. The data demonstrated that the presence of ARGs was relatively independent of their respective antibiotic inducer. In addition to antibiotics, toxic heavy metals, such as Hg, Cu, and Zn, exerted a strong selection pressure and acted as complementary factors for ARG abundance.

Reference	Country	Sample Number	Bacterial species	Susceptibility to PTM and antimicrobial agents	Conclusion
(Rahube and Yost, 2012)	Canada	Swine manure slurry obtained from a southern Saskatchewan pork producer; this slurry was later applied as fertilizer onto a research field at the Canada Saskatchewan Irrigation Diversification Centre.Two hundred and fifty grams of soil was collected in triplicates at soil depths of 0–10, 10–20 and 20–30 cm, from the four different sites of the field (North East, NE; North West, NW; South East, SE; South West, SW). The soil samples collected before and following manure application.	Plasmids were isolated from manure and transformed into *E. coli* competent cells.	Hg, Cr Erythromycin, tetracycline, gentamycin, streptomycin, kanamycin, neomycin, ampicillin, streptomycin, spectinomycin, rifampicin	The pMC2 plasmid has a tetracycline-resistant core and has acquired additional resistance genes by insertion of an accessory region (12 762 bp) containing macrolide, Hg and Cr resistance genes, which was inserted within the Tn903 / IS102 mobile element.

(Holzel et al., 2012)	Germany	Pig manure samples N = 305	*E. coli* (n=613)	Zn, Cu, Hg, Cd, Pb Beta-lactams, aminoglycosides (total 29 different antimicrobial agents)	Concentrations of heavy metals were determined by atomic spectroscopic methods in pig manure samples and were connected to the phenotypic resistance of *Escherichia coli* against 29 antimicrobial drugs. AMR in the porcine microflora might be increased by Zn and Cu. In contrast, the occurrence of Hg in the environment might, due to co-toxicity, be counter-selective against antimicrobial resistant strains.

Reference	Country	Sample Number	Bacterial species	Susceptibility to PTM and antimicrobial agents	Conclusion
(Caldini et al., 1987)	Italy	A total of 313 *E. coli* strains, of these 111 isolated from healthy human faeces, 103 from animal (pigs), and 99 from sewage sludge waters. N = number sewage samples not stated.	*E. coli* 313 strains, of these 111 isolated from healthy human faeces, 103 from animal (pigs), and 99 from sewage sludge waters.	As, Cu Streptomycin. Tetracycline, Ampicillin, chloramphenicol, Cefuroxime, Cepahlotine, Gentamicin	For *E. coli* isolates from sewage, the frequency of As and Cu markers was over 80%. The study was a methodological study rather that to investigate possible correlation between heavy metal and antibiotic resistance.

Appendix III

Table xx. Concentration (mg/kg) of PTM in manure from different animal species (Serikstad et al., 2012). It should be noted that the analysis is based on low numbers of samples. Some sample numbers stated in the Table.

Bioforsk

11. Vedlegg

11.1 Samletabell for tungmetallinnhold i ulike husdyrgjødselprøver

Tall fra Amundsen & Grønlund (1997a)							
Husdyrgjødsel	Kadmium	Bly	Kvikksølv	Nikkel	Sink	Kobber	Krom
Storfe, middel	0,2	1,7		3,1	195	30,6	1,9
Storfe, Jæren	0,39	5,6		3,1	356	50,8	1,6
Sau, middel	0,2	1,6		5,1	228	28,9	3,7
Gris, Akershus	0,29	2,2		3,7	568	62,8	2,7
Tall fra Daugstad m.fl. 2012							
Fjørfe	0,16	1,044		3,4	343	62	1,53
Min-maks	0,07-0,31	0,76-1,4		1,8-5,6	140-760	28-128	0,25-3,61
Gris	0,27			4,93	637	95	3,25
Min-maks	0,12-0,69			3,4-7	250-1542	47-185	1,3-5,2
Sau	0,16				600	34	0,942
Min-maks	0,09-0,23				250-850	17-48	0,91-0,97
Storfe	0,13			4,31	184	39	1,083
Min-maks	0,08-0,2			4,3	140-220	27-67	0,95-1,2
Tall fra Amlinger m.fl. 2004							
Bløtgjødsel, storfe, øko.	0,11	0,75		2,5	156	32	1,8
Bløtgjødsel, storfe, konv	0,13	0,92		3,6	190	49	2,3
Bløtgjødsel, gris, konv.	0,17	0,95		3,2	635	178	4,1
Fastgjødsel, storfe, øko.	0,13	1,02		3,5	148	29	3,8
Fastgjødsel, storfe,konv.	0,16	0,69		3	174	31	2,8
Fastgjødsel gris, konv.	0,32	1,17		4,4	791	130	7
Tall fra Salomon m.fl. 2006							
Bur, fast gjødsel (3)	0,23	2,1	0,008	4,33	410	40	5,63
Bur, bløt-gjødsel (18)	0,19	2,75	0,007	4,18	409	49,1	4,96
Bur, gylle(4)	0,39	2,42	0,007	4,3	523	73,3	6,08
Frittgående, fast gj.(16)	0,22	2,11	0,008	4,39	417	54,7	5,89
Frittgående, bløtgj.(11)	0,19	2,72	0,007	4,15	377	47,5	5,63
Frittgående, gylle (8)	0,22	4,09	0,013	5,16	431	61,5	6,05
Økologisk, fast gj. (5)	0,18	3,3	0,015	5,1	376	50,6	6,8
Økologisk, bløtgj. (2)	0,12	2,55	0,01	4,8	350	49	5,6
Økologisk, gylle (1)	0,04	2,6	0,014	5,9	420	49	6,7
Tall fra Haraldsen m.fl. (2011)							
Pelletert hønsegjødsel (komm.)	0,4				388	63,5	
Kompostert hønsegjødsel (komm.)	0,9				751-819	108-140	
Binadan (kommersiell)	0,27	0,58	< 0,01	5,7	482	144	3,5
Norsk naturgjødsel (komm.) Analyse fra Eurofins	0,16	0,57	0,002	5,3	430	78	3,9

Serikstad, G.L., McKinnon, K. & Eggen, T. Bioforsk Rapport vol. 7 nr. 28 2012