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ANTIBIOTICS AND ANTIBIOTIC RESISTANCE IN THE ENVIRONMENT

Ülar Allas

Tanel Tenson

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Institute of Technology

University of Tartu

AMR·RITA

1. RELEASE OF ANTIBIOTICS INTO ENVIRONMENT

A substantial fraction of antibiotics that are used to treat infections in humans or animals, for growth promotion in animals, aquaculture or plant production will ultimately end up in the environment. Hence, the environment acts as a dispersal route and reservoir of resistant pathogens, and also as an arena for the evolution of resistance.

1.1. Manufacturing plants

In the World wide scale there is a growing recognition that waste from pharmaceutical factories is a global issue. The highest concentrations of antibiotics and the highest abundances of antibiotic-resistance genes (ARGs) detected in the environment are found in environments polluted by direct discharges from the manufacturing of antibiotics. Antibiotic pollution due to wastewater emissions from manufacturing plants can be staggering, with concentrations reaching into the mg/L range, constituting strong drivers of resistance (Topp et al 2017).

As no antibiotics are produced in Estonia this issue is not directly relevant for the country.

1.2. Wrong disposal of leftovers by general population

About 20% of antibiotics consumed in human health care are used in hospitals and 80% in the households. Outdated medicines can be returned to a pharmacy or handed over free of charge in hazardous waste stations. Unfortunately, a large proportion of people discard unused medicines in household garbage or via the sewage system.

1.3. WWTP

The heavy use of antibiotics has resulted in the presence of large quantities of antibiotic residues in sewage and wastewater treatment plants (WWTPs). In addition to households, WWTPs receive sewage from hospitals which are important sources of antibiotics and their residues. Even low concentrations of antibiotics can cause a selection pressure for antibiotic resistance. Therefore, discharge of treated sewage leads to release of antibiotic resistant bacteria (ARB), ARGs and antibiotic residues to the environment. By now, there are no special technologies designed to directly remove drug residues in WWTPs.

1.4. Farming, application of manure and sludge

Antibiotic use in farming accounts for 70% of the total antibiotic consumption globally and continues to increase. Consumer demand in China and India for meat protein and adoption of commercial production practices in which antibiotics are widely employed have the potential to massively increase the global use of antibiotics in agriculture. Tetracyclines in particular, followed by penicillins and sulfonamides, are the most commonly used antibiotics in animal farming. Antibiotics are administered to animals to treat or prevent infectious diseases or as food supplements to improve livestock productivity. A proportion of antibiotics is absorbed or metabolized by animals, while some of the antibiotics or their metabolites are excreted in feces. When animal manure becomes fertilizer and is applied to agricultural land, antimicrobial residues are transferred to soil, surface water, and ground water. Similarly, fertilization of crop fields with sewage sludge and reuse of wastewater for crop irrigation have the potential to entrain residues of antibiotics into agroecosystems (Topp et al 2017).

Sweden banned the use of antibiotic growth promoters as early as 1986, a move that was followed gradually by the whole European Union. However, the practice of using antibiotics as growth promoters continues in many countries. In several countries antibiotics are also applied in plant agriculture for control of bacterial diseases.

1.5. Aquaculture

The significance of aquaculture as a source of fish and shellfish has increased tremendously in the past decades. The term aquaculture may refer to very different activities with tremendous diversity in antibiotic use. For example, 13 different antibiotics are authorized for use in aquaculture in China, compared with only five in the UK. In Norway, the use of antibiotics was reduced by 99% between 1987 and 2013. In contrast, over 90% of the world aquaculture production is carried out in low- or middle-income countries where the regulation, practices and resources are much more limited. The production of 1 ton of salmon in Norway requires 0.0008 kg of antibiotics whereas 1.4 kg of antibiotics are used per ton of salmon produced in Chile (Topp et al 2017).

Antibiotics are used in aquacultures mainly against fish pathogens. Antibiotic residues can be detected in aquaculture products, and among these oxytetracycline is the one most commonly found. Coastal aquaculture farms often use an open cage system, which allows free transfer of water from the farms to the surrounding water and eventually to the sediment. Therefore, the introduced antibiotics may cause the surrounding water and sediments becoming reservoirs of ARGs due to selection pressure.

2. TOXICITY AND ENVIRONMENTAL IMPACT OF ANTIBIOTIC RESIDUES

The amounts of antibiotics released and accumulating in the environment resulting from human activity are unprecedented, with potential impact on local communities of microorganisms, animals and humans.

2.1. Selection of resistance

Selective pressures associated with antibiotic pollution may accelerate the evolution and dissemination of antibiotic-resistant pathogens. Wastewater, sludge, soil and river water represent environments where bacteria are exposed for long time periods to concentrations of antibiotics that remain below the minimal inhibitory concentration (MIC). Moreover, low antibiotic concentrations are present in human or animal tissues during therapeutic or growth promotion use. Bacteria can still grow at sub-MIC concentrations while they are under selective pressure. Low levels of antibiotics can enrich for pre-existing resistant mutants in a bacterial population due to the differences in bacterial growth rates. In addition, the presence of antibiotics at sub-lethal levels accelerates the development of resistance through the step-wise accumulation of mutations. Even weak selective pressures can cause evolution of high-level resistance. For instance, *Salmonella enterica* exposed to low levels of streptomycin evolved high-level resistance via three small-effect resistance mutations (Wistrand-Yuen et al 2018). Exposure to low levels of antibiotics can increase genetic diversity in microbial population via the direct mutagenic effect on the DNA or via the activation of the bacterial SOS response, resulting in an increased mutation rate throughout the genome. In addition, low levels of antibiotics may enhance horizontal gene transfer. By now, upper boundaries for selective concentrations are estimated for 111 antibiotics. These concentrations can guide implementation of compound-specific emission limits (Bengtsson-Palme, Larsson 2016).

Interestingly, recent study found that the presence of resistance genes can largely be explained by fecal pollution, with no clear signs of selection in the environment, with the exception of environments polluted by very high levels of antibiotics from manufacturing, where selection is evident. This result demonstrates the necessity to take into account fecal pollution levels to avoid making erroneous assumptions regarding environmental selection of antibiotic resistance (Karkman et al 2019).

2.2. Other forms of toxicity

All medicines have side effects on human health and antibiotics are no exception. It is sometimes argued that the levels of antibiotic compounds present in the environment are very low and present negligible risks to humans. However, low concentrations can accumulate via long-term exposure to drinking water, food or consumer goods. For instance, an antimicrobial compound triclosan has been detected in urine and breast milk of people not currently using the antimicrobial.

Selective pressure from antibiotics can act on the overall microbial community composition. Antibiotic pollution in aquatic environments was found to reduce overall microbial diversity, including taxa responsible for carbon cycling and primary productivity. Similarly, the presence of antibiotics in soil was found to alter microbial community structure, leading to a loss of biomass and a reduction in microbial activity including nitrification, denitrification, and respiration (reviewed in Kraemer et al 2019).

Antibiotics have been shown to impact higher organisms by disrupting the microbial populations associated with animal hosts. Disruption of a hosts' microbiome can lead to serious health consequences, including developmental defects, allergies, metabolic diseases or increased susceptibility to pathogens. The effect of antibiotic pollution is especially strong in aquatic environment where animals are constantly exposed to the pollutants. Low concentrations of antibiotics administered as a prophylactic in aquaculture can reduce microbial diversity in the fishes' gut and increase mortality. In addition, low concentrations of macrolides were shown to induce malformations in zebrafish and in an amphibian *Xenopus tropicalis* (Kraemer et al 2019, and references within).

3. RELEASE OF RESISTANT BACTERIA AND RESISTANCE GENES

One of the most noted consequences of antibiotic misuse and antibiotic pollution is the increased frequency of bacteria harboring ARGs in different environments. The environment plays a role both in the transmission of resistant pathogens and as a source for resistance factors that over time are transferred horizontally to pathogens.

3.1. Flow of resistance genes

Both ARB and ARGs may spread from hotspots to pristine environment through several modes of transport, including surface waters, air, humans and animal carriers. Surface waters are considered one of the most important factors of ARG dissemination as they receive sewage from WWTP and pharmaceutical institutions as well as run-off from manure-fertilized fields and animal feeding operations. Besides facilitating ARG transport, surface waters also provide a suitable environment for horizontal gene transfer to take place.

Recent studies have shown that air can also contribute to the spread of ARGs. Bacteria can disperse via wind, fog, and precipitation when attached to particles floating in the air. Analysis of urban air covering 19 world cities revealed the presence of ARGs against seven commonly used antibiotics. Airborne transmission of ARGs has also been registered in proximity of WWTPs and hospitals. Thus, there is a need to redefine current air quality standards in terms with public health (Kraemer et al 2019).

Animals and humans may constitute a reservoir of antibiotic resistance as their microbiomes offer nutrient-rich environments where ARB may amplify or change genetic background. A number of vertebrates and insects have been shown to carry ARB. In addition, the spread of ARB has been associated with international travelers and the transport of goods and food. Studies have demonstrated that human commensals and pathogens carrying ARGs can establish themselves in travelers' microbiome. As a consequence, these bacteria are then transported to travelers' home country.

3.2. Farming, aquaculture, use of antibiotics in general human community

Manure is an important fertilizer, but it may contain diverse ARGs. Therefore, its application to fields may lead to increased abundance of ARGs in the environment. The presence and enrichment of mobile genetic elements in farm sediments may potentially lead to co-selection of other ARGs and facilitate the transfer of ARGs to other bacteria, including human pathogens.

ARB from animals have to cross a species barrier to establish themselves in humans. Therefore, the probability that animal-originated ARB will be transmitted directly to humans is relatively low. Nevertheless, animal-originated ARGs may be transferred into genomes of human commensals or pathogens. Farmers and other workers in direct contact with livestock have been shown to develop increased resistance to antibiotics. Similarly, small rodents trapped on or close to farms carry more ARB than those trapped in other areas (Kraemer et al 2019).

Aquaculture farms have been suggested to be hotspots for ARG enrichment and transfer due to prophylactic and therapeutic use of antibiotics to treat fish infections. Fish farming may also cause enrichment of mobile genetic elements that carry ARGs and can thus pose a risk of transferring ARGs to other bacteria and in some cases to clinically relevant bacteria (Topp et al 2017).

Global human consumption of antibiotics corresponds to several tens of billions of unit doses each year. Beta-lactam antibiotics are currently the most widely used in humans. However, up to half of antibiotics used in humans are unnecessary or inappropriate. For instance, antibiotics are often used to attempt to treat flu and other viral diseases. Another reason for the widespread use of antibiotics is uncontrolled sale in low or middle income countries, where they can be obtained without a prescription, potentially resulting in being used when not indicated.

3.3. WWTP and hospitals as specific hotspots to consider

Health care institutions are regarded as hotspots in the spread of bacterial resistance due to the broad spectrum of antibiotics used and the high risk of human-to-human transmission of selected resistant bacteria. Also, hospitals are often unable to identify the causative organism(s) in time to treat patients presenting with rapidly progressing sepsis or other severe infections, resulting in the overuse of broad-spectrum antibiotics. The intensive use of antibiotics in hospitals provides an environment where resistant bacteria can flourish, largely unhampered by competition from susceptible bacteria. Movement of patients and staff between wards and other hospitals is another potential route for transmission of resistant microorganisms. A few hospitals worldwide use membrane bio reactors in combination with activated carbon, osmosis, nanofiltration or some oxidation process to remove the active pharmaceutical ingredients from the waste water (Casas et al 2015). In addition, application of disinfection technologies (UV light, steam vapor machines, etc) may improve decontamination in healthcare facilities.

Municipal wastewater treatment is one of the major pathways by which ARGs from anthropogenic sources are introduced into natural ecosystems. WWTPs receive sewage and bacteria from various sources, including pharmaceutic industry and hospitals. In addition to antibiotics, waste water also contains disinfectants and metals which can form a selection pressure for antibiotic resistance. The presence of antibiotics, ARB, and ARGs in the same setting creates an environment that selects for antibiotic resistance and provides an opportunity for genetic material housing ARGs to transfer between bacterial species via horizontal gene transfer. Thus, WWTPs can act as reservoirs and environmental suppliers of antibiotic resistance (Karkman et al 2018).

People working in hospitals and WWTPs may easily become in contact with human-evolved ARB and participate in their transmission and dissemination. There is an increased risk that the chain of transmission also extends to workers' family members. Moreover, birds (especially ducks) have been associated with the spread of resistance from WWTPs. Cockroaches and flies have also been implicated with transmissions of ARB from WWTPs, hospitals and farms.

Recently, a study was completed that compared the influent and final effluent of 12 urban WWTPs located in seven European countries. This first trans-Europe surveillance showed that urban WWTP antibiotic resistance profiles mirror the antibiotic resistance gradient observed in clinics. Antibiotic use, environmental temperature, and WWTP size were important factors related with resistance persistence and spread in the environment (Pärnänen et al 2019).

To test the seasonality of ARGs in wastewater, the distribution of prescriptions and different ARGs in the sewer system and WWTP was investigated in Dresden. The results showed a clear seasonal pattern - the higher ARGs levels in autumn and winter coincide with the higher rates of overall antibiotic prescriptions. Neither the transport through the sewers nor the treatment of wastewater resulted in a reduction of the relative level of ARGs. Contrasting to this, there were clear differences in the dominant taxa between wastewater and treated wastewater. This analysis suggests that the bacteria carrying the resistance genes were either not part of the dominant microbial community, or that processes such as horizontal gene transfer disconnected the community and ARGs profiles of the treated wastewater (Caucci et al 2016).

3.4. Transfer mechanisms (transformation, conjugation, transduction)

Bacteria are able to exchange their genetic material. This process, known as horizontal gene transfer, is the primary mechanism for the spread of ARGs in bacteria. Genes can be passed from one bacterium to another by three different mechanisms: transformation, conjugation and transduction. Transformation is a phenomenon in which bacteria acquire naked DNA from the surrounding environment. Such extracellular DNA comes from lysis of dead bacteria or secretion by live bacteria. Conjugation is a form of horizontal gene transfer in which DNA transfer occurs by direct cell-to-cell contact between bacterial cells or by a bridge-like connection between two cells. Unlike transformation, conjugation enables the transfer of large DNA fragments, such as conjugative plasmids that are regarded as key contributors in the dissemination of ARGs and promoters of multi-drug resistance. Finally, transduction is a mechanism of DNA transfer that is mediated by bacteriophages (a group of viruses that parasite on bacteria). When bacteriophages infect bacteria, they harness host cells to produce new viral particles. During this process, bacterial DNA can be packed into freshly synthesized bacteriophage particles. New bacteriophages then continue to infect other bacteria. Upon next infection, phage-delivered bacterial DNA may be recombined into the chromosome of another bacterium. The length of DNA transferred by transduction is limited by the size of the bacteriophage head. Horizontal gene transfer is common among bacteria and can occur between distantly related species (Kraemer et al 2019).

3.5. Effect of metals (and other chemicals) on the spread of antibiotic resistance

Accumulating evidence shows that metal residues can enhance environmental selection of antibiotic resistance. Bacteria are generally very sensitive to metal pollution. However, they can evolve a variety of resistance mechanisms, mediated by chromosomal mutations or by the uptake of resistance genes carried by mobile genetic elements.

Metals can co-select for resistance to clinically relevant antibiotics by several mechanisms, including co-resistance (metal resistance- and antibiotic resistance genes are located on the same mobile genetic element) and cross-resistance (the same genetic determinant mediates resistance to both antibiotics and metals).

Metals are used in animal farming together with antibiotics. For instance, copper and zinc are widely used to complement antibiotics in order to promote animal growth or alleviate diarrhea. Metals persist for long periods in soil, and they consequently accumulate in agricultural soils receiving animal manure.

Manure-amended soils will thus contain elevated levels of copper and zinc. This increases the likelihood for selection of mobile genetic elements conferring resistance to both metals and antibiotics.

Study of the abilities of copper and zinc to co-select for tetracycline resistance in soil bacterial communities demonstrated that both metals induced a persistent selection pressure thus facilitating co-selection for antibiotic resistance. The observed co-selection threshold concentrations were high, but environmentally realistic. The authors suggest that metals may in some cases exert a stronger selection pressure for environmental selection of resistance to an antibiotic than the specific antibiotic itself. Thus, metal residues in soil must be considered a significant environmental issue (Song et al 2017).

Besides antibiotics and metals, there is an evidence that widely used biocides have a potential to co-select for resistance, primarily through co- and cross-selection mechanisms. In addition to providing selective advantages, low concentrations of certain antimicrobials may increase resistance development by promoting mutagenesis or through stimulation of horizontal gene transfer, as shown for both triclosan and chlorhexidine (Jutkina et al 2018).

4. SURVEILLANCE POLICY

The environment plays an important role both in the transmission of antibiotic resistant pathogens and in their evolution. Environmental surveillance of antibiotics and antibiotic resistance could contribute towards the protection of human, animal and ecosystem health. Integrated antibiotic resistance surveillance is one of the objectives of the World Health Organization global action plan on antimicrobial resistance. Unfortunately, many low- and middle-income countries lack the capacity to establish and maintain systems to collect and make use of data on antimicrobial consumption.

Regulatory requirements for antibiotic manufacturing sites and emission control worldwide vary from being non-existent to the application of high standards. The production, trade and consumption chain of pharmaceuticals is complex and involves many actors with different interests. In fact, 33 different actor-types, their main stakes and interest have been identified together with incentives that can motivate them to improve the situation, as well as disincentives that may undermine such motivation (Nijsingh et al 2019).

Recently, a conceptual framework for the environmental surveillance of antibiotics and antibiotic resistance was proposed that envisages integration of environmental, human and animal surveillance. Five objectives for surveillance were defined: 1) to address the risk of transmission of already antibiotic-resistant bacteria to humans via environmental routes; 2) to address the risk for accelerating the evolution of antibiotic resistance in pathogens through pollution with selective agents and bacteria of human or animal origin; 3) to address the risks antibiotics pose for aquatic and terrestrial ecosystem health, including the effects on ecosystem functions and services; 4) to identify the population-level resistance prevalence; 5) to identify population-level antibiotic use. Moreover, the framework outlines the choice of markers for each objective together with sites where monitoring would be particularly informative (Huijbers et al 2019).

At a workshop held in Gothenburg in 2017, scientists from 14 countries identified critical knowledge gaps that need to be filled in order to more efficiently manage the emergence and spread of antibiotic resistance in the environment. These key areas are: 1) the relative contributions of different sources of antibiotics and antibiotic resistant bacteria into the environment; 2) the role of the environment, and particularly anthropogenic inputs, in the evolution of resistance; 3) the overall human and animal health impacts caused by exposure to environmental resistant bacteria; and 4) the efficacy and feasibility of different technological, social, economic and behavioral interventions to mitigate environmental antibiotic resistance. Researchers, funding organizations, policy makers and other relevant stakeholders are encouraged to take the steps needed to fill these knowledge gaps (Larsson et al 2018).

5. STUDIES IN OUR REGION

5.1. Freshwater and groundwater

In 2008, samples were taken from Estonian river Emajõgi in order to test the extent of resistance among cultivable freshwater bacteria. 760 isolates were collected that were resistant to at least one antibiotic. The phylogeny of the isolates covered a wide range of Proteobacteria, Actinobacteria and Bacteroidetes. The isolates were tested on six antibiotics in order to determine the extent of multiresistance. It was concluded that there is no massive spread of multiresistance determinants in the examined freshwater environment. The results suggested that multiresistance happens by chance and that there is no selection for it (Voolaid et al 2012). Further studies demonstrated, that resistance genes from the *ampC* class, important resistance genes in medicine, can be found in the river Emajõgi. All detected genes belonged to the FOX family of *ampC* genes. The most numerous species found to contain these genes were members of the *Aeromonas* genus (Voolaid et al 2013).

As a Member State of European Union, Estonia is obliged to carry out the monitoring of the chemical status of groundwater. In 2018, Estonian Environmental Research Center revealed a report concerning the presence of potentially dangerous substances in groundwater. The most commonly detected antibiotic in groundwater samples was dimetridazole, found in 6 out of 40 study sites. Sulfamethoxazole was detected in 4 and ofloxacin in 2 study sites. Groundwater is the main source of drinking water supplies in many Estonian regions (Eesti Keskkonnauuringute Keskus, 2018).

5.2. WWTP

There is a rising concern that conventional WWTPs increase the relative abundance of ARGs during processing because they do not employ technologies that target the removal of genetic elements. To confirm this idea, both raw wastewater and final effluent water samples were collected during 2010 to 2011 from three city WWTPs located in Helsinki, Tallinn and Tartu. Several ARGs were detected in most wastewater and effluent samples. However, the purification process caused no significant change in the relative abundance of ARGs. Thus, the studied WWTPs didn’t seem to be important sites for changes in the relative abundance of ARGs at the whole community level (Laht et al 2014).

Another study examined the abundance and proportion of seven ARGs in microbial communities in the influent, effluent, and wetland media biofilm of horizontal subsurface flow filter mesocosms of a constructed wetland, located in Nõo village, Estonia. ARGs were detected in influent, effluent as well as in media biofilm. Nevertheless, the abundance and the proportions of ARGs in the microbial community were found to reduce during the wastewater treatment process. The authors proposed that constructed wetlands can serve as alternatives to conventional treatment for ARG removal (Nõlvak et al 2013).

Sewage sludge is an inevitable by-product of wastewater treatment. In Estonia, about 360,000 − 500,000 tons of sewage sludge is created annually. According to studies, is possible to enhance the degradation rate of several pharmaceuticals through the intelligent selection of sewage sludge composting conditions. It was shown, for instance, that the degradation of fluoroquinolones and sulphonamides takes place during sewage sludge co-composting with sawdust, peat and straw. The composting period should last at least six months (Haiba 2017).

Lillenberg and others have developed a reliable method for simultaneous determination of fluoroquinolones, sulfonamides and tetracyclines in sewage sludge from WWTPs. The compounds are extracted from sewage sludge by pressurized liquid extraction. This novel method was used to analyze sludge from sewage treatment plants of Tallinn and Tartu. The results showed that antibiotics are present in Estonian sewage sludge and their content may exceed the relevant trigger values for manure. Therefore, application of sewage sludge compost as a fertilizer and may cause contamination of agricultural plants (Lillenberg et al 2009, Lillenberg 2011). Nevertheless, environmentally secure utilization of composted waste products is possible. Sewage sludge can be used more effectively by target-applying it to the exact spots where it is needed instead of spreading it uniformly over the area (Järvis 2018).

5.3. Soil and manure

An antibiotic resistance study conducted in Estonia between 2011 and 2014 identified three *E. coli* strains harboring a plasmid carrying the colistin resistance gene *mcr-1*. All three strains were found in a single pig slurry sample originated from a farm located in North Estonia. *mcr-1* was the only ARG on a 33,311-bp plasmid, with the other genes mainly coding for proteins involved in conjugative DNA transfer. This plasmid could cause serious problems as colistin is an antibiotic used as a last-resort for multidrug-resistant Gram negative infections. These particular *E. coli* strains are not likely to be problematic from an antibiotic treatment viewpoint. Still, considering that the strains were genetically very different, it is probable that the plasmid is highly mobile. It could be transferred into strains resistant to a wide range of antibiotics, thereby creating strains that are very hard to treat (Brauer et al 2016).

Nõlvak and colleagues have examined the effect of mineral fertilizer (NH_4NO_3), cattle slurry and cattle slurry digestate amendment on the abundance and proportion dynamics of five ARGs (*sul1*, *tetA*, *blaCTX-M*, *blaOXA2* and *qnrS*) and two classes of integron-integrase genes (*intI1* and *intI2*) in agricultural grassland soil. It was found that both cattle slurry and cattle slurry digestate are considerable sources of ARGs, as well as integron-integrases. Fertilization of agricultural grassland soil substantially affected its ARG content. Mineral fertilizer usage significantly enhanced *tetA* abundance in soil. The application of organic fertilizers, on the other hand, significantly enhanced *sul1, intI1* and *intI2* abundance in soil. The results indicate that *sul1, intI1* and *intl2* genes are mainly entrained to soil via cattle slurry and its digestate fertilization (Nõlvak et al 2016).

Data from Finland indicates that ARGs are disseminated even in regions with comparably low antibiotic use. The effects of manure storage and application to fields on the abundance of ARGs were studied in Finnish production animal farms. Analysis of samples collected during the cropping season of the year 2013 revealed that the relative abundance of ARGs increased about fourfold in soil after manure application. Genes encoding carbapenemase were found despite the lack of on-farm use. The relative abundance of ARGs increased in stored manure compared with fresh manure roughly fivefold (Ruuskanen et al 2015).

Another study in Finland focused on the resistome in dairy and swine farms where use of antibiotics is limited to treating bacterial infections and manure is only applied from April to September. The results of this study suggest that increases in ARG abundances after fertilizing are temporary under agricultural practices that restrict antibiotic use. The abundance and richness of the resistance determinants decreased substantially by 6 weeks after manure application. However, the authors warn that the threat arising from the possible enrichment of resistance determinants during storage of manure used as land-applied fertilizer should not be underestimated (Muurinen et al 2017).

5.4. The Baltic Sea

It is necessary to collect information about the prevalence of antibiotic resistance in the Baltic Sea, the world's largest inland brackish sea. In addition to being a transport channel and recreational region, the Baltic Sea serves as a drainage area for approximately 85 million people in 14 countries that differ from one another in antibiotic use and sewage treatment policies. To characterize the bacterioplankton community and ARGs in the Baltic Sea, samples were collected from four different regions during 2008 - 2009. The abundance and proportion of seven ARGs encoding resistance to four major antibiotic classes was determined. The results showed that all the resistance genes targeted in the study were detectable from the bacterioplankton. The most numerous ARG detected was the *tetA* gene and this gene also had the highest proportion in the whole microbial community (Tiirik et al 2014).

Several studies have assessed the presence of ARGs in Baltic Sea fish farms. During 2006−2009, sediment samples were collected from four aquaculture farms located in the archipelago area in the northern Baltic Sea in order to monitor the presence of tetracycline-resistance genes together with the amount of tetracyclines. Results showed greater copy numbers of tetracycline resistance genes at the farms compared to pristine sites. However, no resistance genes were found in samples collected 200 m from any of the farms. None of the farms were using tetracycline at the time of the sampling and none of the samples contained therapeutically active concentrations of tetracyclines. These data suggest that aquaculture maintains elevated levels of tetracycline resistance genes at the marine sediments below the farms even when antibiotic use is stopped (Tamminen et al 2011). In general, fish farm resistomes were enriched in transposon and integron associated genes and in ARGs encoding resistance to antibiotics which had been used to treat fish at the farms (Muziasari et al 2016b). The resistomes of the intestinal contents of farmed fish contribute to the enrichment of ARGs in sediments below fish farms (Muziasari et al 2017a).

REFERENCES

Bengtsson-Palme, J., & Larsson, D. G. (2016). Concentrations of antibiotics predicted to select for resistant bacteria: Proposed limits for environmental regulation. *Environ Int, 86*, 140-149.

Brauer, A., Telling, K., Laht, M., Kalmus, P., Lutsar, I., Remm, M., Kisand, V., & Tenson, T. (2016). Plasmid with Colistin Resistance Gene mcr-1 in Extended-Spectrum-β-Lactamase-Producing Escherichia coli Strains Isolated from Pig Slurry in Estonia. *Antimicrob Agents Chemother, 60*(11), 6933-6936.

Casas, M. E., Chhetri, R. K., Ooi, G., Hansen, K. M., Litty, K., Christensson, M., … Bester, K. (2015). Biodegradation of pharmaceuticals in hospital wastewater by staged Moving Bed Biofilm Reactors (MBBR). *Water Res, 83*, 293-302.

Caucci, S., Karkman, A., Cacace, D., Rybicki, M., Timpel, P., Voolaid, V., Gurke, R., Virta, M., & Berendonk, T. U. (2016). Seasonality of antibiotic prescriptions for outpatients and resistance genes in sewers and wastewater treatment plant outflow. *FEMS Microbiol Ecol, 92*(5), fiw060.

Haiba, E. (2017). *Optimization of Sewage Sludge Composting: Problems and Solutions.* Tallinn University of Technology. Retrieved from: https://digikogu.taltech.ee/et/Download/9c93f769-c4b1-4eb3-83fa-80a789ca4ae2

Huijbers, P. M. C., Flach, C. F., & Larsson, D. G. J. (2019). A conceptual framework for the environmental surveillance of antibiotics and antibiotic resistance. *Environ Int, 130*, 104880.

Järvis, J. (2018). *Novel Technology for Utilization of Solid Organic Waste in Recultivation of Abandoned Mining Areas.* Tallinn University of Technology. Retrieved from: https://digikogu.taltech.ee/et/Download/82e3a0ef-68af-4b4c-8b50-eea87855a0fb

Jutkina, J., Marathe, N. P., Flach, C. F., & Larsson, D. G. J. (2018). Antibiotics and common antibacterial biocides stimulate horizontal transfer of resistance at low concentrations. *Sci Total Environ, 616-617*, 172-178.

Karkman, A., Do, T. T., Walsh, F., & Virta, M. P. J. (2018). Antibiotic-Resistance Genes in Waste Water. *Trends Microbiol, 26*(3), 220-228.

Khan, S., Beattie, T. K., & Knapp, C. W. (2017). The use of minimum selectable concentrations (MSCs) for determining the selection of antimicrobial resistant bacteria. *Ecotoxicology, 26*(2), 283-292.

Kraemer, S. A., Ramachandran, A., & Perron, G. G. (2019). Antibiotic Pollution in the Environment: From Microbial Ecology to Public Policy. *Microorganisms, 7*(6).

Laht, M., Karkman, A., Voolaid, V., Ritz, C., Tenson, T., Virta, M., & Kisand, V. (2014). Abundances of tetracycline, sulphonamide and beta-lactam antibiotic resistance genes in conventional wastewater treatment plants (WWTPs) with different waste load. *PLoS One, 9*(8), e103705.

Larsson, D. G. J., Andremont, A., Bengtsson-Palme, J., Brandt, K. K., de Roda Husman, A. M., Fagerstedt, P., . . . Wernersson, A. S. (2018). Critical knowledge gaps and research needs related to the environmental dimensions of antibiotic resistance. *Environ Int, 117*, 132-138.

Lillenberg, M. (2011). *Residues of some pharmaceuticals in sewage sludge in Estonia, their stability in the environment and accumulation into food plants via fertilizing.* Estonian University of Life Sciences. Retrieved from http://hdl.handle.net/10492/146

Lillenberg, M., Yurchenko, S., Kipper, K., Herodes, K., Pihl, V., Sepp, K., Lõhmus, R., & Nei, L. (2009). Simultaneous determination of fluoroquinolones, sulfonamides and tetracyclines in sewage sludge by pressurized liquid extraction and liquid chromatography electrospray ionization-mass spectrometry. *J Chromatogr A, 1216*(32), 5949-5954.

Muurinen, J., Stedtfeld, R., Karkman, A., Pärnänen, K., Tiedje, J., & Virta, M. (2017). Influence of Manure Application on the Environmental Resistome under Finnish Agricultural Practice with Restricted Antibiotic Use. *Environ Sci Technol, 51*(11), 5989-5999.

Muziasari, W. I., Pitkänen, L. K., Sørum, H., Stedtfeld, R. D., Tiedje, J. M., & Virta, M. (2016a). The Resistome of Farmed Fish Feces Contributes to the Enrichment of Antibiotic Resistance Genes in Sediments below Baltic Sea Fish Farms. *Front Microbiol, 7*, 2137.

Muziasari, W. I., Pärnänen, K., Johnson, T. A., Lyra, C., Karkman, A., Stedtfeld, R. D., Tamminen, M., Tiedje, J. M., & Virta, M. (2016b). Aquaculture changes the profile of antibiotic resistance and mobile genetic element associated genes in Baltic Sea sediments. *FEMS Microbiol Ecol, 92*(4), fiw052.

Nijsingh, N., Munthe, C., & Larsson, D. G. J. (2019). Managing pollution from antibiotics manufacturing: charting actors, incentives and disincentives. *Environ Health, 18*(1), 95.

Nõlvak, H., Truu, M., Kanger, K., Tampere, M., Espenberg, M., Loit, E., Raave, H., & Truu, J. (2016). Inorganic and organic fertilizers impact the abundance and proportion of antibiotic resistance and integron-integrase genes in agricultural grassland soil. *Sci Total Environ, 562*, 678-689.

Nõlvak, H., Truu, M., Tiirik, K., Oopkaup, K., Sildvee, T., Kaasik, A., Mander, Ü., & Truu, J. (2013). Dynamics of antibiotic resistance genes and their relationships with system treatment efficiency in a horizontal subsurface flow constructed wetland. *Sci Total Environ, 461-462*, 636-644.

Pazda M., Kumirska, J., Stepnowski, P., & Mulkiewicz, E. (2019) Antibiotic resistance genes identified in wastewater treatment plant systems – A review. *Sci Total Environ,* 679.

Pärnänen, K. M. M., Narciso-da-Rocha, C., Kneis, D., Berendonk, T. U., Cacace, D., Do, T. T., . . . Manaia, C. M. (2019). Antibiotic resistance in European wastewater treatment plants mirrors the pattern of clinical antibiotic resistance prevalence. *Sci Adv, 5*(3), eaau9124.

Ruuskanen, M., Muurinen, J., Meierjohan, A., Pärnänen, K., Tamminen, M., Lyra, C., Kronberg, L., & Virta, M. (2016). Fertilizing with Animal Manure Disseminates Antibiotic Resistance Genes to the Farm Environment. *J Environ Qual, 45*(2), 488-493.

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, 51*(5), 3040-3047.

Eesti Keskkonnauuringute Keskus. (2018). Kvaternaari põhjaveekihtidest moodustatud põhjaveekogumites ja maapinnalt esimestest aluspõhjalistest põhjaveekihtidest moodustatud põhjaveekogumites ohtlike ainete sisalduse uuring. Retrieved from https://www.envir.ee/

Tamminen, M., Karkman, A., Lõhmus, A., Muziasari, W. I., Takasu, H., Wada, S., Suzuki, S., & Virta, M. (2011). Tetracycline resistance genes persist at aquaculture farms in the absence of selection pressure. *Environ Sci Technol, 45*(2), 386-391.

Tiirik, K., Nõlvak, H., Oopkaup, K., Truu, M., Preem, J. K., Heinaru, A., & Truu, J. (2014). Characterization of the bacterioplankton community and its antibiotic resistance genes in the Baltic Sea. *Biotechnol Appl Biochem, 61*(1), 23-32.

Topp, E., Larsson, D. G. J., Miller, D. N., Van den Eede, C., & Virta, M. P. J. (2018). Antimicrobial resistance and the environment: assessment of advances, gaps and recommendations for agriculture, aquaculture and pharmaceutical manufacturing. *FEMS Microbiol Ecol, 94*(3).

Voolaid, V., Jõers, A., Kisand, V., & Tenson, T. (2012). Co-occurrence of resistance to different antibiotics among aquatic bacteria. *BMC Microbiol, 12*, 225.

Voolaid, V., Tenson, T., & Kisand, V. (2013). Aeromonas and Pseudomonas species carriers of ampC FOX genes in aquatic environments. *Appl Environ Microbiol, 79*(3), 1055-1057.

Wistrand-Yuen, E., Knopp, M., Hjort, K., Koskiniemi, S., Berg, O. G., & Andersson, D. I. (2018). Evolution of high-level resistance during low-level antibiotic exposure. *Nat Commun, 9*(1), 1599.