OCCURRENCE OF GENES INVOLVED IN ANTIBIOTIC RESISTANCE IN SIX DANISH STREAMS

Scientific Report from DCE – Danish Centre for Environment and Energy No. 554 2023

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Data sheet

Series title and no.:	Scientific Report from DCE – Danish Centre for Environment and Energy No. 554
Category:	Scientific advisory report
Title:	Occurrence of genes involved in antibiotic resistance in six Danish streams
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Institution:	Department of Environmental Science, Aarhus University
Publisher:	Aarhus University, DCE – Danish Centre for Environment and Energy ©
URL:	http://dce.au.dk/en
Year of publication:	May 2023
Editing completed:	May 2023
Referee(s):	Niels Bohse Hendriksen
Quality assurance, DCE:	Kirsten Bang
External comments:	No external comments
Financial support:	World Animal Protection, Denmark
Please cite as:	Johansen, A., & Zervas A. 2023. Occurrence of genes involved in antibiotic resistance in six Danish streams. Aarhus University, DCE – Danish Centre for Environment and Energy, 18 pp. Scientific Report No. 554.

Abstract: DCE has been commissioned by World Animal Protection Denmark to quantify antibiotic-resistance genes (ARGs) in water and sediment samples from six streams located in agricultural landscapes in Denmark. The analyses showed that ARGs related to tetracycline and macrolides were detected in all water and sediment samples. However, their association with the nearby farms cannot be confirmed by the present study

Keywords:	Antibiotic resistance, tetracycline, macrolide, gene, water, sediment, penicillin
Front page photo:	Colorbox (the location on the photo is not related to the present study)
ISBN:	978-87-7156-777-9
ISSN (electronic):	2244-9981
Number of pages:	18

Contents

Preface

The present study was conducted for World Animal Protection (WAP) in Denmark. WAP presented in 2021 the report "Silent superbug killers in a river near you", which has participation from WAP offices in Thailand, United States, Canada, and Spain. According to this report [1], the obtained data indicates a connection between pig farm effluent discharge and presence of genes associated with antibiotic resistance (ARGs) in the nearby waterways. To complement this report, WAP Denmark commissioned DCE to quantify ARGs related to bacterial resistance to tetracycline, penicillin, and macrolides in six streams near Danish conventional pig farms during spring 2022.

Summary

The purpose of the present survey was to quantify the occurrence of five ARGs associated with resistance to tetracycline, penicillin, and macrolides in six streams located in agricultural landscapes in Denmark.

World Animal Protection Denmark (WAP DK) collected the water samples from streams, upstream and downstream of locations with neighboring pig farms in order to find out whether enhanced levels of ARGs in downstream water might indicate a release of ARGs from the farm into the stream. At all sampling locations, additional sediment samples were collected to assess whether the microbiota in the stationary sediment contained different levels of ARGs compared to the microbiota in the non-stationary water phase.

DCE was commissioned by WAP DK to perform the extraction of DNA from the water and sediment samples, quantify antibiotic-resistance genes by qPCR and subsequent analyze the obtained data.

The analyses showed that ARGs related to tetracycline and macrolides were detected in all water and sediment samples from the six streams investigated, while ARGs associated with resistance to penicillin was only detected in very low numbers. In general, occurrence of ARGs per unit of total DNA was higher in the sediment than in the water. There was no clear relation between ARGs occurrence in samples taken upstream and downstream, respectively, and hence, no indications that the close surroundings of the farms contributed to the occurrence of ARGs in the nearby streams.

Common for all the five investigated ARGs is that the natural background level of these genes is unknown. It is therefore not possible in the present study to assess whether the measured ARGs occurred at higher densities than would be expected in a pristine environment.

1 Introduction

Resistance to antibiotics is a huge global health problem, impacting millions of humans due to decreased possibilities to treat bacterial, parasitic, and fungal infections. Bacterial resistance to antibiotics, alone, is estimated to be responsible for 1.3 million deaths in 2019 [2]. Accordingly, WHO declares antimicrobial resistance to be on the top ten of global public health threats also hindering the fulfillment of the UN Global Development Goals (SDGs), namely regarding health and well-being (SDGs 3, 6), poverty reduction (SDG 1), food security (SDGs 2, 12), environment and economic growth (SDGs 6, 8, 12) [3,4,5].

The precondition leading to antibiotic resistant bacteria (ARBs) is due to bacterial ARGs with different genetic traits which might lead to resistance to specific antibiotics rendering them ineffective to control the pathogens carrying them. Bacteria may acquire antibiotic resistance through mutations or by transfer of ARGs from other bacteria in possession of them. It is a general understanding that the overuse of antibiotics creates an evolutionary pressure on bacteria to acquire ARGs due to conditions with enhanced concentrations of antibiotics e.g., at hospitals, doctors' generous prescriptions to patients, and in the agricultural sector when antibiotics are used both to treat sick animals as well as growth enhancers [6]. In Denmark most antibiotics are used only for treating diseased farmed animals, especially pigs and their use as growth enhancers has been banned since 2000 [7]. However, bacteria resistant to antibiotics can also be found in pristine environments with no exposure to antibiotics released through human activities. In these cases, ARGs are naturally occurring, probably conferring resistance to antibiotics released by other microorganisms or may have a pleiotropic role and serve other functions besides antibiotic resistance [8,9]. Antibiotics and antibiotic resistance have always coexisted in the microbiota in all types of environments. However, there are many indications that the use and release of antibiotics to the environment is a driver for development and increased occurrence of ARBs and ARGs in many places [6,8,9].

In animal husbandry, antibiotics are used to treat diseased animals. However, a proportion of these antibiotics are passing through the animals and, hence, found in the manure [10]. This poses a risk that the antibiotics are spread when using the manure as fertilizer. In parallel, bacteria with ARGs may be present in the gut system of the treated animals, as well as in the resulting manure, where they may exchange ARGs with bacteria from the same or other species [11].

In all, the appearance and exchange of ARGs among members of the microbiota in the gut system of farmed animals, manure, and in the environment is extremely complex and far from understood. It is important to know the pool of ARGs in the environment as it may indicate which drivers are determining the number and diversity of ARGs - e.g., as a consequence of spreading manures originating from antibiotic-treated domestic animals.

The purpose of the present survey was to quantify the occurrence of five ARGs associated with resistance to tetracycline, penicillin, and macrolides in six streams located in agricultural landscapes in Denmark. These specific antibiotics were chosen, as they are commonly used antibiotics in pig production

in Denmark and they were also common to several of the WAP studies conducted in other countries [1,7].

WAP DK collected water samples from streams, upstream and downstream of locations with neighboring pig farms to find out whether enhanced levels of ARGs in downstream water might indicate a release of ARGs from the farm into the stream. At all sampling locations, additional sediment samples were collected to assess whether the microbiota in the stationary sediment contained different levels of ARGs compared to the microbiota in the non-stationary water phase.

DCE was commissioned by WAP DK to perform the extraction of DNA from the water and sediment samples, quantify antibiotic-resistance genes by qPCR and subsequent analyze the data obtained.

2 Materials and methods

2.1 Sampling of water and sediments

The survey was carried out in May-July 2022, a period where manure is often spread by farmers in Denmark. Water and sediment samples were collected in six Danish streams, as decided and sampled by WAP DK, located on Zealand and Fyn. The streams were located close to farms with conventional pig production and known to have a high level of antibiotic use in 2021-2022 [12]. A high level of antibiotics was defined as values exceeding the Danish Veterinary and Food Administrations (DVFA) issued threshold for antibiotic use for at least one of the age groups of farmed pigs [12]. Upstream and downstream samples were defined by WAP DK as collecting samples 300 m upstream and 300 m downstream of the farm buildings, respectively. In total, 72 samples from six streams as collected by WAP DK (Table 1).

Table 2.1. Overview of samples

Number of locations sampled	6
Number of sampling sites at each location (up-/downstream)	2
Number of sample type (sediment/water)	2
Number of replicates	3
Total number of samples	72

In each stream, 1-L water samples were collected 10 cm below the water surface. The samples were collected in sterile glass bottles that were sealed right after sampling. At the same locations, 50 ml sediment was sampled in sterile Cellstar tubes that were filled and emptied several times to ensure a pure sediment sample free of the water phase. The samples were kept on ice until processed in the lab (within 12 hours). The sediment samples were kept at -20°C until extraction of DNA. The water samples were filtered through a 0.22-µm filters (47 mm diameter, Fisherbrand) that was kept at -20°C until extraction of DNA.

2.2 Treatments of samples (DNA extraction)

DNA was extracted by DCE from filters and sediments using the DNeasy PowerSoil Kit (QIAGEN) according to the manufacturer's instructions. DNA was measured using a Qubit fluorometer (Thermo Fischer Scientific) in all samples, in concentrations as high as 2-26 ng and 7-50 ng per µl of elution buffer, in water and sediment samples, respectively.

2.3 Enumeration of antibiotic-resistance genes by qPCR

Five genes (Table 2), considered associated with antibiotic resistance in bacteria, were quantified by qPCR in the DNA extracted from the individual samples. The qPCR method is a common method used to estimate levels of ARGs in the environment, e.g. like in the WAP survey [1]. The qPCR method was adopted by Department of Environmental Science, Aarhus University, in terms of choice of primers (Table 2).

Table 2.2. Antibiotic-resistance genes and DNA primers* use for qPCR enumeration

Antibiotic	ARG		Primers (forward/reverse)
Tetracycline	*tetA*	F	CTCACCAGCCTGACCTCGAT
		R	CACGTTGTTATAGAAGCCGCATAG
Tetracycline	*tetB*	F	AGTGCGCTTTGGATGCTGTA
		R	AGCCCCAGTAGCTCCTGTGA
Beta-lactam	*blaTEM*	F	CGCCGCATACACTATTCTCAG
		R	GCTTCATTCAGCTCCGGTTC
Beta-lactam	*blaCTX-M*	F	GCGATAACGTGGCGATGAAT
		R	GTCGAGACGGAACGTTTCGT
**MLSB	*erm(E)*	F	GTCACGCAGCTGGAGTTCG
		R	CGGTGAAGCACAGCTCGAC

* Reference for all primers [13]
** Macrolide-lincosamide-streptogramin B

qPCR setup for each reaction

10 µl qPCRBIO SyGreen Blue Mix Lo-ROX – 2000 mastermix

0.8 µl for each primer

3 µl template

5.4 µl water

Real-time cycling conditions included a 2-min enzyme activation at 95°C followed by 40 cycles at 95°C for 5 sec and 60°C for 30 sec. A no-template control was included in all assays, and all reactions were run in triplicates, in parallel with serial dilutions of the standards.

Standard curves based on DNA extracted from isolates containing the respective resistance gene are reported in appendix 1. Standards were prepared by diluting the extracted DNA to 1 ng μl^{-1} and performing five times serial dilutions in PCR water. They all showed a strong positive linear relationship between the number of gene copies and the threshold values.

3 Results and discussion

DNA was successfully extracted from all samples, in sufficient amounts to ensure proper qPCR measurements. In general, the water samples contained less DNA than their respective sediment samples, with an average total amount of 361 and 908 µg of DNA extracted from water and sediment samples, respectively (data not presented).

The qPCR analysis revealed that ARGs were detected in all streams, where especially genes associated with tetracycline (tetA) and MLSB (ermE) were prominent (Figures 1, 2). ARG tetA was identified in the water phase of all streams at relative abundances (i.e. copy number per ng extracted DNA) of minimum 3.6×10^2 ng^{-1} DNA (downstream, stream B) and up to 9.7×10^3 ng^{-1} DNA (upstream, stream F). The tetA copy numbers ng^{-1} DNA were rather similar in upstream and downstream water and sediment samples from the individual streams, although with a slightly enhanced level in upstream water compared to downstream water (ANOVA main effect, P=0.04). In general, abundances of tetA were higher (5-10 times) in the sediment than in the water column, reaching 1.3×10^4 ng^{-1} DNA in sediment from stream G – the highest value of relative copy number ng^{-1} DNA measured in the present study.

In streams A, C, E, G, the relative abundance of ermE in the water phase was comparable to values of tetA, while in streams B and F ermE relative copy numbers were approximately ten times higher or lower, respectively, compared to tetA. The relative abundance of ermE in water samples was similar in upstream and downstream water. In sediment samples, ermE relative abundance was slightly increased in downstream water compared to upstream samples (ANOVA main effect, P=0.01).

ARGs tetB and blaCTX-M were typically detected in relative low copy numbers between 10 and 50 copies ng^{-1} extracted DNA, with tetB reaching 112 and 103 relative copy numbers in upstream sediment from stream F and G, respectively.

Although at a low level, the relative copy number of tetB was slightly enhanced upstream, compared to downstream water, in both water (ANOVA main effect, P=0.01) and sediment (ANOVA main effect P=0.001). With blaCTX-M the same tendency was observed for water samples (ANOVA main effect, P=0.045), while for sediment samples there was no difference. These differences must be taken with precaution, taking the low copy numbers into consideration.

ARG blaTEM was not detected in any of the water or sediment samples.

Hence, ARGs considered associated with bacterial resistance to tetracycline antibiotics (tetA) were measured in relative copy numbers between 3.6×10^2 and 9.7×10^3 ng^{-1} DNA in water and between 1.8×10^3 and 1.3×10^4 ng^{-1} DNA in sediment. ARGs associated with resistance to macrolide antibiotics (ermE), were measured in relative copy numbers ranging between 1.2×10^3 and 2.3×10^3 ng^{-1} DNA in water and between 7.1×10^2 and 2.0×10^3 ng^{-1} DNA in sediment. ARGs associated with antibiotics within the beta-lactamase group were detected in lower numbers.

Common for all the five investigated ARGs is that the natural background level of these genes is unknown and it is not possible to evaluate if the measured occurrence of ARGs is exceeding that.

For the ARGs associated with tetracycline, beta-lactam and macrolides, there was no clear relation between their abundance and samples taken 300 m upstream and downstream, respectively, of the farm buildings. Hence, there is no indication that the close surroundings of the farms located near the streams influenced the abundance of these ARGs, neither in the water nor in the sediment. In addition, in the present study the management of animal manures, e.g. usage as fertilizer to the surrounding fields, is not known. A more thorough investigation is needed to confirm whether the farms contribute to the discharge of ARGs in nearby streams.

ARGs are detected in all environments, globally. In a recent survey, along an ocean transect from South Korea to Antarctica, ARGs were found in high numbers and with a high diversity [10] where beta-lactamase- and tetracycline-related resistance mechanisms were the most prominent. Likewise, pristine Antarctic soils contained a wide array of ARGs, including genes for tetracycline resistance, indicating that a natural background of antibiotic resistance mechanisms must be taken into account when interpreting data like in the present study [14, 15]. Tetracycline ARGs are commonly found in the environment, e.g. in river water and associated drinking water treatment plants along the Yellow River in China [16], with tetA found in copy numbers of 10^3-10^4 per ml water. In this case a contribution from human-related activities (e.g. farming, human excreta) seemed obvious, although the natural background remained unknown.

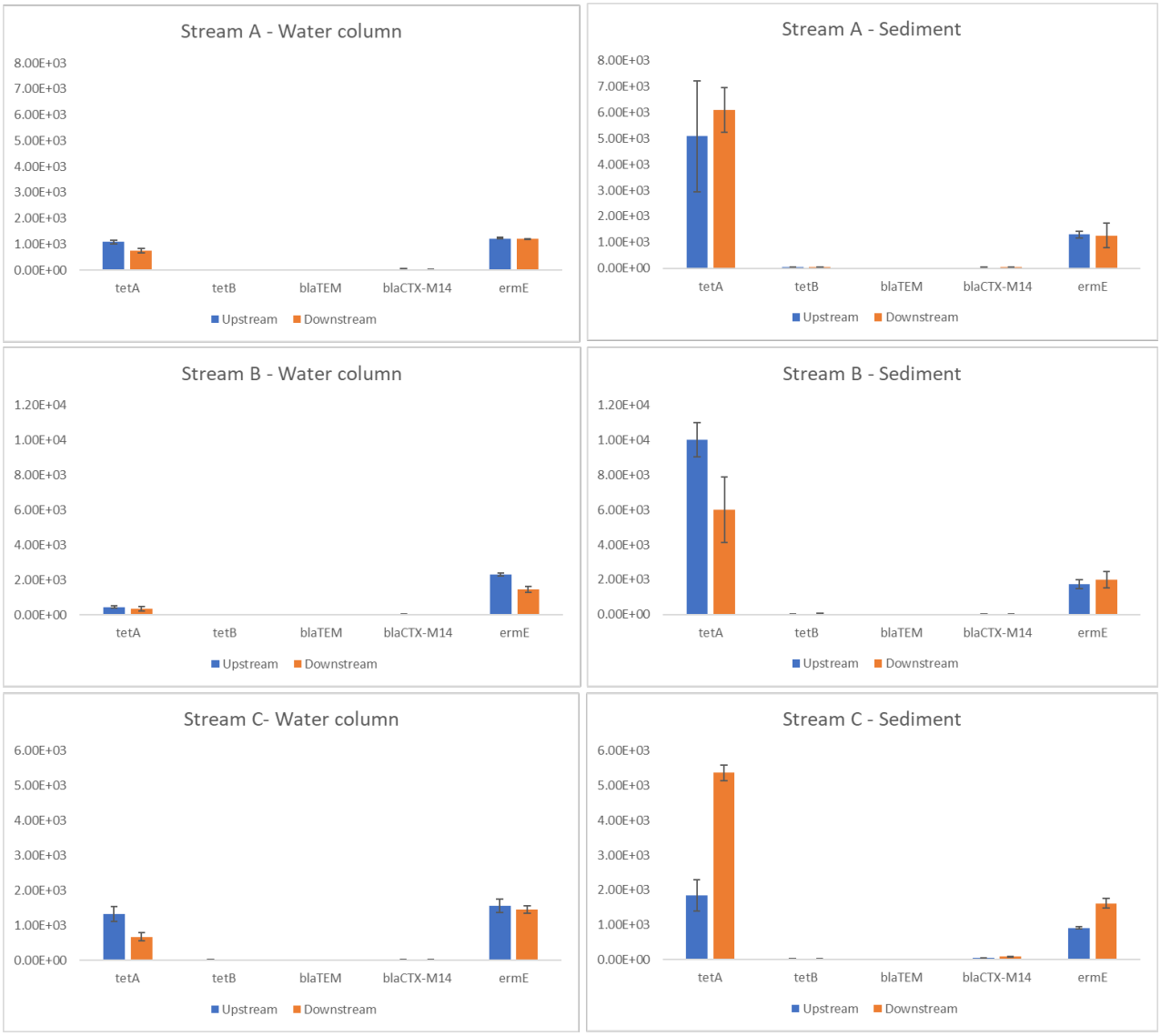

Figure 3.1. Relative abundance of ARGs per ng DNA (stream A, B, and C) in water column and sediment. Samples were taken 300 m upstream and 300m downstream from conventional pig farms located close to the stream. (n=3, bars indicate standard error of the mean).

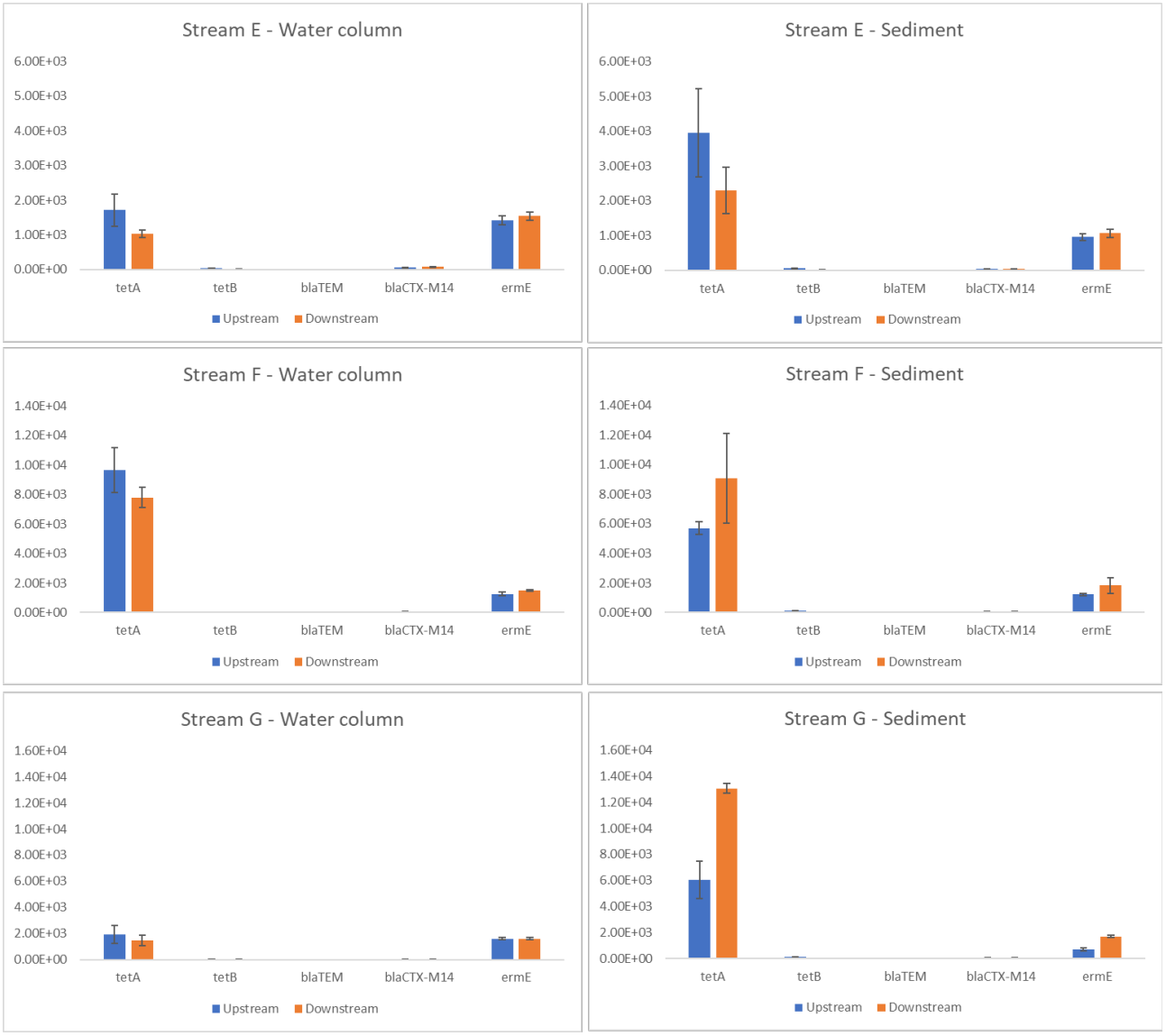

Figure 3.2. Relative abundance of ARGs per ng DNA (stream E, F, and G) in water column and sediment. Samples were taken 300 m upstream and 300 m downstream from conventional pig farms located close to the stream. (n=3, bars indicate standard error of the mean).

4 Conclusion

It can be concluded that ARGs considered associated with bacterial resistance to tetracycline and macrolide antibiotics were measured in all the six streams. ARGs associated with antibiotics within the beta-lactamase group were not detected in significant numbers. However, the natural background level of the ARGs is unknown.

For the ARGs associated with tetracycline and macrolides, there was no clear relation between ARGs abundance in upstream and downstream water or sediment samples at the individual streams and thus no indication that the close surroundings of the farms located near the streams contributed to the abundance of these ARGs in neither the water nor in the sediment.

More knowledge about the natural abundance of ARGs in pristine environments, as well as the emission patterns of antibiotics and the respective ARGs from farms, is required in future studies aiming at clarifying farming contributions to emission of ARGs into streams.

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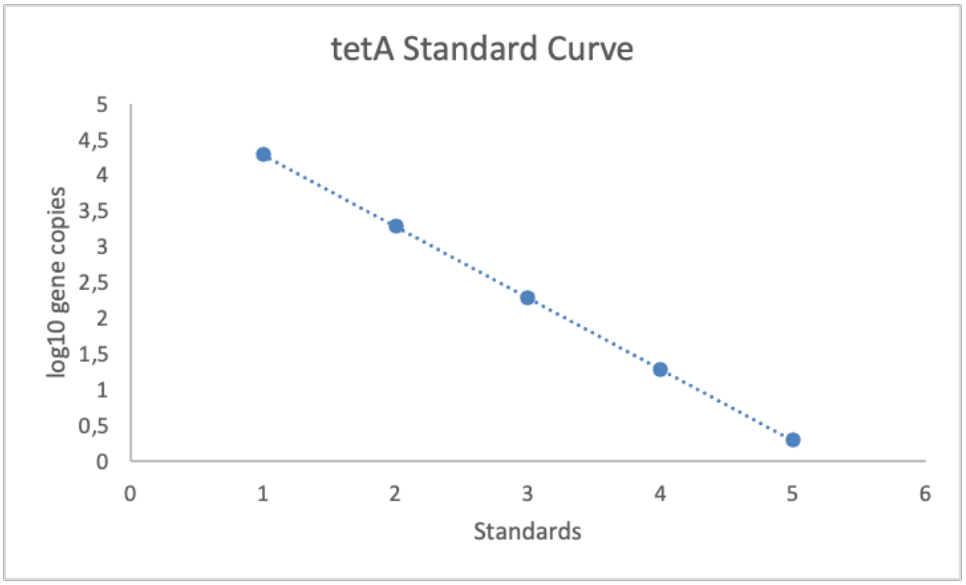
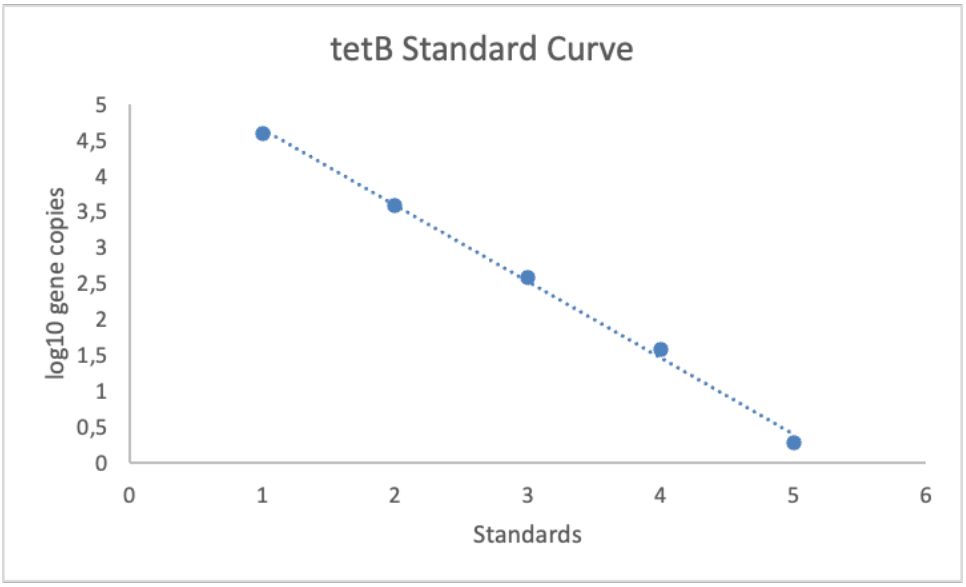
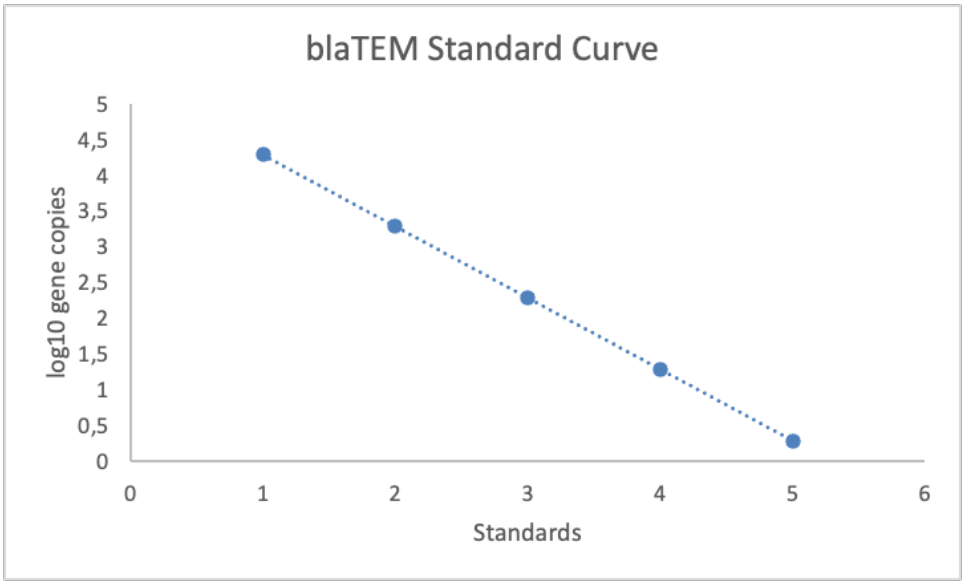
Appendix 1 – Standard curves for qPCRs

Representative qPCR-standard curves for *tetA*, *tetB*, *blaTEM*, *blaCTX-M14*, and *ermE* primers, all with correlation values of at least 0.997.






blaTEM Standard Curve
log10 gene copies
5
4,5
4
3,5
3
2,5
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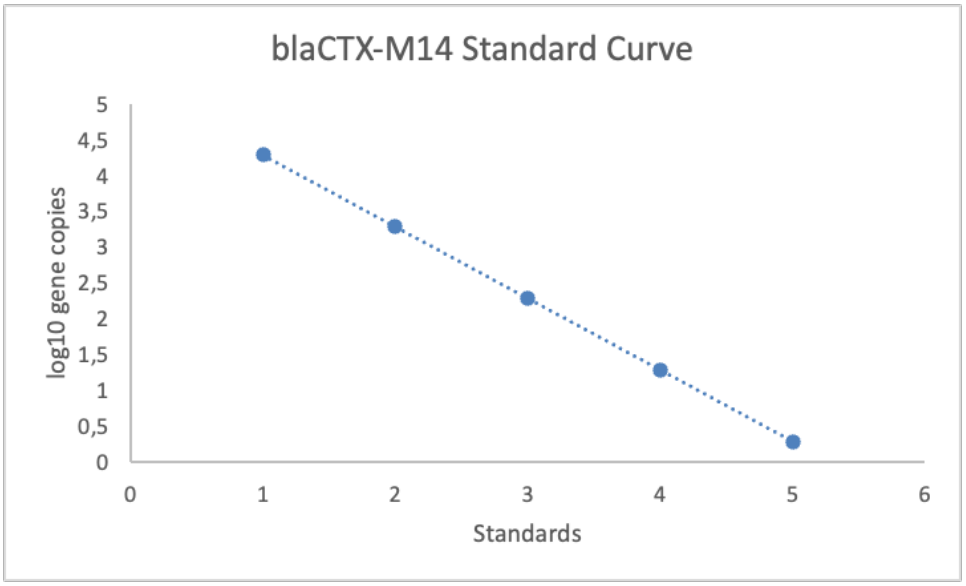
1,5
1
0,5
0
0
1
2
3
4
5
6
Standards


blaCTX-M14 Standard Curve
log10 gene copies
5
4,5
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3,5
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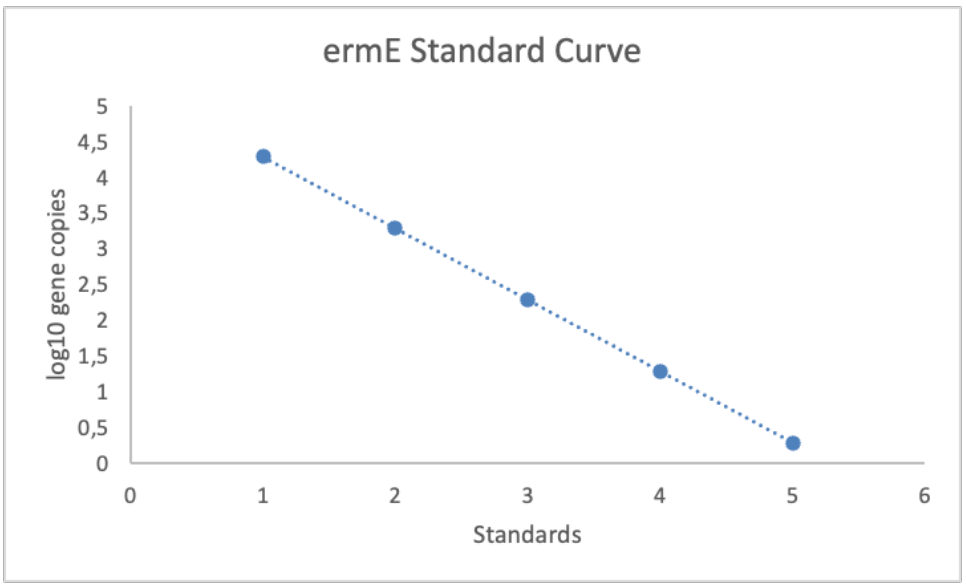
2,5
2
1,5
1
0,5
0
0
1
2
3
4
5
6
Standards


ermE Standard Curve
log10 gene copies
5
4,5
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3,5
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1
0,5
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0
1
2
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4
5
6
Standards

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DCE has been commissioned by World Animal Protection Denmark to quantify antibiotic-resistance genes (ARGs) in water and sediment samples from six streams located in agricultural landscapes in Denmark. The analyses showed that ARGs related to tetracycline and macrolides were detected in all water and sediment samples. However, their association with the nearby farms cannot be confirmed by the present study.

ISBN: 978-87-7156-777-9
ISSN: 2244-9981