RNA - DNA Extraction and Expression of Antibiotic Resistance Genes

The Research Background

It has been observed that antibiotic resistance genes are persistent in the sediment below aquaculture farms even in the absence of selection pressure (Tamminen, 2011). However, HPLC measurement results showed a high amount of OTC concentration on aquaculture sediment of year 2011. Therefore, RNA-DNA extraction will be conducted by using Jacobs method thus the expression and quantification of antibiotic resistance genes will be monitored through RT-PCR.

The Research Goals

The main objective of this study is to extract RNA and DNA included monitoring the expression and quantification of antibiotic resistance genes from sediment below marine aquaculture farms and fish feed samples.

The Research Methods

Sampling. Samples (2-5cm of surface sediment) were collected from marine aquaculture and pristine areas in the northern Baltic Sea during the summers of 2007-2011 using Limnos probe (Limnos Ltd., Turku, Finland). The sediment samples were stored on -80°C and transferred on dry ice. Fish feed samples are white fish (Silver) and rainbow troat (Hercules) pellets which were obtained from the farms and keep at room temperature.

Sediment nucleic acids extraction. 500 mg of dry sediments and fish feeds (Freeze Drying method) was extracted using Jacobs method as described as follows: cell lyses were conducted on BIO101 Fast prep FP120, 2 x 20s (with 60s on ice in between) at speed 5 inside Ceramic Bead Tube (MOBIO) + G1 (DNA treated which has been patented by GEUS) and addition of 0,5mL Phenol-chloroform-isoamyl alcohol (lower phase) followed by 0,5mL CTAB extraction buffer (one volume 10% CTAB, Sigma 52365 in 0,7M NaCl, DEPC treated mixed with one volume of 240mM phosphate buffer pH 8,0, DEPC treated). After centrifugation at 16.000g for 5min at 4˚C, supernatant was slowly mixing with equal volume of chloroform-isoamyl alcohol (24:1), Sigma 25666 and re-centrifugated. Precipitation of total nucleic acids from aqueous phase with two volume 30% PEG (polyethylene glucol 6000 in 1,6M NaCl, Sigma 81253, DEPC treated) and 1uL glycogen (Roche 10 901 393 001 20 mg/ mL) on ice for 2 hrs. Collect the pellet by centrifugation at 16.000g for 30min at 4˚C. The pellet resuspended in 150uL dH_2O-DEPC treated by shaking in eppendorf shaker at 1400rpm for 10min at 4˚C. Clean up the nucleic acids based on protocol from kit (Macherey Nagel). Nucleic acids divided half for DNA, keep at -80˚C and another half for RNA, directly continue with DNase treatment.

DNase treatment. To collect pure RNA from the nucleic acids extraction RTS DNase kit (MOBIO) was used. 6uL nucleic acids extracted was added with 1uL RTS DNase enzyme, 2uL 10X RTS DNase buffer and dH_2O-DEPC treated up to 20uL. The reaction was incubated for 20min at 37˚C. Removed the DNase by using 5 uL RTS DNase Removal Resin and resuspended every 1-2min for 10min at RT. RNA used for Reverse Transcription after centrifugation at 13.000g for 1min.

Reverse Transcription. 6uL of DNase treated RNA samples mixed with reverse transcriptase reaction from RevertAid Premium double-stranded cDNA Synthesis Kit (Fermentas) to produce cDNA. PCR machine (BioRad iCycler-thermal cycler) used for incubation as follow 10min at 25˚C,

for 30min at 50˚C and for 5min at 85˚C. Double stranded cDNA is ready for RT-PCR analysis. However, there is not available method to discern the cDNA integrity instead RNA integrity will be used to analyse the quality of produced cDNA.

RNA integrity. To analyse the integrity of RNA extraction, Bioanalyzer (Agilent 2100 total prokaryote RNA 6000 pico kit was used. Follow the instruction from manufacture.

Real Time-PCR. To monitor the expression and the quantification of resistance genes, BioRad iCycler-optical module and software program iQ5 were used. Primers of tetracycline resistance genes (*tetA* and *tetH*) were obtained from reference 1 and primers of *rpoB* gene, a household gene which responsible for making RNA polymerase was used to discern the recovery of mRNA from extracted RNA, were obtained from GEUS-Geochemistry Lab. PCR reactions contained Maxima SYBR Green qPCR Master Mix (Fermentas), 8pmol of each primers, and 1uL of 1:10 dilutions of double stranded cDNA in reaction volume of 20uL. 1uL of 1:5 dilutions of DNase treated RNA was used as negative control of reactions. The quantification standard used pUC57 vector and the fragments of *tetA* and *tetH* from reference 1 and E. coli cells for resistance genes and *rpoB* gene, respectively. In addition, SYBR Premix DimerEraser (TaKaRa) and DyNAmo Flash SYBR Green kit (Finnzymes) were used in order to find the best reaction for the negative control.

The Research Results and Discussions

RNA-DNA extraction. Jacobs method, a method which was developed in GEUS-Denmark to efficiently extract RNA-DNA from soil, was also successful to extract RNA-DNA in the sediment below marine aquaculture. Unfortunately, not for the fish feed samples.

RNA integrity was not able to be reported due to technical matter on the bioanalyzer hardware while the experiments held. There was an issue of high dye concentration which showed as an early peak analysis time range before 40s and 45s for 16S and 26S RNA, respectively (Figure 1 and 2).

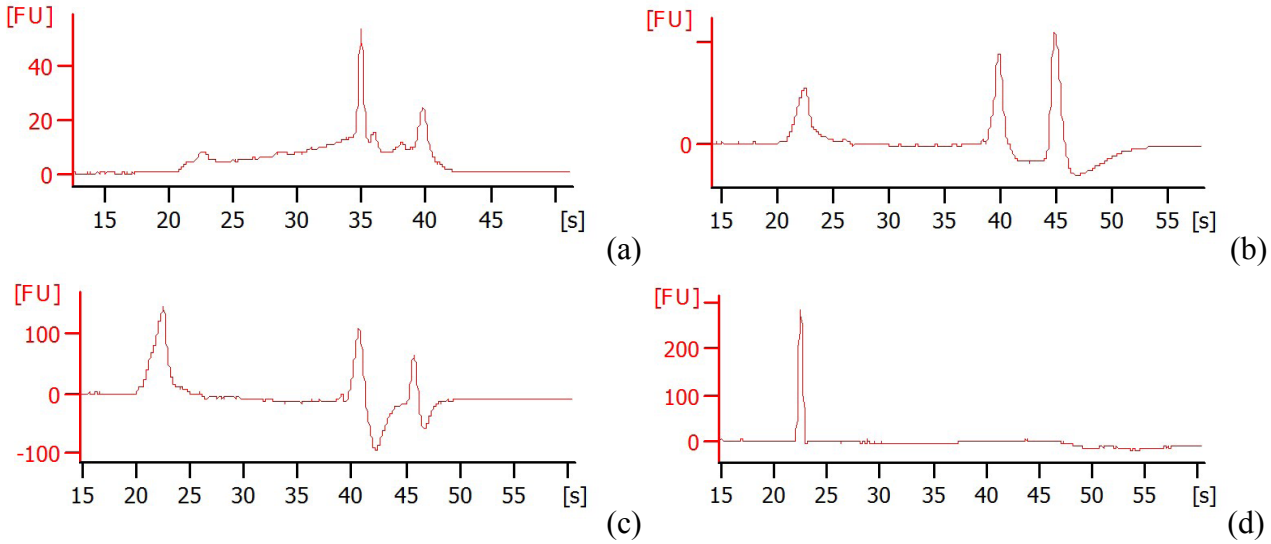
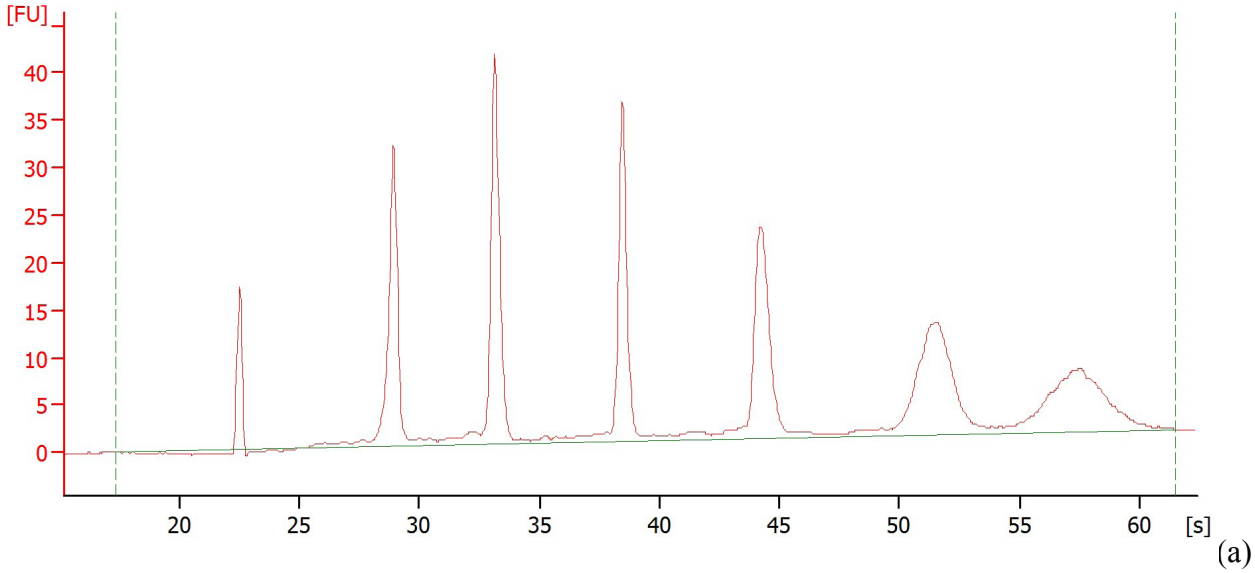
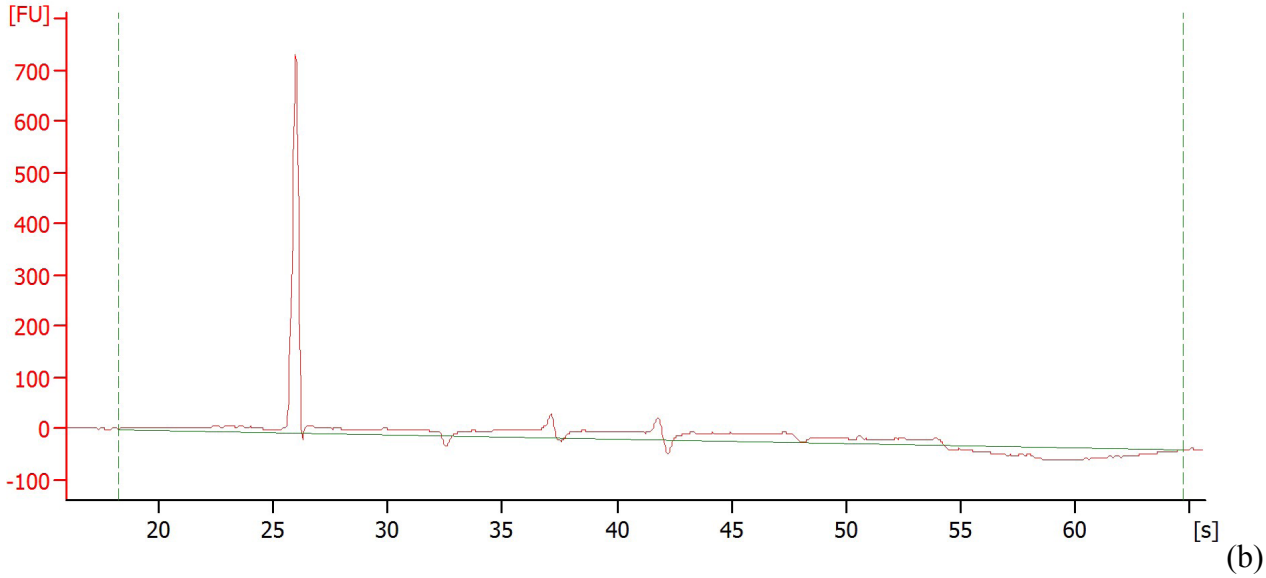


Figure 1. Samples Electropherogram summary of Bioanalyzer; (a) a sample was run on 8.3.2012 which not showed an early peak before 16S (40s) and 26S (45s) however, increasing of baseline changed the range time of ribosomal peaks; (b) a sample run on 12.3.2012, and (c) on 15.3.2012 started having a technical matter which showed an early peak on range 20s-25s; (d) the analyse was run without sample on 19.3.2012 which also showed an early peak on range 20s-25s. Bioanalyzer technical support suggested that it was caused by high concentration of dye on analyses.



(a)



(b)

Figure 2. Ladder Electropherogram summary of Bioanalyzer; (a) Ladder was normally run on 8.3.2012; (b) Ladder was run on 19.3.2012 also which was run on 12.3.2012 and 15.3.2012 showed the early high peak (more that 700 FU) which either covered the ladder peak (up to 50 FU) or a ladder degradation was occurred.

Here, only Electrophoresis summary of Bioanalyzer analysis will present the quality of extracted RNA (Figure 3). The electrophoresis summary result is a crucial preliminary step to choose a proper negative control and produced cDNA template on RT-PCR analysis. We selected the samples which clearly showed two bands around analysis time range 40s (16S) and 45s (26S). To be noted, the analysis time range for 16S and 26S may change depended on starting point of analysis range time and the baseline. Beside than those bands, the RNA might be degraded during the sampling or technical matter of extraction process. Based on Figure 3, there were 19 of 34 samples successfully extracted from sediment below marine aquaculture (table 1).

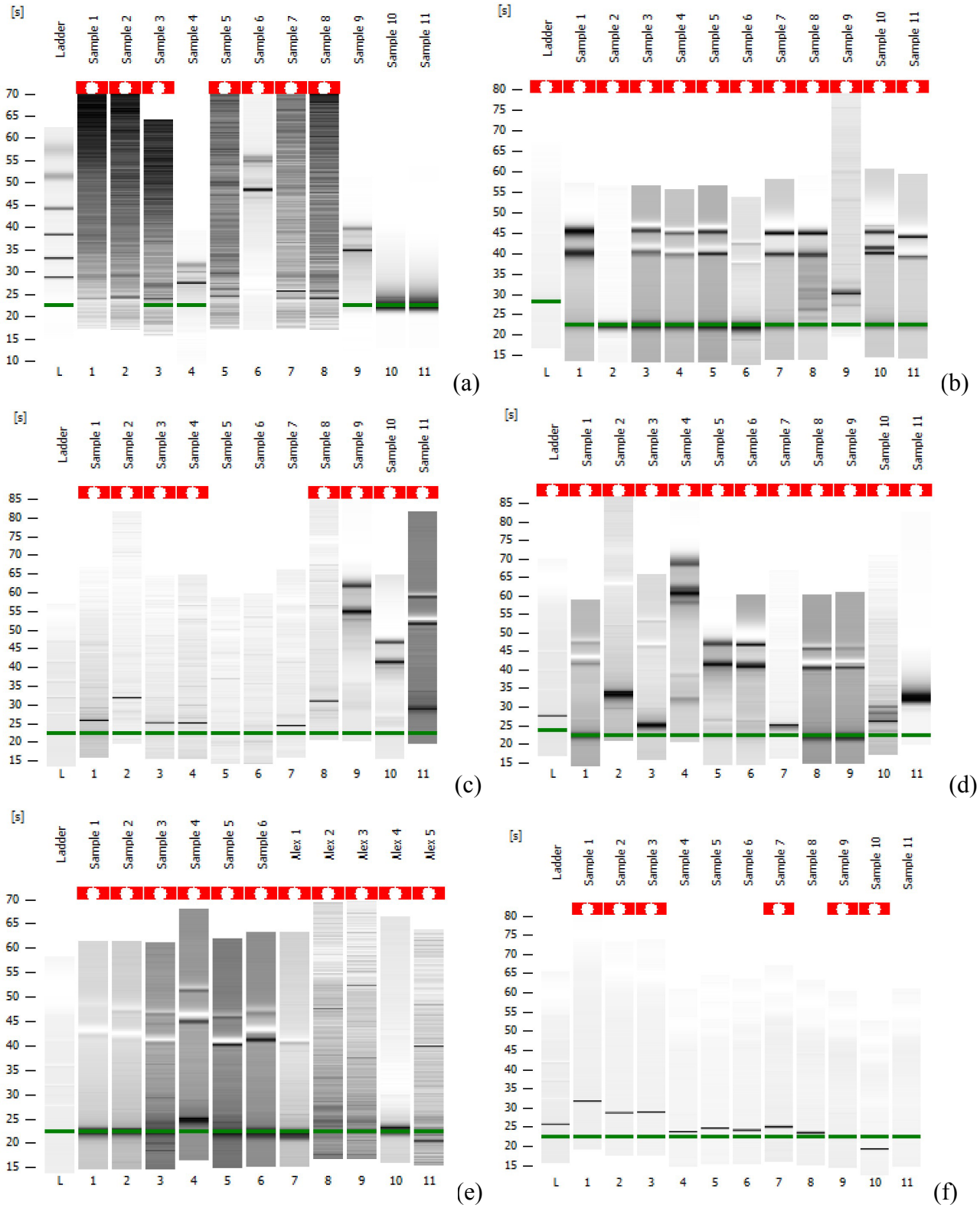


Figure 3. Electrophoresis summary from Bioanalyzer. (a) Run on 8.3.2012, sample 1-8: belonged to Shinjini. M from oil polluted soil, sample 9: FIN2-2011(3A), sample 10-11: fish feeds; (b) Run on 12.3.2012_a, sample 1: FIN1-11(1B), sample 2: FIN1-pristine-11(2B), sample 3: FIN2-11(3B), sample 4: FIN1-09, sample 5: FIN2-09, sample 6: FIN1-08, sample 7: FIN2-08, sample 8: FIN1-07, sample 9: FIN2-07, sample 10: FIN2-pristine-07, sample 11: FIN1-11(1A); (c) Run on 12.3.2012_b, sample 1-8: belonged to Shinjini. M, sample 9: FIN1-pristine-11(2A), sample 10: FIN2-08, sample

11: FIN2-11(3A); (d) Run on 15.3.2011, sample 1: FIN1-11(1C), sample 2: FIN1-pristine-11(2C), sample 3: FIN2-11(3C), sample 4: FIN1-11(1C), sample 5: FIN1-pristine-11(2C), sample 6: FIN2-11(3C), sample 7: FIN1-pristine-11(2B), sample 8: FIN1-08, sample 9: FIN2-07, sample 10: FIN2-22(3C), sample 11: fish feed; (e) Run on 20.3.2012, sample 1: FIN1-pristine-11(2C), sample 2-4: FIN2-11(3A), sample 5: FIN2-11(3B), sample 6: FIN2-11(3C), sample 7-11: belonged to Alejandro from permafrost soil; (f) Run on 19.3.2012, sample 1-11: without samples.

Nevertheless, there were difficulties to extract good quality RNA from sediment sample which has high OTC contamination (FIN2-2011-3A) RNA-DNA was successfully extracted from all the sediment samples of each year as shown below. The RNA from 5 years sediment sample was not degraded because the sediments carefully storage on -80˚C.

Table1. Sediment samples list

Sample name	Sample No. (Bioanalyzer)	Running analysis (Bioanalyzer)
FIN1-2011(1A)	11	(b) 12.3.2012_a
FIN1-2011(1B)	1	(b) 12.3.2012_a
FIN1-2011(1C)	1	(d) 15.3.2012
FIN1-pristine-2011(2A)	9	(c) 12.3.2012_b
FIN1-pristine-2011(2B)	-	-
FIN1-pristine-2011(2C)	5	(d) 15.3.2012
FIN2-2011(3A)	9	(a) 8.3.2012
FIN2-2011(3B)	3/ 5	(b) 12.3.2012_a/ (e) 20.3.2012
FIN2-2011(3C)	6/ 6	(d) 15.3.2012/ (e) 20.3.2012
FIN1-2009	4	(b) 12.3.2012_a
FIN2-2009	5	(b) 12.3.2012_a
FIN1-2008	6/ 8	(b) 12.3.2012_a/ (d) 15.3.2012
FIN2-2008	7/ 10	(b) 12.3.2012_a/ (c) 12.3.2012_b
FIN1-2007	8	(b) 12.3.2012_a
FIN2-2007	9	(d) 15.3.3012
FIN2-pristine-2007	10	(b) 12.3.2012_a

Expression of antibiotic resistance genes. Here, RT-PCR result from *tetH gene* is reported (figure 4). RT-PCR results showed that the *tetH* gene was expressed under detection limit. The (-) control was the RNA samples and the (+) control was the DNA samples of each analyzed cDNA samples. The (-) controls didn't show any amplification which means there was enough good quality of RNA samples with less contamination of genomic DNA. The (+) controls were elevated for all sediment samples excluded pristine area as also reported on previous research (Tamminen, 2010). Low expression of *tetH gene* was suggested due to low bioavaibility of tetracycline in the sediment below aquaculture as also previous reported by Tamminen et al. To be noted, there are several of tetracycline resistance genes are found persistent (tetA, tetE, tetM, tetC, tetW, tetG) in the sediments which may also one of the resistance genes that was actually expressed by bacteria.
The extraction method to successfully recover mRNA was also considered need to be confirmed on RT-PCR by quantify the copy number of a household gene which is always expressed on bacteria. We will further analyse using *rpoB gene* which responsible for making RNA polymerase.

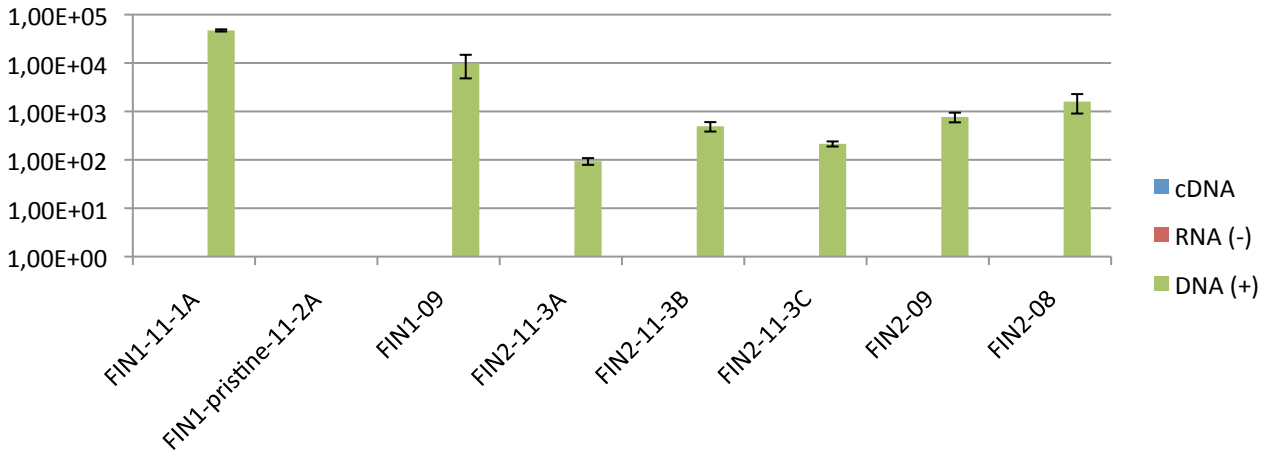


Figure 4. Copy number of *tetH gene*

Acknowledgment

This research was supported by NENUN travel grant of PhD training on molecular microbial ecology at GEUS, Copenhagen, Denmark. I am indebted to Carsten, Pia, Morten, Jacobs and Shinjini for all the experiments that had been done and thank you for the warm hospitality in Division Geochemistry-GEUS, Copenhagen.

References

Tamminen, M., Karkman, A., Lõhmus, Muziasari, W.I., A., Takasu, H., Wada, S., Suzuki, S. & Virta, M. 2011. *Environmental Science and Technology*. 45:286-391.