Abstract

Antibiotic Resistance Gene Transfer in Oysters as a Result of Fecal Pollution.

Antibiotic resistance is an increasing problem in many species of bacteria today, with pathogens an important focus. Fecal contamination of shellfish is already a concern due to potential pathogens. This report examines the possibility of resistance gene transfer between microbes, due to fecal bacteria, within the oyster. In this study, the transfer of a tetracycline resistance gene, *tet*Q, in a quasi-natural environment is examined. A donor strain of *Bacteroides thetaiotaomicron*, a species that can be found in feces, successfully transferred a *tet*Q containing conjugative transposon to a recipient *B. thetaiotaomicron* strain. Oysters were exposed to various treatments and controls, and examined for the presence of transconjugant microbial colonies. Transconjugant colonies were recovered from the bodies of experimental oysters, and verified via growth on selective media and PCR amplification. The preliminary work in this report indicates that fecal bacteria could initiate resistance gene transfer between microbes within oysters.

Thesis approved:_____Date:_____
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Antibiotic Resistance Gene Transfer in Oysters as a Result of Fecal Pollution

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A thesis submitted to Oregon State University in partial fulfillment of the requirements for the degree of Bachelor of Science in the Bioresource Research Program, Biotechnology Option.

Acknowledgements

I would like to thank Nadja B. Shoemaker and the lab of Dr. Abigail Salyers for providing strains of *B. thetaiotaomicron.* I would also like to thank the Bioresource Research program and all those affiliated with it. Additionally, the members of Dr. Katharine Field's lab have aided my research immensely.

This work was funded in part by grants from the Oregon State University research office in the form of Undergraduate Research Innovation Scholarship and Creativity (URISC) grants. I would like to thank the URISC committee for funding in part this research.

In addition I would like to thank Dr. Katharine G. Field and Dr. Chris Langdon for agreeing to participate in the undergraduate Bioresource Research Program as my faculty mentors.

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Introduction

Antibiotic resistance genes are produced by a variety of bacteria today, and have traditionally been borne by microbes desiring defense against competitors and predators. Today antibiotic resistance is an increasing threat. Suggested causes for the increase include the medical industry's over-prescription of still useful antibiotics for non-bacterial illnesses; contributions from industrial runoff; agricultural use to promote animal growth; and the abundance of antibiotics in household products [14]. These points may be valid, especially the latter. Low levels of some antibiotics in the environment induce and increase the frequency of resistance transfer [20]. Studies tracking the spread of resistance genes have found that the colon of animals is a highly conducive environment for horizontal gene transfer [19]. Horizontal gene transfer is a main method of resistance gene transfer, with conjugative events making a major contribution [11,15].

Bacteroides is a numerically dominant member of the fecal flora of warm-blooded animals such as mammals [14,15,17]. Aero-tolerant, but strictly anaerobic, this genus and *Prevotella*, a closely related genus, have been used to detect non-point source fecal pollution in waterways from both humans and cows [2,3,23]. Unique *Bacteroides* related bacteria often form host species based phylogenetic groupings that share common sequences. Species from different hosts share

common sequences, as well as having unique areas of their genetic sequence specific to host species groups [2, 3].

Bacteria in the colon and feces generally contain plasmids and other conjugative elements; *Bacteroides* is not an exception, carrying mobile genetic elements called conjugative transposons [15,17]. Conjugative transposons are integrated elements, frequently carrying antibiotic resistance genes for one or more antibiotics. When these elements are integrated in the bacterial chromosome they are expressed as other genes are, by the host's cellular machinery, and confer resistance to the microbe. When conjugative transfer is induced, the conjugative transposon forms a circular intermediate and proceeds to conjugate with the adjoining cell. The replicated conjugative transposon and the original re-integrate into the chromosome[15].

The promiscuous action of these conjugative transposons has influenced the spread of resistance genes such as *tetQ*, an inducible ribosome protection variety of tetracycline resistance [11]. The conjugative transposon in question, in addition to carrying resistance genes such as *tetQ*, may carry an adjoining tetracycline sensor [11]. Thus, low ambient levels of tetracycline in the environment aid transfer of *tetQ*. This leads to a high rate of *tetQ* transfer and co-resident plasmid mobilization in the presence of low levels of tetracycline. The sequences of *tetQ* genes found in various fecal flora species are virtually identical, indicating a high level of horizontal transfer[19]. According to recent studies, the horizontal transfer of this gene takes place primarily in the colon, and transfer between livestock and humans has been noted[14]. The colon is an anaerobic area of highly

concentrated cells and plentiful nutrients. The high concentration of cells aids the conjugative events.

Certain species of *Bacteroides* can be induced to transfer genes such as *tetQ* from a resident conjugative transposon to related as well as unrelated species in conditions similar to those in the colon [1, 11, 19]. In the laboratory, conjugation can be easily and reliably induced between two strains of *Bacteroides thetaiotaomicron*. One of these strains carries a conjugative transposon carrying the *tetQ* gene (Bt 4109). The other strain does not carry any conjugative transposon, but is resistant to rifampicin (Bt 4001). Transfer can be initiated and completed by low levels of tetracycline. The resulting organisms are tetracycline and rifampicin resistant, a version of Bt 4001 with a conjugative transposon.

Antibiotic resistance transfer has also been suspected to occur in natural environments other than the colon [13]. Typically antibiotic resistance is found in non-pathogenic species of bacteria. The concern arises when transfer is initiated to organisms with pathogenic potential[16]. Antibiotic resistant species and genes have been obtained from the environment in water samples collected from estuaries, rivers, and lakes and near areas of runoff from industrial pollution [24,13]. The actual frequencies of transfer in many natural locations would likely be too low to detect, though the aftermath of transfer can be observed. This leads to the question of what natural environments other than the colon could facilitate conjugative transfer? Are all antibiotic resistant species found in environments such as waterways the result of fecal pollution events? Or are there natural

environments capable of facilitating transfer between fecal species and resident microbial life? For detectable transfer of antibiotic resistance, the environment would have to have a relatively high level of useable nutrients and have high cell concentration. In addition, a low environmental level of antibiotics would encourage species such as *Bacteroides*, which contain conjugative transposons, to initiate transfer [20].

One natural environment that could fit these requirements is the stomach and other internal regions of oysters exposed to fecal pollution. Fecal pollution events lead to detectable *Bacteroides* and *tetQ* DNA in the water and presumably in oysters as well. DNA from *Bacteroides* persists in river water at 14°C for four to five days, and cells are degraded largely due to predation [9]. Oysters are filter feeders and scavenge particulates in their environment. Additionally, oysters are known to concentrate bacteria internally [5,12]. The concentration of bacterial colony forming units, for example, could be up to 100 times greater than the concentration of colony forming units in the surrounding waters [5]. Younger oysters, oyster spat, are more active and filter better than adults in an unnatural environment such as the laboratory under experimental conditions[10]. Fecal pollution is already a concern for oyster farms due to the incidence of pathogens that are either contained in feces or can arise as a result, such as with eutrophication [12]. A fecal pollution event would cause the filtering oysters to not only take in *Bacteroides* and other fecal species, but the nutrients contained in the fecal particulates as well. This could encourage transfer of the resistant genes between species as well as genera. These antibiotic resistant

genes could potentially become resident after the pollution event had passed, and reside in the oysters' flora. The ingestion of oysters at a later date could reintroduce the resistance genes to intestinal flora if conditions permitted.

The goal of this study was to determine if antibiotic resistance transfer can be facilitated by oysters in a cold, saline environment; one designed to mimic a natural habitat. If oysters could be shown to facilitate transfer of *tetQ* into a selectable recipient strain of *Bacteroides* in an artificial habitat, then the possibility is open for this occurring naturally.

Primers designed to target sequences on 16S rRNA specific to *Bacteroides* species and related elements allow the fecal contamination of waters to be detected [2,3,22]. By using the touch down polymerase chain reaction (TDPCR), very small amounts of target DNA can be adequately amplified and viewed [6]. This procedure allows increase in both specificity and yield of the PCR, and, the sensitive detection of very small amounts of *Bacteroides* specific sequences as well as *tetQ* specific sequences. This procedure allowed testing of oysters and water for both *Bacteroides* and *tetQ* at a very sensitive level. For this study, cow feces acted as the contaminating element. Both human and cow fecal flora, and many others, carry a detectable gene for tetracycline resistance [13,18]. While primers used for PCR detection of *Bacteroides* and *tetQ* are sensitive, it was possible that PCR would be unable to detect a potentially small number of transconjugants in comparatively large experimental DNA samples. If there were less gene copies than the amount required for amplification in the aliquot of DNA used as template then no positive result would be observed, though

transconjugants may be present. To counteract this possibility, material from the oyster's bodies was plated on selective media to observe any transconjugants.

A LH-PCR (Length Heterogenicity PCR) profile of bacterial rDNA genes allowed comparison of the bacterial communities in the experiments from day to day [2]. The various experimental treatments and controls were tracked and visualized throughout the sampling period and correlated with the PCR results from the same DNA samples. The purpose of this portion of the experiment was to visually track the presence and fluctuations of the *Bacteroides* strains added to the microcosms over the treatment period. This procedure measured persistence and fluctuations of various DNA's in the experimental environment, as well as showing the effects of fecal pollution on bacterial diversity in an artificial oyster habitat.

Fecal pollution events, indicated by high numbers of colony-forming units obtained from water samples in oyster farming areas, lead to the closure of the farming area to harvesting until more than 14 days have passed after contamination is no longer detected [21]. A colony forming unit is a viable cell obtained from environmental or laboratory samples. On average, one cell gives rise to one colony, and is used as an indication of the microbial density of the sample. The number of colony forming units obtained from shellfish waters must be below 14 CFU/ 100ml [21].

The initial fecal pollution event, however, could initiate gene transfer in the concentrated, anaerobic, nutrient rich stomachs of the oysters. Even after the oysters are safe to consume, the effects of the fecal pollution event, such as

newly formed antibiotic resistant species, could persist. Though the oysters will rid themselves of contamination, newly resistant bacterial species from the gut or feces of the oyster may be released into the environment [21].

Bacteroides specific primers can detect concentrations of *Bacteroides,* indicative of feces, much lower than can be detected by traditional methods of coliform counting. Theoretically, conjugation and transfer of resistance genes could be occurring at fecal contamination levels previously considered safe for harvest and consumption.

The idea behind this series of experiments is that oysters are an ideal medium for facilitation of conjugative transfer. There are many contributing elements in this variety of transfer. Contributors include low levels of environmental tetracycline from feces and agricultural wastes [14], fecal pollution events contributing to the nutrients and bacterial content of the oyster's intake, and the oyster's mechanism of feeding, which allows concentration and a suitable anaerobic environment. That an organism routinely consumed raw could be facilitating low levels of conjugative transfer of resistance genes is surprising.

Materials and Methods

Media:

All cultures and strains were grown in supplemented brain heart infusion (BHI) [7]. The supplements included 5 grams of yeast extract (Difco) per liter, 500 milligrams cysteine-HCL per liter (Sigma), 1 ml of 5mg/100ml hemin stock solution per liter (Sigma), 200μl of vitamin K1 stock (150μl vitamin K1/30ml 95% ethanol) per liter, four milliliters of resazurin stock (10mg/mL) per liter, and one milligram per liter thymidine [20]. The organisms were incubated anaerobically in a Plas-Labs anaerobic chamber filled with a mixture of 85% nitrogen, 10% carbon dioxide, and 5% hydrogen. The chamber was maintained at a temperature of 37°C. All culture manipulations were done on the bench.

Bacterial Strains:

Bacteroides thetaiotaomicron strains, derivatives of Bt 4100, Bt 4109 and Bt 4001, were provided by Dr. A. Salyers, and were grown from pure culture overnight at 37°C. To originally initiate transfer between strains, the conjugative transposon containing strain, Bt 4109, and the chromosomally rifampicin resistant strain, Bt 4001, were mixed prior to filtration and filtered through a 47mm 0.2μm filter (Gelman Supor) [1,2]. The method used was similar to that in the reference. After completion of filtration, the filter was aseptically placed cell side up in a sterile petri dish and one milliliter of tetracycline containing supplemented BHI (1μg/mL) was placed in the dish. The filter was then incubated overnight in an anaerobic chamber at 37°C. The filter was then aseptically spread on solid

media (supplemented BHI) with 3 μg/ml freshly made tetracycline and 50 μg/ml rifampicin. This was also allowed to grow anaerobically for 2-3 days at 37°C. Colonies were then picked and tested for the presence of *tetQ* and *Bacteroides* via PCR in a buffer that allowed maximum amplification from single colonies [8]. The bacterial strains were added from liquid culture grown overnight to a concentration of $2.3X10^7$ per culture.

Oysters and Water:

The oysters were placed in ten gallon (29.9L) buckets, which had been washed with a 10% solution of HCl. In each bucket microcosm there were approximately fifteen oysters of about 2.5-3.5 cm in size. Ten liters of natural seawater from Newport Harbor, Oregon was placed in each microcosm. The seawater in the buckets had been filtered through a sand filter prior to collection, and was tested and found negative for both *Bacteroides* and *tetQ* DNA. The oysters were aerated via small electric air pumps and an attached air stone, and the buckets were covered with plastic (Saran Quick Covers™) to prevent evaporation and contamination. The oysters in buckets were placed in a cold lab maintained at 15°C. This lab was dark except when sampling. The oysters were fed algal paste (algae diet C7, Coast Oysters) daily. Oysters were removed from the water using clean utensils for each treatment. Water and oysters were sampled and extracted every day for seven days. The contaminated seawater was siphoned out and replaced with new (collected from the same source) on days three and five of the experiment. The water was changed to maintain the health of the oysters through the duration and conditions of the experiment.

Treatments:

For the experiment, the quasi-natural environments consisting of oysters, ocean water, and various additives were subjected to nine distinct treatments (Table 1). Treatments were added directly to the microcosms. Treatments were either experimental or controls. All of treatments I-VII contained oysters and sodium montmorillonite, which prevented illness of the oysters from toxin buildup[10].

Treatment I was a control. This treatment received no additives, and was only oysters, seawater, and algal paste. Treatment I was used to determine a baseline for results received over the sampling period. Treatment II received thymidine at a concentration of 10mg/L. This treatment was also a control designed to observe the effect of thymidine on oysters and seawater microbes. Treatment III, a control designed to examine the effect of tetracycline on the microbes in oysters and water, received tetracycline at a concentration of 1mg/L. Treatment IV was an experimental treatment designed to show the interaction of feces and thymidine, without tetracycline to induce transfer on both oysters and a recipient strain of *B. thetaiotaomicron*. This treatment received 10^{-2} grams of feces per liter and 10mg/L thymidine, which is necessary for the growth of both donor and recipient stain of *B. thetaiotaomicron*, in addition to recipient culture. Treatment V also contained feces, thymidine and recipient culture, as well as tetracycline at a concentration of 1mg/L. Treatments V and VII were designed to show the effect of elements believed necessary for transfer of the conjugative transposon. Treatment VI contained no feces or tetracycline, but instead

contained thymidine and both donor and recipient cultures. This treatment was done to examine the effects of oysters on the two strains, which were able to transfer and receive conjugative transposons in the laboratory. Treatment VII was the same as treatment VI, with thymidine, and both cultures, but tetracycline was also added as a potential inducing agent for transfer of the conjugative transposon from one strain to another. Treatment VIII had no oysters, and was otherwise identical to treatment V. Treatment IX did not include oysters either, and was otherwise the same as treatment VII. Treatments VIII and IX were necessary to show the role of oysters in facilitating conjugative transfer. Table 1 shows additives to various treatments and treatments that yielded transconjugant colonies.

The water in each treatment containing oysters was replaced with fresh seawater from the same location after the third and the fifth day. The oysters and water were exposed to the individual treatments for 48 hours and the water was changed. Sixty mL of water from each bucket, and one oyster from each bucket were collected daily. These samples were processed appropriately and DNA was extracted as described.

DNA Extraction:

DNA was extracted from oysters using Qiagen DNEasy kits. The oysters' shells were cracked and entire bodies removed from shells for DNA extraction. The oysters were incubated overnight in tissue lysis buffer as directed by the manufacturer. The DNA from the oysters was then extracted according to kit

directions and stored at -20°C until use. Water was sampled with sterilized 60ml syringes and filtered through 47 mm, 0.2μm filters (Gelman) using a Barnant brand vacuum pressure pump and Fisherbrand filtration apparatus [22]. The filters were then placed in sterile 15 mL Falcon tubes containing 0.5ml of GITC buffer (5 M guanidine thiocyanate, 100mM EDTA, pH 8, and 0.5% sarkosyl). The DNA was then extracted according to a modified Qiagen DNEasy protocol. The modified protocol was previously optimized in this laboratory (results not included). Individual colonies were picked and amplified in a PCR buffer containing 500mM Tris-HCL pH 8.2, 100mM KCL, 20mM $MgCl_2$, 10% DMSO, 10mg/mL BSA, and 10% betaine (Sigma) [8]. This unique buffer was used to enhance amplification from small or recalcitrant samples.

TD-PCR:

The DNAs from each sample were used as template in touchdown (TD) PCR in the following program: Stage 1: 1 cycle of 94°C for 2:00. Stage 2: 10 cycles of 94°C for 0:20, 55°C for 0:20, and 72°C for 0:45. Stage 3: 20 cycles of 94°C for 0:20, 55°C for 0:20, with a decrease of 0.5°C every cycle, and 72°C for 0:45 Stage 4: 10 cycles of 94°C for 0:20, 50°C for 0:20, 72°C for 1:00. Stage 5: 1 cycle of 72°C for 10:00. DNA was extracted from samples and subjected to TD PCR to detect the presence of the *tetQ* gene using primers TetQ1 F, and TetQ1R ($^{5'}$CATGGATCAGCAATGTTCAATATCGG $^{3'}$, $^{5'}$CCTGGATCCACAATGTATTCAGAGCGG $^{3'}$) and *Bacteroides* 16S rRNA using primers Bac32F and Bac708R

(5'AACGTCAGCTACAGGCTT 3', 5' CAATCGGAGTTCTTCGTG 3') [2] in amounts not necessarily detectable by conventional PCR. Touchdown PCR can increase both specificity and yield of PCR [6].

LH-PCR:

LH-PCR was done to visually track the fluctuation of Bacteroides strains, as well as Bacteroides present in feces in the oysters and water over the duration of the experiment. Prior to any treatment, DNAs were extracted as previously described from both oysters and water to obtain a eubacterial LH-PCR profile. Eubacterial DNA from each oyster and water sample was also analyzed in this method. DNAs were used as templates in eubacterial amplification with a fluorescent eubacterial primer 6 FAM-Eub B (27F- 5'AGAGTTTGATCMTGGCTCAG3') and 338R (5'GCTGCCTCCCGTAGTAGT 3') [2,3]. After a two-minute denaturation at 94°C, the program for amplification was comprised of 30 seconds at 94°C, one minute at 55°C, and one minute at 72°C repeated for a total of 35 cycles. Following the completion of the cycle, the samples were subjected to a ten-minute 72°C elongation. Experimental DNA samples were subjected to LH PCR and analyzed via Genescan to detect the unique identifying peaks that indicated various treatments in the microbial population. These unique identifying peaks (fig. 2) were followed through the various treatments to oysters and water to observe the effect of the additives on the microbial population.

Transconjugants:

The transconjugant strains were obtained by spreading oyster contents on selective media. Proper aseptic technique was used for all samples. The media consisted of BHI as previously described, with 50μg/mL rifampicin and 3μg/mL tetracycline. This media was prepared fresh and sterilized prior to addition of antibiotics. Oyster contents were spread on the media before DNA was extracted from the oyster as described. Colonies were allowed incubation for 24-48 hours at 37°C in an anaerobic environment as previously described. The colonies were picked, re-streaked on fresh selective media for preservation and used directly as a template for PCR's. Specialized PCR buffer was used for maximum efficiency in amplification [6].

Table 1. Experimental and Control Treatments and Recovery of Transconjugants.

Treatment	I	II	III	IV	V	VI	VII	VIII	IX
Oysters [A]	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	No
Thymidine [B]	No	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes
Tetracyline [C]	No	No	Yes	No	Yes	No	Yes	Yes	Yes
Feces [D]	No	No	No	Yes	Yes	No	No	Yes	No
Donor Culture [E]	No	No	No	No	No	Yes	Yes	No	Yes
Recipient Culture	No	No	No	Yes	Yes	Yes	Yes	Yes	Yes
Transconjugants Recovered [F]	No	No	No	No	No	Yes	Yes	No	No

A. Each bucket contained approximately 15 oysters in ten liters of water.

B. Thymidine was added to a concentration of 10mg/L.

C. Tetracycline was added to a concentration of 1mg/L.

D. Fresh bovine feces were added to a concentration of 10^{-2}g/L.

E. Cultured cells were added to the indicated treatments to a concentration of $2.3X\ 10^{-7}$ for treatments with only one culture added, or $5X10^{-7}$ cells per mL for treatments with two cultures.

F. Transconjugants are described further in the results section.

Results

Transconjugants:

Conjugative transfer of a *tetQ* containing conjugative transposon contained in *Bacteroides thetaiotaomicron* was observed to be facilitated by an environment containing oysters. Strain 4109 was able to transfer a conjugative transposon of the variety CTn7853 to strain 4001 in this quasi-natural environment. Oysters apparently facilitated the conjugative transfer of *tetQ,* as an identical treatment, except without oysters, did not yield transconjugants. Treatments VII and IX differed only in the presence of oysters. Treatment VII yielded transconjugants from oysters from both day five and day seven of the experiment. No treatment containing feces demonstrated transconjugant colonies. Although transconjugant recovery was greater in an environment containing tetracycline, tetracycline was apparently not necessary for transconjugation to take place, as one successful treatment contained tetracycline, and one did not (table 1). Fecal organisms did not appear to serve as donors for transfer of the *tetQ* gene, as no treatments containing feces yielded transconjugant colonies. Entire oyster contents were spread on media selective for transconjugants and the resulting colonies verified via PCR for the presence of *Bacteroides* 16S rRNA genes and the *tetQ* gene. Water was tested for the presence of *tetQ* and *Bacteroides* with the idea that discrepancies would be

detectable. There were no persistent instances in which *tetQ* was detectable when *Bacteroides* was not in the experimental treatments (appendix B). Only seven colonies were obtained from all days sampled. The days and treatments that generated colonies were day three, treatment VII, day five treatment VI and day seven treatment VI (table 1). Of these seven colonies, five tested positive for the presence of *tetQ* as well as for *Bacteroides*. PCR verifications of one colony from day three (3B and 3T), two colonies from day five (5B1, 5T1, 5B2 and 5T2) and two colonies from day seven (7B1, 7T1, 7B2, and 7T2) are represented in figure 1.

Figure 1: Verification of colonies obtained from experimental oysters.

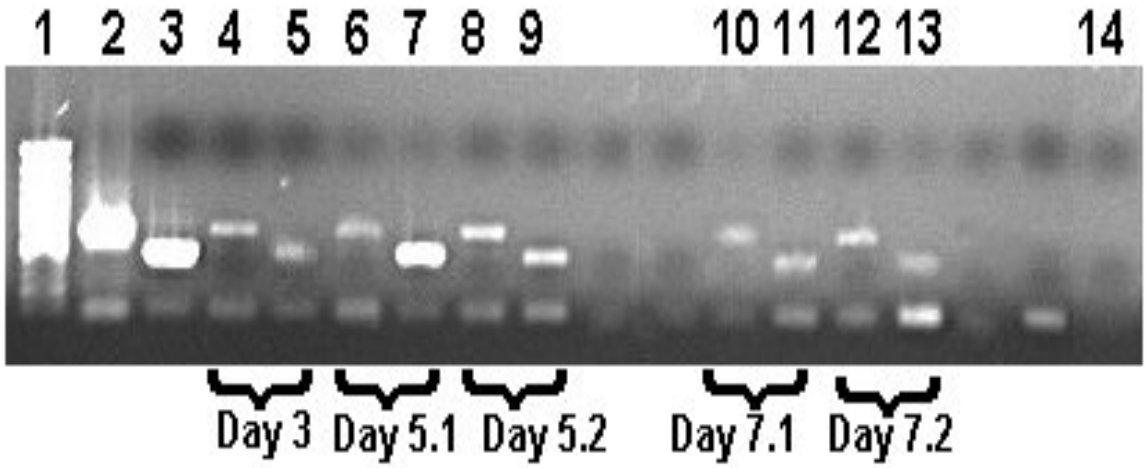


FIG. 1: These are PCR verifications of putative transconjugant colonies recovered from experimental oyster material grown on selective media containing 3 μg/mL tetracycline and 50μg/mL rifampicin. Numbers indicate lanes of interest; 1 is a 100 bp ladder, 2 is the ~700bp fragment indicating the presence of *Bacteroides* 16S rDNA, 3 indicates the ~ 460 bp fragment indicating the presence of the tetQ gene. The experimental samples are grouped by day and colony with *Bacteroides* and *tetQ* PCR verifications of colonies recovered grouped together. Lanes 5, 7, 9, 11, and 13 indicate the presence of *tetQ*. Lanes 4, 6, 8 10, and 12 indicate the presence of *Bacteroides* 16S rDNA. Results from a single colony for both tetQ and Bacteroides are shown in adjacent wells, such as 4 and 5. Each colony has a unique identity, for example, 5.1 and 5.2 being different colonies obtained from the same experimental sample on the same day. Number 14 indicates negative control lane.

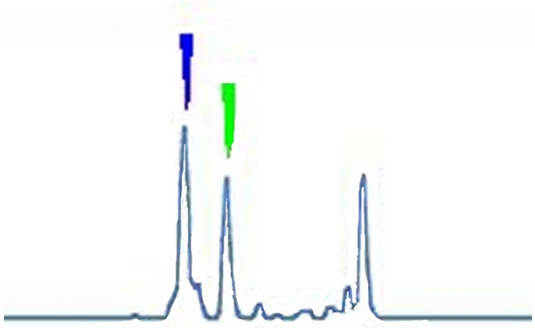
LH-PCR and Genescan:

Genescan images were generated from daily DNA samples. Over seven days, with 16 samples per day, approximately 120 samples were generated. These were grouped by treatment and are available for examination in appendix A. Suspected components of the samples, such as algal paste, untreated oysters, untreated water, and donor and recipient cultures were analyzed for unique and identifying peaks (Fig. 2). The unique peaks allowed tracking of components in the experimental samples. Of those, treatments that generated transconjugant colonies were examined in greater detail. As mentioned previously, LH-PCR was done to visually track the presence of treatments, specifically the added *Bacteroides* cells from culture and feces. A contrast between oysters and water of treatment VII and water of treatment IX illustrates differences in two treatments that are similar, one of which demonstrated transconjugant colonies, and one of which did not (Fig. 3). As can be seen in figure 3, the two water treatments seem similar in community structure through day four. After day four, divergence of microbial flora is visible, though similarities can still be seen.

Figure 2:

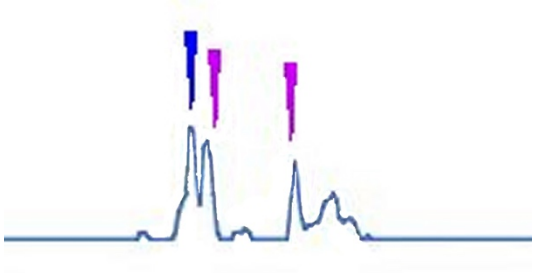
Genescan images of Experimental samples component DNA.

A.



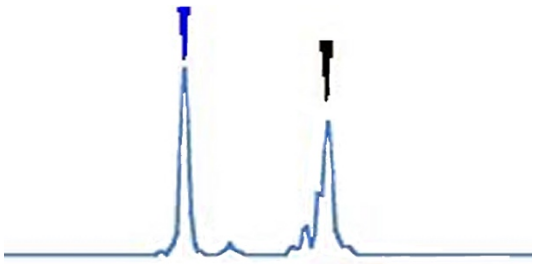
A. **Algal paste (oyster food).**The left-hand (blue) arrow indicates a PCR fragment of 317 base pairs common to all constituent Genescan images, as well as to most of the experimental samples. Unless otherwise indicated, the left most peak on any sample is the 317 base pair fragment. The right hand (green) arrow on this diagram indicates a peak specific for the algal paste at 326 base pairs. All samples were amplified with FAM labeled eubacterial general primer 27F and unlabeled 338R

B.



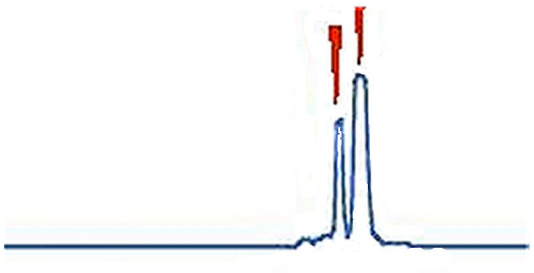
B. **Oysters.** The two right-hand (purple) peaks indicate specific peaks amplified from the microbial flora of the oyster. These peaks reside at 320 and 340 base pairs respectively.

C.



C. **Water.** The common peak at 317 is also present in the sand filtered ocean water, as well as a unique peak (black) at 348 base pairs.

D.

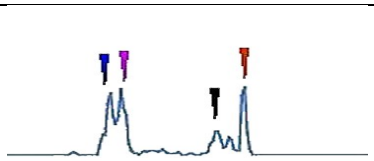
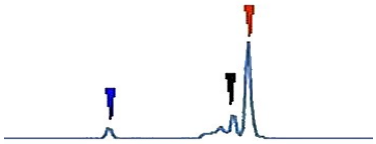
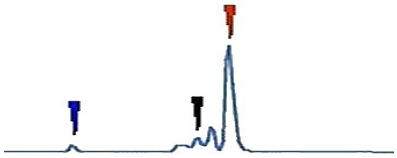
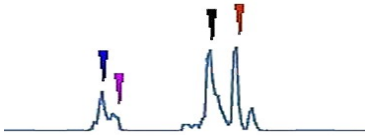
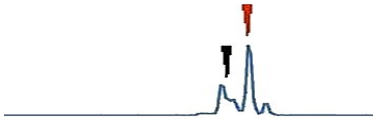
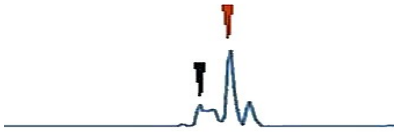
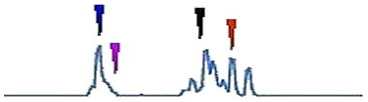
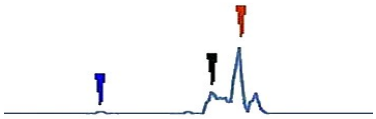
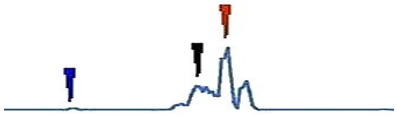
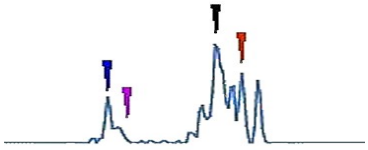
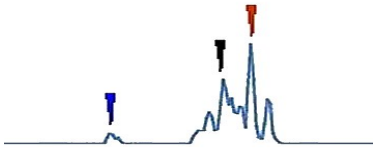
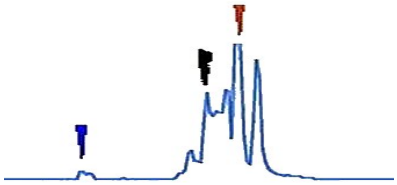
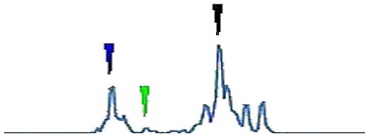
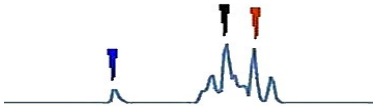
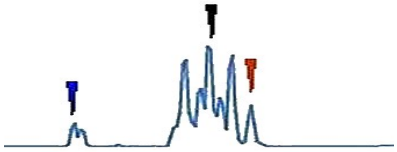
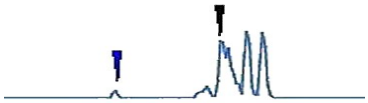
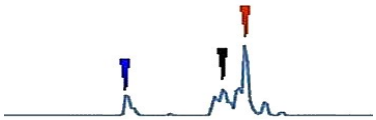
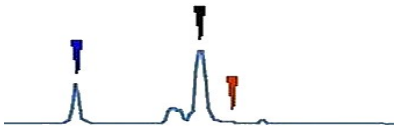
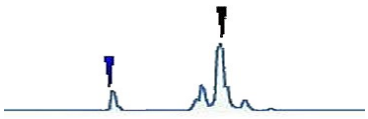
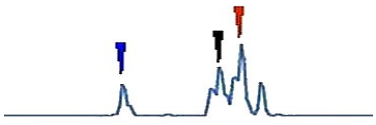
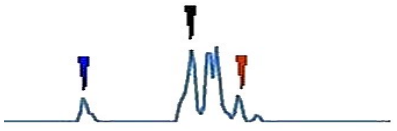


D. ***Bacteroides.*** These two peaks (red) were common to the *Bacteroides* strains used as donor and recipient of the conjugative transposon. The 317 base pair peak is not present in this image as this sample was generated from a pure culture of *B. thetaiotaomicron*. The two identifying peaks for this organism lie at 353 and 356 base pairs respectively. These values were also used as markers for *Bacteroides* present in feces.

Figure 3: Genescan Images from Treatment VI Microbes, Oysters and Water.

Here treatments VII and IX are contrasted. Treatment VII produced multiple transconjugant colonies (see figure 1), where treatment IX did not. Though treatment VII produced several putative transconjugants, there was no visible *Bacteroides* peak in the Genescan images for those days.

Blue arrows indicate a PCR fragment of 317 base pairs. This fragment was amplified from nearly every sample. Purple arrows indicate fragments of either 320 or 340 base pairs. These fragments were amplified specifically from oysters. Green arrows indicate a specific peak obtained from algal paste used to feed the oysters. Black arrows indicate a fragment of 348 base pairs. This fragment was amplified from the water, and consequently was found in the oysters. Red arrows indicate a *B. thetaiotaomicron* specific peak. These fragments are 356 base pairs in length.

Treatment VII			Treatment IX
	Oysters	Water	Water
Day	300 320 340 360	300 320 340 360	320 340 360
1			
2			
3			
4			
5			
6			
7			

Bacteroides and *tetQ* PCR Results:

Experimental samples are arranged by treatment, and grouped with all days of the same treatment. For example treatment VI, days one through seven are grouped so that the change over time, and the variation in identifying peaks can be observed throughout the sampling period. In addition, each DNA sample was tested for the presence of *Bacteroides* 16S genes and the *tetQ* gene. These results in full can be seen in appendix B. An abridged version corresponding to treatment VI, oyster, days one through seven, and treatment VI, seawater, days one through seven, are shown in table 2. In oysters, the DNA from *Bacteroides* and *tetQ* becomes undetectable in a matter of days, while in water, after seven days the DNA has variable detection limits, and is generally more persistent. Water was changed for the treatments on days three and five and this can be observed in several PCR results (appendix B). Differences can be observed between samples taken from oysters and water on the same day from the same treatment. Specific peaks in both cases are most likely due to members of the community flora. In the case of oysters, a specific peak found only within oysters could be a species only able to exist within the stomach, whereas a peak found both inside and outside of the oyster might be a species that travels through the digestive system. I would expect to find some peaks common to both oysters and water, being that the oysters are marine creatures and scavenge the water for food. Differences and similarities should be present between the samples due to individual variation, identity of the sample, and treatment.

Table 2: Persistence of *Bacteroides* and *tetQ* DNA in Oysters and Water, Treatment VI.

	Treatment VI, Oyster		Treatment VI, Water	
Day	**Bacteroides**	**TetQ**	**Bacteroides**	**TetQ**
1	+	+	+	+
2	+	+	+	+
3	+	+	+	+
4	+	+	+	+
5	-	-	+	+
6	-	-	+	+
7	-	-	+	+

Treatment VI, Oyster: *Bacteroides* and *tetQ* were detectable until day five for this treatment. Even though the Bacteroides detection limit was sensitive, samples that yielded transconjugant colonies (days five and seven) show no sign of *Bacteroides* or *tetQ* in this case.

Treatment VI, Water: *Bacteroides* and *tetQ* DNA in the water persisted throughout this treatments duration.

Only one oyster, or 60 mL of water, was sampled per treatment, per day, each sample receiving a score of positive or negative. All samples contained eubacterial 16S DNA. The water was changed in all treatments with oysters on days noted. The water changes may have affected the persistence of *Bacteroides* and *tetQ*. On day five the oysters were removed, and water sampled several hours after the water change. In some cases, treatment IV for example, Bacteroides was not present on day five, yet reappeared on day six, and then disappeared on day seven. This fluctuation may have been due to the water change. See appendices A & B for details.

Discussion

In the initial conception of the experiment, a *B. thetaiotaomicron* strain containing a conjugative transposon carrying the *tetQ* gene was to be mated to *Enteroccus faecalis* to demonstrate inter-genus transfer [4,11]. The conjugative transposon was apparently able to transfer but was extremely unstable and transfer was never definitively proven as has been done by other researchers [4]. Therefore, we chose to use strains of *B. thetaiotaomicron* as both the donor and recipient. Both strains were thymidine dependent. This was used as an additional selective element. The donor strain, Bt 4109, contained the conjugative transposon that conferred resistance to tetracycline at concentrations above 3μg/mL [20]. The recipient, Bt 4001, did not contain the conjugative transposon, but had a chromosomal resistance to rifampicin [20]. In the laboratory, the donor readily transferred the conjugative transposon to the recipient in a concentrated environment with the presence of low levels of tetracycline. Conjugative transfer effectiveness was not tested for in the absence of tetracycline. After mating via the filter method (see previous description) as well as within the oysters, the resulting transconjugant strains were rifampicin resistant, with an acquired and PCR detectable resistance to tetracycline. Donors, recipients and resulting transconjugant strains all tested positive via PCR for *Bacteroides* 16S rRNA genes, confirming their identity as *Bacteroides*.

There are some distinct differences between the laboratory and natural seawater microcosms procedures and conditions that resulted in induced transfer of conjugative transposons. To test for transfer initially, the cultures were mixed in equal proportions, filtered, and transconjugants were isolated with selective media [7, 20]. This entire process, save for the bench top manipulation, was done at 37°C, and the cultures were allowed to grow anaerobically. Within the oysters, though the environment was presumably anaerobic, the temperature was only 15°C, and the nutrient quality was questionable. These factors may have led to varying efficiency of the transfer rate. From the oysters only five of seven colonies were recovered that met the requirements for transconjugants. This is in contrast to more than twenty colonies recovered from the initial conjugation events observed in the laboratory.

My initial hypothesis was that if a conjugation event occurred, *tetQ* DNA would be detectable in oysters or water, and *Bacteroides* DNA would not be. This hypothesis assumed that transfer would occur to non-*Bacteroides* species only. In hindsight this was a naïve assumption. By this standard, the DNA samples taken from the water and oysters show no verifiable conjugation event. In every situation that the *tetQ* gene was detectable in the oysters, the *Bacteroides* 16S rRNA gene was also detectable. In the water there was greater variability, in some cases *tetQ* being present when *Bacteroides* was not detectable, (see appendix B). Treatments containing feces may have contained *tetQ* genes of non-*Bacteroides* origin[18]. Because other fecal bacteria are known to carry the *tetQ* gene, this suggests that any transfer occurring within contaminated oysters

would be of such a low frequency, that without specific selection, the event might go unnoticed while contributing to the spread of antibiotic resistance.

In some cases, PCR verification detected *Bacteroides* 16S rDNA, but no corresponding peak was seen on Genescan images of the sample. This is not surprising as the primers used to detect *Bacteroides* are highly specific, being able to detect as little as 10^5 gene copies per liter, or about 200 cells per mL^3; therefore it is possible that the less specific Eubacterial primers did not amplify the *Bacteroides* peak in some cases. As expected, laboratory procedures have indicated that *tetQ* genes are amplified at a lesser magnitude than *Bacteroides* genes from a given sample (data not included).

Oysters are a possible natural medium for the transfer of antibiotic resistance via conjugative transposons due to their method of feeding and the concentration of cells that occurs within the oyster as a result of that feeding [5], though digestive enzymes in the oysters stomach may inhibit accumulation of foreign species [10].Fecal pollution in a natural environment with agents such as water currents would tend to recirculate and dilute the water and associated pollution until the particulates have come to rest or been absorbed, while in the laboratory environment, aeration is present, but the water is re-circulated and undiluted until removed. The laboratory environment allows for a longer potential exposure to the contaminating elements than would be found in nature. The filter feeding of oysters, in either a natural or laboratory environment, allows the concentration of microbiological, organic and chemical additives in one place. The filter mating procedure performs much the same function, concentrating the

cells together for exposure to the chemicals and necessary nutrients. The main concern, I believe, is that after a pollution event, oysters may have mediated the creation of new antibiotic resistant strains.

Although in my experiment this did not occur from actual raw fecal contamination, conjugation within the oysters did occur from a bacterial species associated with fecal pollution events, *B. thetaiotaomicron.*

This experiment revealed a previously unknown medium of antibiotic resistance transfer. While antibiotic resistance transfer has been documented as occurring between livestock and humans and in the human colon [14,19], transfer is now documented as occurring in a more unfavorable environment. Whereas previously, transfer was believed to occur only in such favorable areas as the colon, the transfer of the antibiotic resistance genes could be tracked from a contaminating source to transfer in a commercial consumable product, oysters. In our experiments, the transferred conjugative transposon was frequently unstable in the recipient cell, and the recipient cell did not retain the conjugative transposon if selection with tetracycline was not maintained. This response to the mobile element may change after several generations, creating a stable strain of antibiotic resistant bacteria. This implies that constant selection in environments conducible to conjugative transposition may create and maintain these antibiotic resistant strains.

Sampling ceased after seven days because *Bacteroides* and *tetQ* from the initial exposure to the treatments were no longer detectable in any of the oysters. To continue the experiment any longer would not have provided any further information about conjugative events within the oyster.

Conclusions

This experiment revealed that oysters are a suitable medium for antibiotic resistance gene transfer. A larger scale experiment with more natural surroundings, such as flowing water, would allow a more accurate assessment of this potential problem. Though the frequency of conjugative transposition within the oysters was at the lower limit of detection limits, this experiment showed that in some conditions it is possible and must be taken into account. Conjugative transposons are highly promiscuous, and conditions permitting, transfer could potentially occur to pathogenic bacteria.

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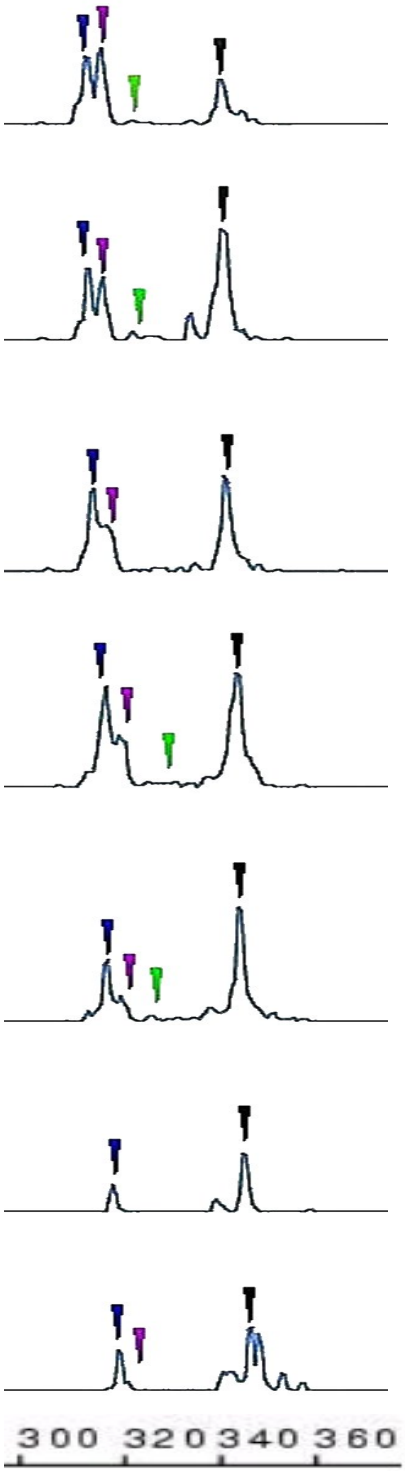
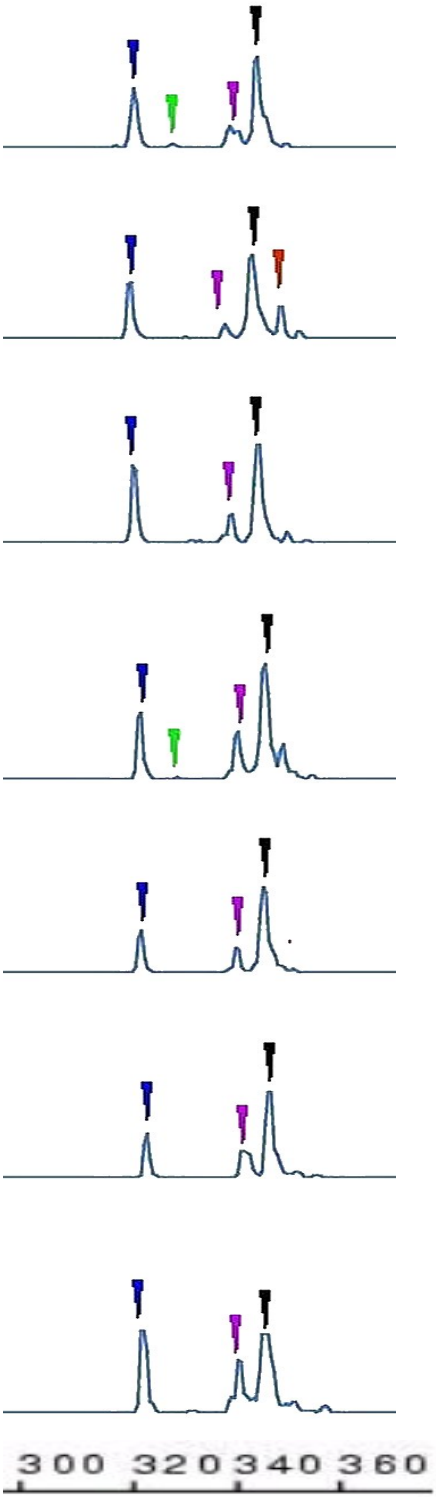
Appendix A: Genescan images.

As noted in results section, blue arrows indicate a peak of 317 base pairs that was common to all water, oyster, and algal paste samples. There are two sizes of purple arrows, one at 320 base pairs, and one at 340 base pairs. The 320 base pairs peak was related to organisms found internal to the oyster, while the 340 base pairs peak was related to organisms external to the oyster as it was commonly found in untreated, oyster containing water. A green arrow, at 327 base pairs, indicates a peak specific to algal paste microbes. A black arrow indicates a peak at 348 base pairs. This peak was unique to the seawater used in the experiment. Naturally, this peak was also found in oysters. A red peak indicates the presence of a *Bacteroides* specific peak. This peak was located at 356 base pairs.

Treatment I, Oyster	Treatment I, Water
	

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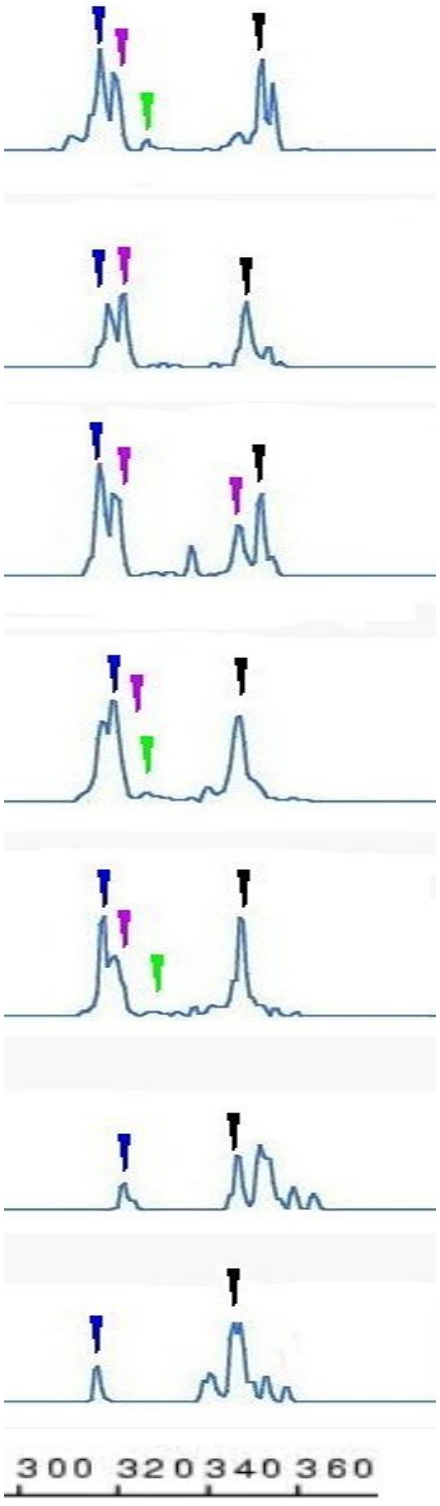
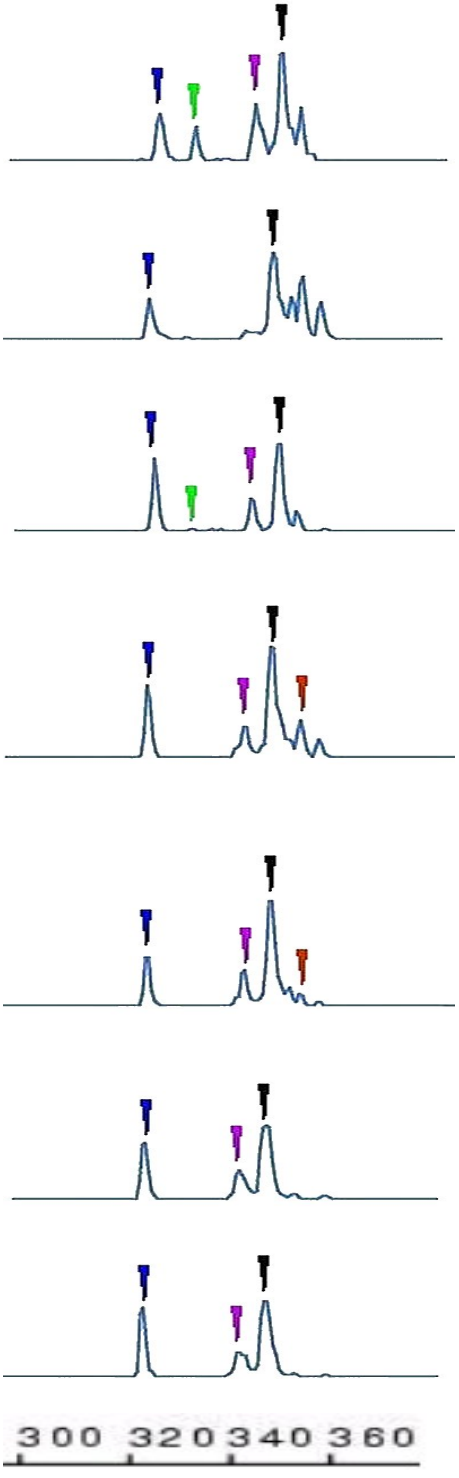
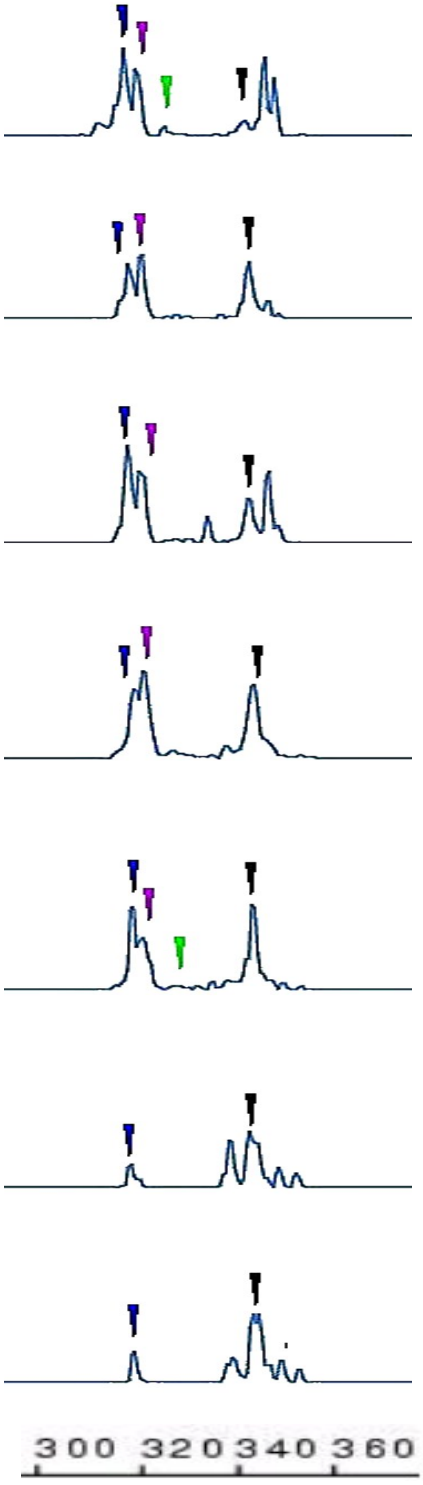
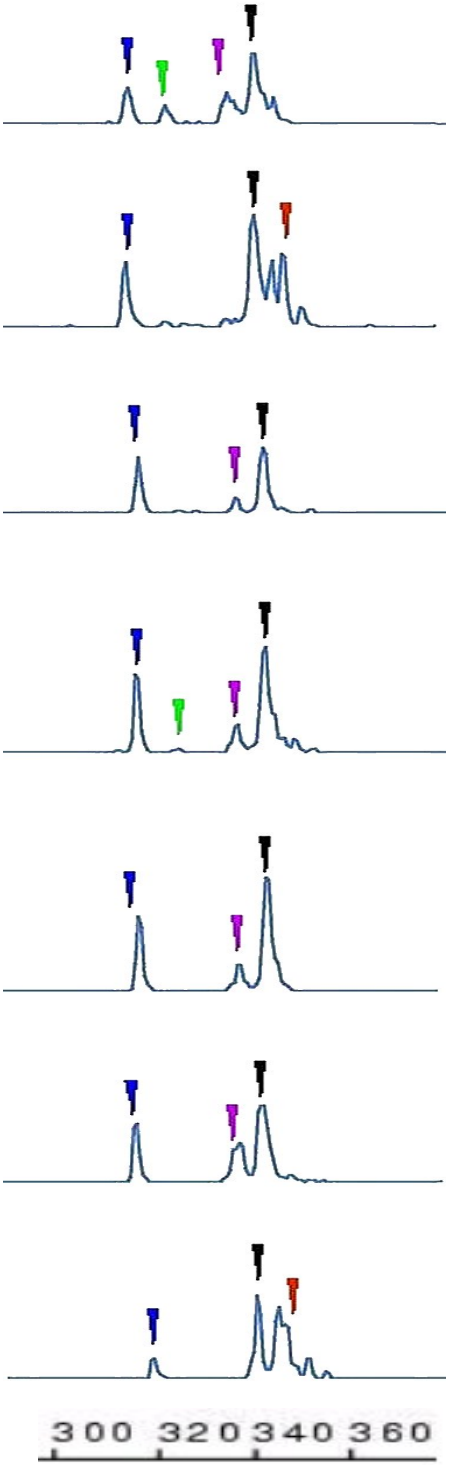
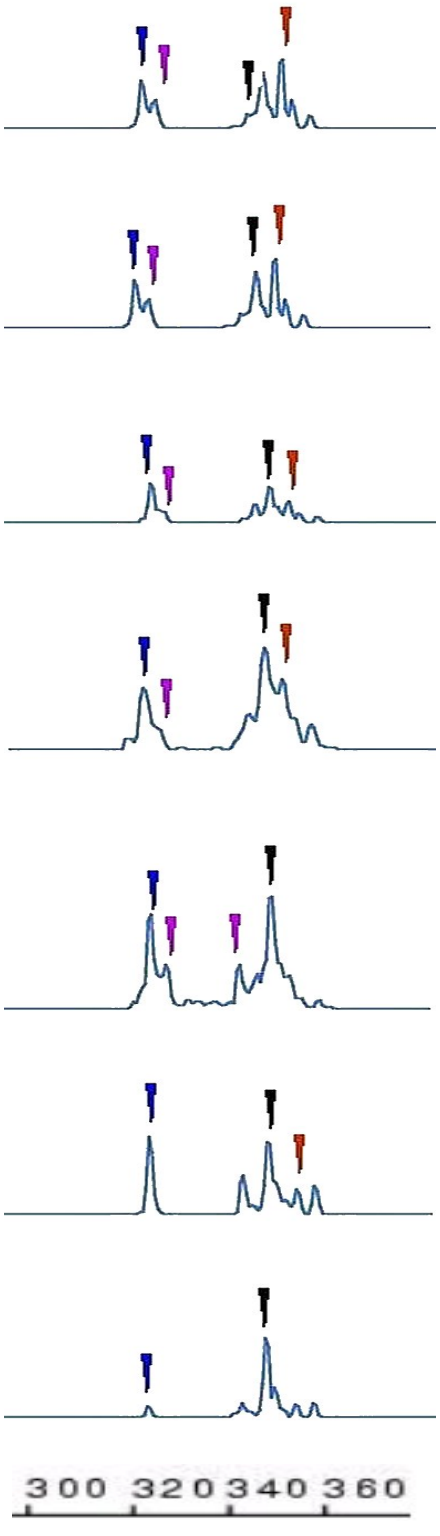
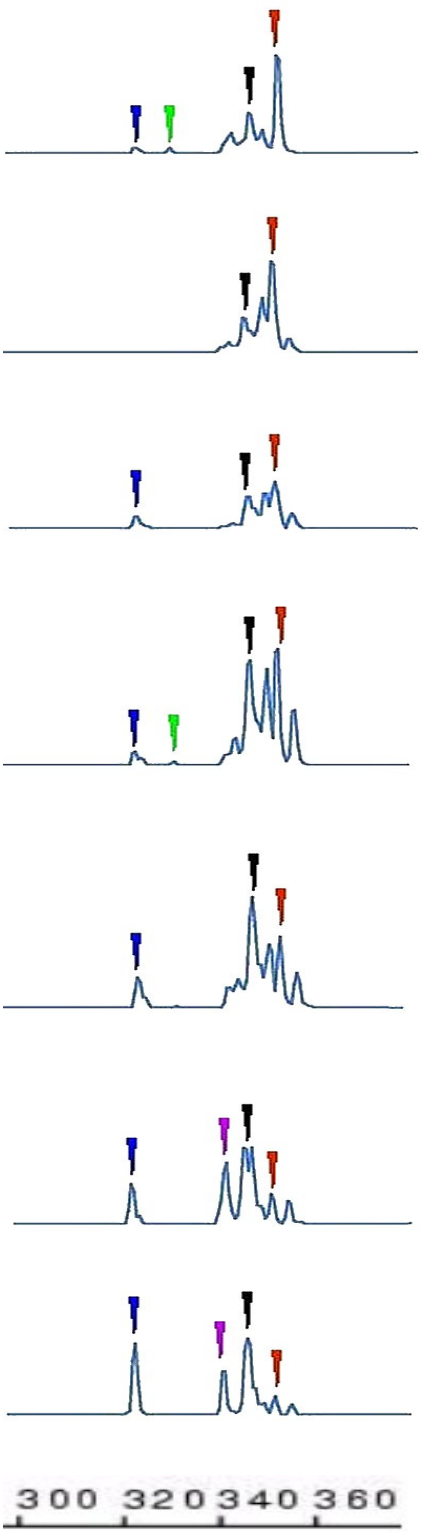
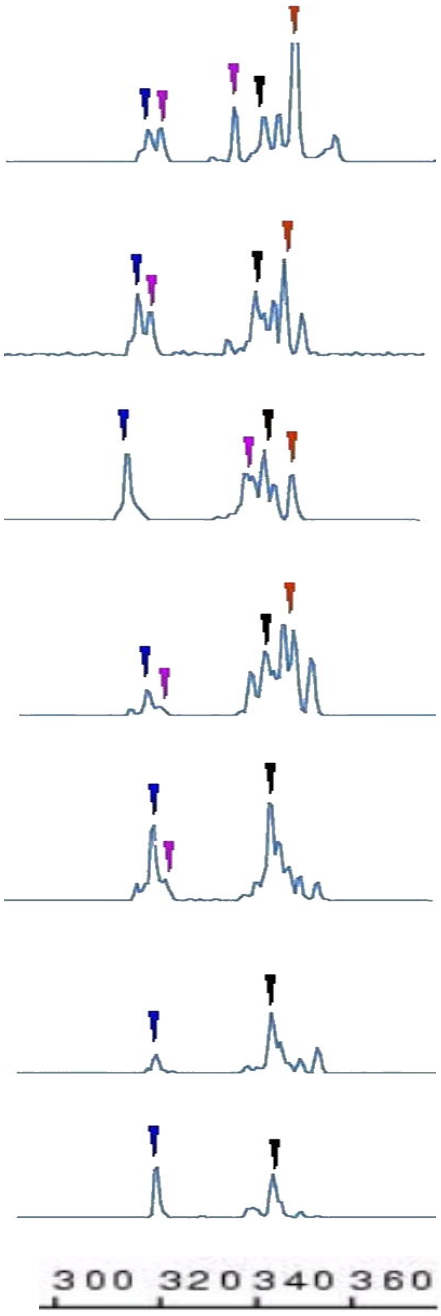
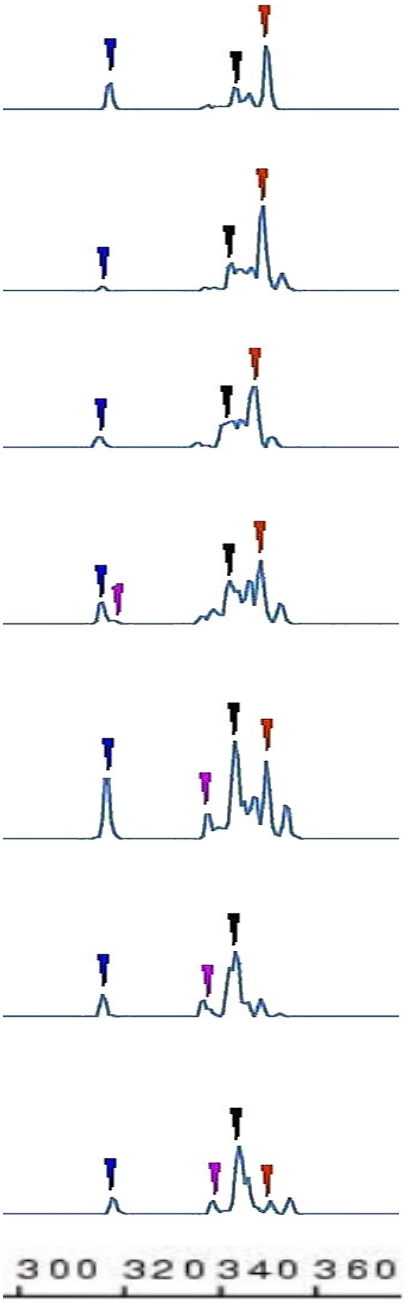
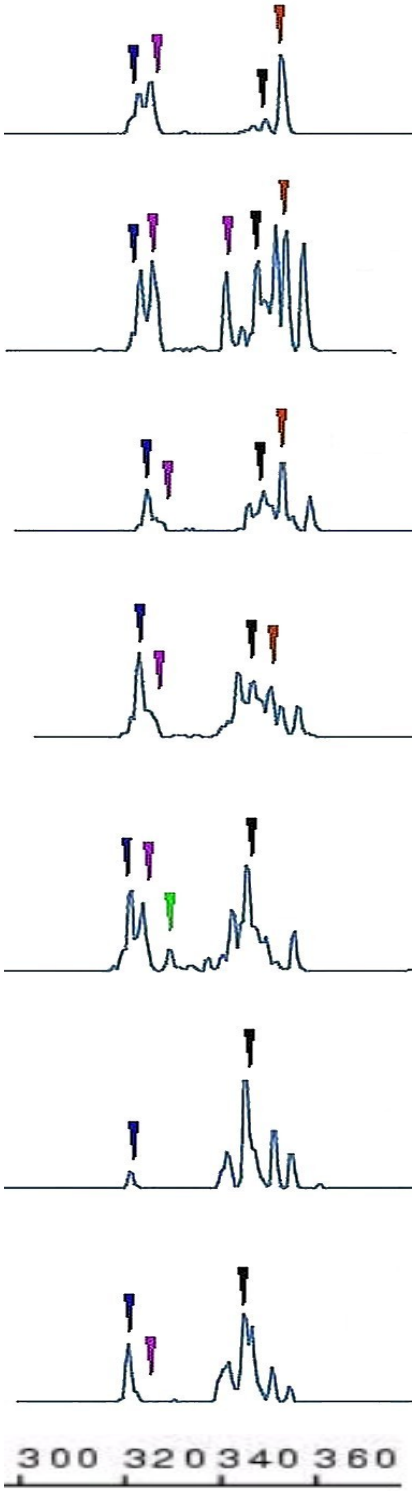
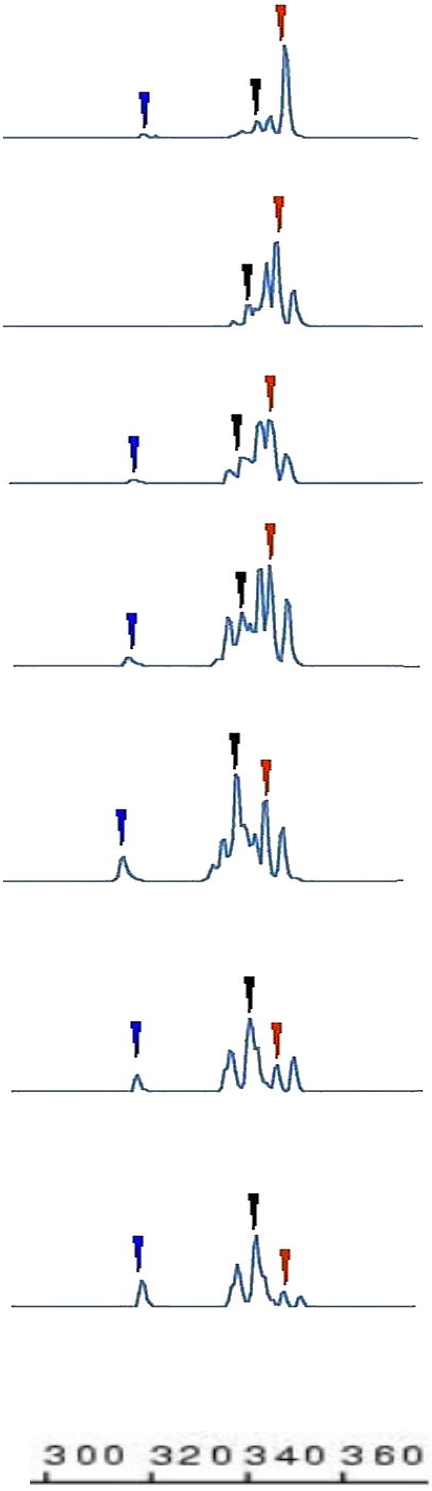
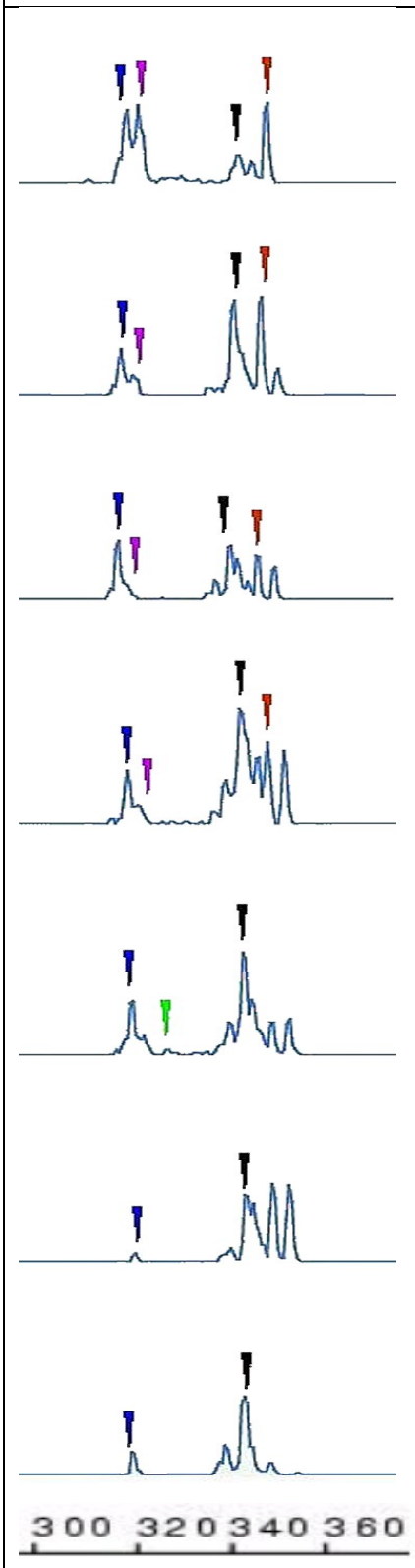
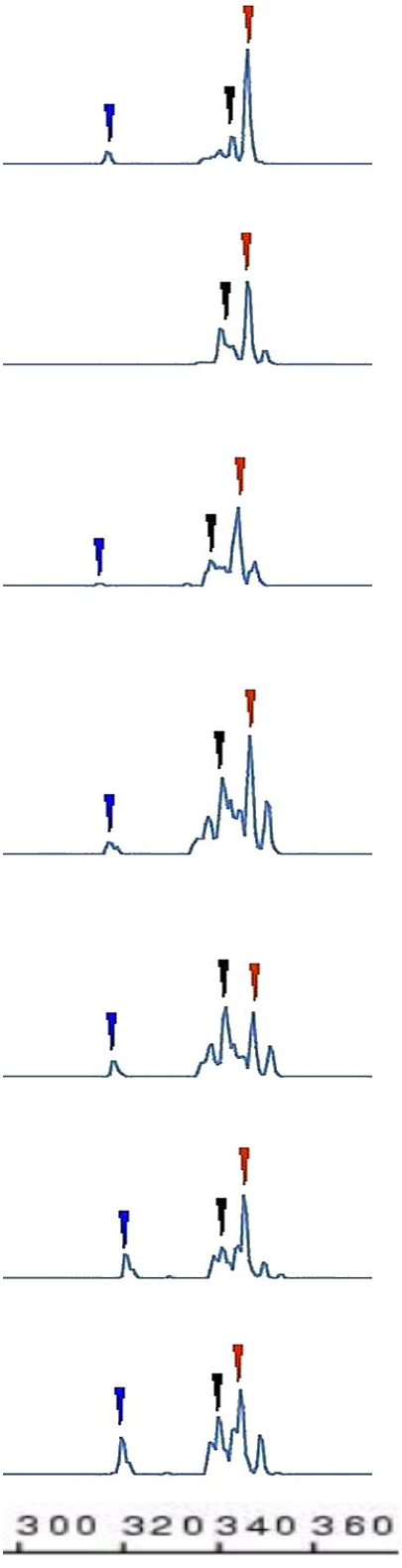
	

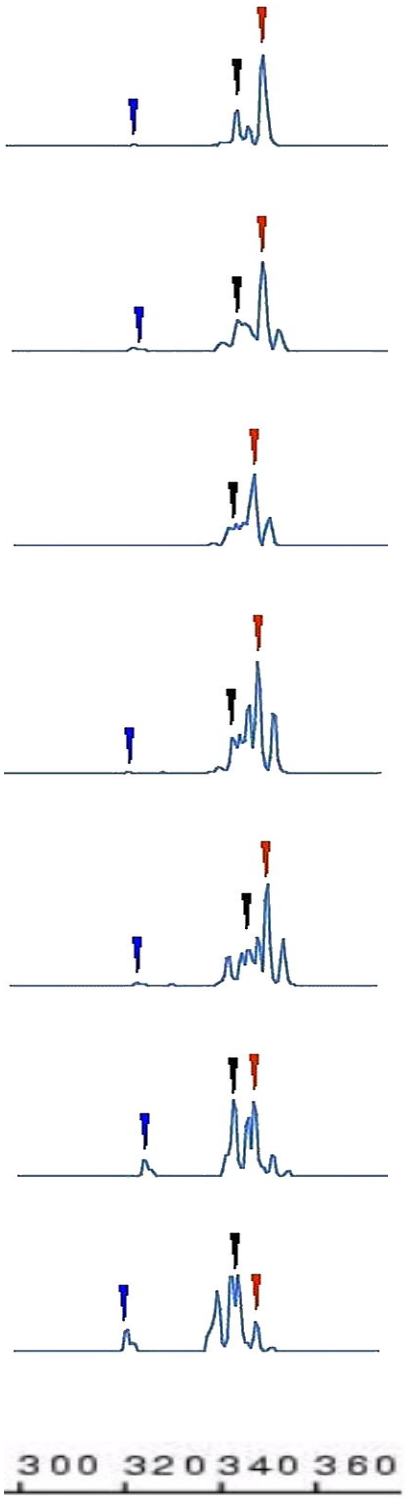
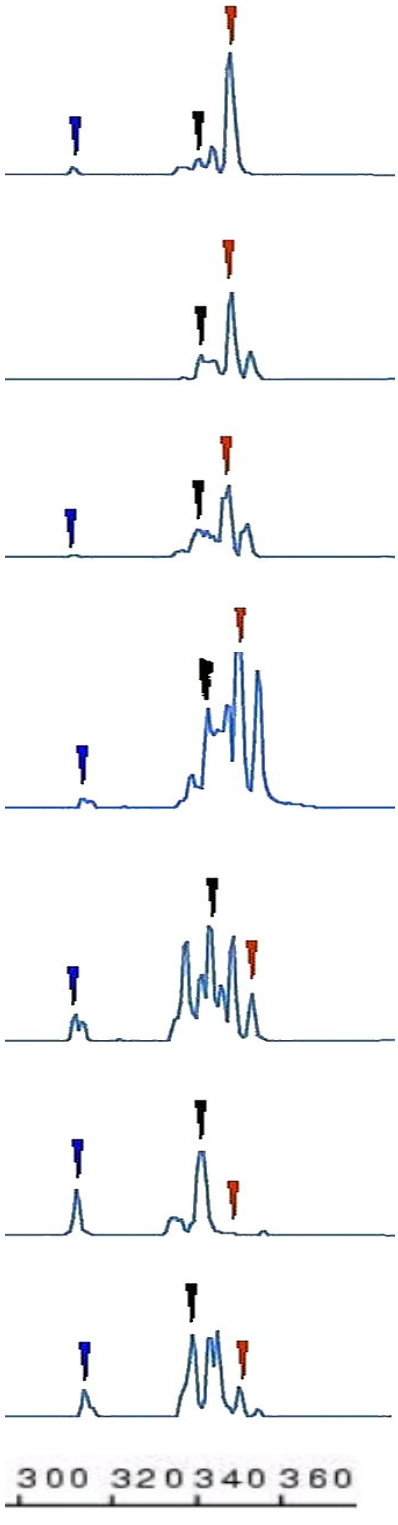
Treatment III, Oyster	Treatment III, Water
	

Treatment IV, Oysters	Treatment IV, Water
	

Treatment V, Oysters	Treatment V, Water
	

Treatment VI, Oysters	Treatment VI, Water
	

Treatment VII, Oysters	Treatment VII, Water
	

Treatment VIII, Water	Treatment IX, Water
	

Appendix B: *Bacteroides* and *tetQ* PCR results.

Treatments	Bacteroides	TetQ
I Oyster	0	0
I Oyster	0	0
I Oyster	+	0
I Oyster	+	0
I Oyster	0	0
I Oyster	0	0
I Oyster	0	0

Treatments	Bacteroides	TetQ
I Water	0	0
I Water	+	+
I Water	+	+
I Water	+	+
I Water	0	+
I Water	+	+
I Water	+	0

II Oyster	0	0
II Oyster	+	0
II Oyster	+	0
II Oyster	+	+
II Oyster	0	0
II Oyster	0	0
II Oyster	0	0

II Water	0	0
II Water	0	+
II Water	+	0
II Water	+	+
II Water	+	+
II Water	0	0
II Water	+	0

III Oyster	0	0
III Oyster	+	0
III Oyster	+	0
III Oyster	+	0
III Oyster	0	0
III Oyster	0	0
III Oyster	0	0

III Water	0	0
III Water	0	0
III Water	+	0
III Water	+	+
III Water	+	+
III Water	+	0
III Water	0	0

IV Oyster	+	0
IV Oyster	+	0
IV Oyster	+	0
IV Oyster	+	0
IV Oyster	0	0
IV Oyster	+	0
IV Oyster	0	0

IV Water	+	+
IV Water	+	+
IV Water	+	+
IV Water	+	+
IV Water	+	+
IV Water	+	+
IV Water	+	0

V Oyster	+	0
V Oyster	+	0
V Oyster	+	0
V Oyster	+	+
V Oyster	0	0
V Oyster	0	0
V Oyster	0	0

V Water	+	+
V Water	+	+
V Water	+	+
V Water	+	+
V Water	+	+
V Water	0	+
V Water	+	0

VI Oyster	+	+	**VI Water**	+	+
VI Oyster	+	+	**VI Water**	+	+
VI Oyster	+	+	**VI Water**	+	+
VI Oyster	+	+	**VI Water**	+	+
VI Oyster	0	0	**VI Water**	+	+
VI Oyster	0	0	**VI Water**	+	+
VI Oyster	0	0	**VI Water**	+	+
VII Oyster	+	+	**VII Water**	+	+
VII Oyster	+	+	**VII Water**	+	+
VII Oyster	+	+	**VII Water**	+	+
VII Oyster	+	+	**VII Water**	+	+
VII Oyster	0	0	**VII Water**	+	+
VII Oyster	0	0	**VII Water**	+	+
VII Oyster	0	0	**VII Water**	+	+
VIII Water	+	+	**IX Water**	+	+
VIII Water	+	+	**IX Water**	+	+
VIII Water	+	+	**IX Water**	+	+
VIII Water	+	+	**IX Water**	+	+
VIII Water	+	+	**IX Water**	+	+
VIII Water	+	0	**IX Water**	+	+
VIII Water	+	0	**IX Water**	+	+

The properties of the various treatments can be viewed in table 1 in the materials and methods section. These results were obtained via PCR using primers specific for *Bacteroides* 16S rRNA [2], or primers specific for the detection of the *tetQ* gene. A score of (+) indicates a positive result, while a score of zero indicates a negative result.