

Watershed-Related Antibiotic Resistant
Genes in Planktonic and Benthic Habitats
of the Sapelo NERR and Bioaccumulation
of *tet(D)* in Microbiota of *CRASSOTREA*
VIRGINICA.

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The Background

- Fecal waste discharge into waterways and estuarine environments causes invasion of exogenous and often harmful microorganisms into oyster harvesting areas and oysters .
- The spatial domain of oysters in Georgia waters has been reduced from the 1930's when it sustained a thriving shellfish industry to its current status of a negligible economic harvest.
- Source trackers are desired to address point-and non-point source fecal contamination in oyster beds.

The Background

- The fecal coliform bacterial indicator is the current regulatory standard (**below 70 cells per 100 ml of water**) for determining bacterial water quality in oyster beds throughout the U.S., and this standard is not epidemiologically-based.
- Tetracycline resistance genes and integrons may be used as additional source trackers due to their abundance in fecal bacteria and the complexity of their profiles.
- Antibiotic resistance genes themselves were recently (2007) recognized as **emerging environmental contaminants**.
- Pandemic migration of antibiotic resistance genes from microbial to the human population *via* food chains has been also recognized.

The Sites...



The People....



Methodology

- Water column, sediment, and oyster samples were collected between November of 2007 and December of 2008 from seven stations varied on their connection to watershed .
- Water parameters were measured for each site and each trip.
- Coliform contents were in-house evaluated by MI agar and Petrifilm® methods, and by A1 method performed at the Coastal District Health District 9-1 Laboratory for quality control.
- Community DNA was extracted from sediments, filter-collected water column microorganisms, oyster liquid, and oyster tissues using appropriate Mo Bio (US., California) kits.
- The presence and concentrations of *tet* and *int1* genes were determined by PCR and qPCR, respectively.
- The abundance of antibiotic resistant bacteria in surveyed microbial communities was determined by the ratio of particular tet and int1 genes to a housekeeping

How reliable are coliforms?

Table 1. Coliform Content as Measured by Three Methods

Station #	11/07		05/08		10/08			12/08			02/09		
	MI agar	Petrifilm	MI agar	Petrifilm	A1	MI agar	Petrifilm	A1	MI agar	Petrifilm	A1	MI agar	Petrifilm
1	1515±	467±	463±	4400±	16±	7±	13000±	33±	617±	2067±	20±	40±	1778±
	985	412	80	954	6	9	4198	0	266	1948	9	37	1824
2	2774±	5033±	343±	14733±	183±	317±	12266±	N.D.	N.D.	N.D.	52±	13±	356±
	997	4128	89	451	120	448	4152				21	9	156
3	2550±	10167±	451±	22933±	71±	500±	7400±	47±	320±	3733±	93±	193±	1022±
	981	1112	58	1537	42	185	1424	27	194	1161	26	273	932
4	902±	400±	291±	9300±	N.D.	N.D.	N.D.	114±	377±	400±	18±	193±	800±
	50	351	76	287				90	170	283	4	73	628
5	884±	667±	201±	19233±	37±	223±	15800±	32±	227±	800±	11±	30±	1956±
	125	511	68	2401	6	73	4109	12	88	0	2	22	1473
6	N.D.	N.D.	N.D.	N.D.	24±	320±	25000±	210±	367±	600±	18±	0	22±
					7	85	12084	29	125	63	11		31
7	N.D.	N.D.	N.D.	N.D.	17±	153±	1467±	31±	360±	67±	<1.8±	0	224±
					9	31	411	14	91	94	0		157

Table 2. Water Parameters at the Stations

Station #	Trip	Major Fecal Input	pH	Salin. promil	Cond. (s/m)	Turb. (NTU)	dO2 (g/L)	TDS (g/L)	ORP (mV)	T (C°)
1. Doboy Sound 1	1107	Farms	6.68	36	5.49	5.0	6.22	33	91	19.3
	208		7.57	17	2.60	63	8.30	16	38	15.1
	508		7.50	29	4.4	0.1	7.76	27	232	23.9
	1008		6.40	40	6.7	250	5.7	38	232	23.5
	1208		7.74	24.3	31.7	77.1	12.3	19.3	220	14.4
	209		7.82	24.7	31.0	38.8	12.3	18.8	243	12.9
2. Doboy Sound 2	1107	Farms	7.14	24	3.80	5.0	7.20	23	32	20.1
	208		7.03	20	0.43	390	8.40	2.7	47	16.0
	508		7.33	29	4.5	6.3	6.5	26	214	24.8
	1008		6.70	40	7.6	280	5.5	48	223	24.1
	1208		N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	209		7.78	17.1	23	41	12.4	14.3	259	12.9
3. Champney-cut	1107	Farms Septic tanks	7.11	26	4.03	153	7.64	25	99	19.3
	208		7.30	5	0.89	200	8.00	5.6	117	15.6
	508		7.64	11	1.9	23.9	8.6	12	213	23.9
	1008		6.70	40	6.8	300	6.6	4.6	222	23.8
	1208		7.62	11.4	16.9	130	20.1	10.5	212	14.7
	209		7.70	12.3	17.4	28.4	20.1	10.8	253	12.7
4. Hudson Creek	208	Municip.	7.04	24	3.7	9	7.20	23	132	15.6
	508		6.9	27	4.2	0.1	4.56	25	258	25.1
	1008		N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	1208		7.78	22.5	35.7	8.6	9.7	21.5	220	13.1
	209		7.57	25.2	31.5	20.9	9.7	19.3	247	12.9
	208		Not known	7.97	23	3.7	130	7.70	23	91
508	7.77	10		1.8	0.1	6.5	11	198	24.6	
1008		6.40		40	6.1	460	4.7	35	233	25.6
1208		7.64	28.8	36.6	59.9	11.3	22.3	226	14.3	
209		7.74	25.9	32.2	144	11.3	19.7	273	12.9	
6. Duplin River	1008	None	6.40	39	5.9	110	4.9	35	235	25.5
	1208		7.56	29.9	36.6	15.3	8.4	22.3	184	14.2
	209		7.65	25.7	31.8	26.1	8.4	19.4	260	12.5
7. Duplin River	1008	None	6.70	37	5.6	73	4.0	33	223	25.9
	1208		7.60	28.6	36	21.6	10.8	22	232	14.9
	209		7.69	25.7	32.3	89.3	10.8	19.7	254	13.2

Why *tet* genes?

- There are 43 genes encoding resistance to tetracycline.
- Those genes encode either efflux pump proteins, ribosomal protection proteins, or tetracycline modifying proteins.
- Those mechanisms are effective not only against tetracycline but also against other related antibiotics.
- They are carried by Gram positive and Gram negative microorganisms.
- They have been found abundant in bacteria associated to animal and human feces.
- Their profiles are reflective of their origin.

Table 3. *Tet* and *Int1* genes targeted and primers used

<u>Gene</u>	<u>Primer (Sequence 5'-3')</u>	<u>Amplicon</u>	<u>Reference</u>
<i>Tet</i> (A)	fw- GCTACATCCTGCTTGCCTTC rv- CATAGATCGCCGTGAAGAGG	210 bp	Ng et al, 2001
<i>Tet</i> A(P)	fw- GCTACATCCTGCTTGCCTTC rv- CATAGATCGCCGTGAAGAGG	676 bp	Ng et al, 2001
<i>Tet</i> (B)	fw- TTGGTTAGGGGCAAGTTTTG rv- GTAATGGGCCAATAACACCG	659 bp	Ng et al, 2001
<i>Tet</i> (C)	fw- CTTGAGAGCCTTCAACCCAG rv- ATGGTCGTCATCTACCTGCC	418 bp	Ng et al, 2001
<i>Tet</i> (D)	fw- AAACCATTACGGCATTCTGC rv- GACCGGATACACCATCCATC	787 bp	Ng et al, 2001
<i>Tet</i> (E)	fw- CTTGAGAGCCTTCAACCCAG rv- ATGGTCGTCATCTACCTGCC	278 bp	Ng et al, 2001
<i>Tet</i> (G)	fw- GCTCGGTGGTATCTCTGCTC rv- AGCAACAGAATCGGGAACAC	468 bp	Ng et al, 2001
<i>Tet</i> (K)	fw-ACAGAAAGCTTATTATATAAC rv-TGGCGTGTCTATGATGTTAC	169 bp	Ng et al, 2001.
<i>Tet</i> (L)	fw-TCGTTAGCGTGCTGTCATTC rv-GTATCCCACCAATGTAGCCG	267 bp	Ng et al, 2001
<i>Tet</i> (M)	fw- GTGGACAAAGGTACAACGAG rv- CGGTAAAGTTCGTACACAC	406 bp	Ng et al, 2001
<i>Tet</i> (O)	fw-AACTTAGGCATTCTGGCTCAC rv-TCCCACTGTTCCATATCGTCA	515 bp	Ng et al, 2001
<i>Tet</i> (Q)	fw-AGAATCTGCTGTTTGCCAGTG rv-CGGAGTGTCAATGATATTGCA	904 bp	Ng et al, 2001
<i>Tet</i> (S)	fw- CATAGACAAGCCGTTGACC rv- ATGTTTTTGAACGCCAGAG	667 bp	Ng et al, 2001
<i>Tet</i> (T)	fw- AAGGTTTATTATATAAAAAGTG rv- AGGTGTATCTATGATATTTAC	169 bp	Aminov et al, 2001
<i>Tet</i> (W)	fw- GAGAGCCTGCTATATGCCAGC rv- GGGCGTATCCACAATGTTAAC	168 bp	Aminov et al, 2001
<i>Tet</i> (X)	fw- CAATAATTGGTGGTGGACCC rv-TTCTTACCTTGGACATCCCC	468 bp	Ng et al, 2001
<i>Int1</i>	fw- CCTCCCGCACGATGATC rw- TCCACGCATCGTCAGGC	280bp	Goldstein et al, 2001
<i>Int1</i> 2	fw- TTATTGCTGGGATTAGGC rw- ACGGCTACCCTCTGTTATC	203bp	Goldstein et al, 2001
<i>Int1</i> 3	fw- AGTGGGTGGCGAATGAGTG rw- TGTCTTGTATCGGCAGGTG	600bp	Goldstein et al, 2001

Table 4. *Tet* gene profiles in the water column, sediment and oysters collected from seven oyster beds

Station/ Sample	<i>tet</i> A(P)	<i>tet</i> A	<i>tet</i> B	<i>tet</i> C	<i>tet</i> D	<i>tet</i> E	<i>tet</i> G	<i>tet</i> K	<i>tet</i> L	<i>tet</i> M	<i>tet</i> Q	<i>tet</i> S	<i>tet</i> X
St. 1													
sedm					1-3		1-3						
water	2	1		1,2	1-3		1-3		4			2	
oysters			3,5		1-3 5		1-3	3,4,5			4	4	1
St. 2^a					1-3		1-3						
sedm		2			1-3		1-3						
water				2	1-3		1-3						
oysters			2		1-3		1-3					2	
St. 3													
sedm		2			1-3		1-3		4				
water		2			1-3		1-3						
oysters	2,4		2,5	4,5	234		1-3	3,4	4	2	4	2,4	
St. 4^b													
sedm		2			2,3		2,3						
water	2	2			2,3		2,3					2	
oysters	2		2		2,3		2,3			2			
St. 5^b													
sedm					2,3		2,3						
water	2	2			2-4		2,3		4			2	
oysters			2,5	4,5	235		2,3	4,5			4	4	
St. 6^c													
sedm													
water													
oysters			5		5	5		4,5			4	4	
St. 7^c													
sedm								4	4				
water								4					
oysters	4		5	4,5	5			4,5	5		4	4	

Numbers in the Table indicate the dates as follows: 1: 11/07, 2: 2/08, 3: 05/08, 4: 10/08, 5: 12/08. Superscripts: a, b, and c - no 10/08, 11/07 data; and only 10/08 and 12/08 data are available, respectively.

Where are these genes from?

Table 5. A summary of *tet* gene profiles for four animal farms* with *tet*(B) being indicative of AB-usage

	<i>tetA</i>	<i>tetA</i> (P)	<i>tetB</i>	<i>tetC</i>	<i>tetD</i>	<i>tetE</i>	<i>tetG</i>	<i>tetK</i>	<i>tetL</i>	<i>tetM</i>	<i>tetO</i>	<i>tetQ</i>	<i>tetS</i>	<i>tetT</i>	<i>tetW</i>	<i>tetX</i>
Farm 1	+++	+++	+++	+++	+		+++		+++	+++	+++	+++	+++		+++	++
Farm 2	+++	+++	+	+++	++	++	+		+++	+++	+++	+++	+		+++	+++
Farm 3	+++	+++	++	++		+	+++		+++	+++	+++	+++	++		+++	+++
Farm 4	n.a.		n.a.	n.a.	n.a.		n.a.	++	+++	+++	+++	+++		+++	+++	+++

* Three samplings were performed for each farm, the number of crosses indicates the number of samplings in which the gene was detected. N.a. – no analysis performed.

How do they get there?

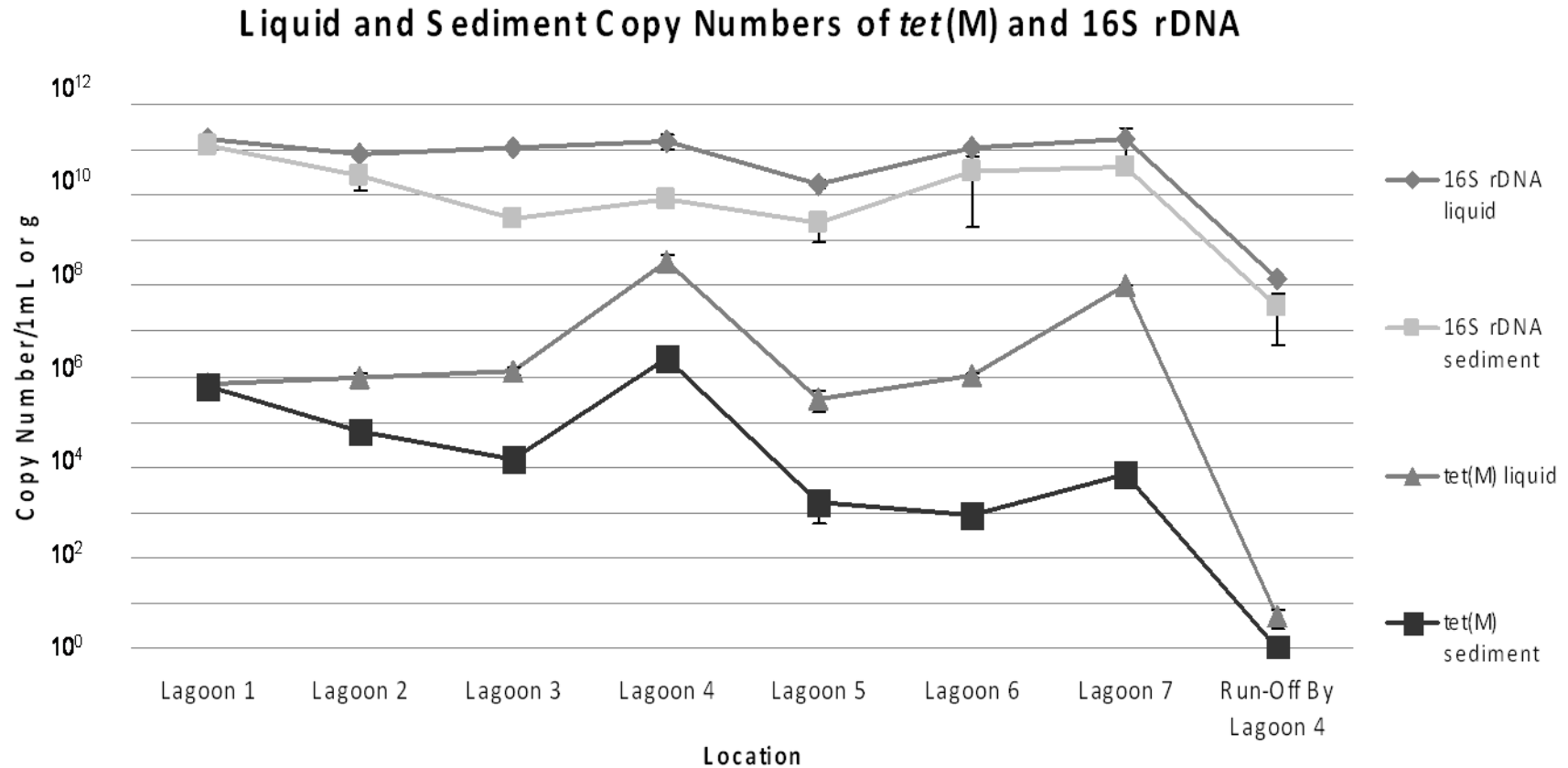


Fig. 1. The progression of *tet(M)* and 16S rRNA genes through the waste lagoons, and the discharge of *tet(M)* to a creek (Farm 3, Dec.'07).

How do they get there?

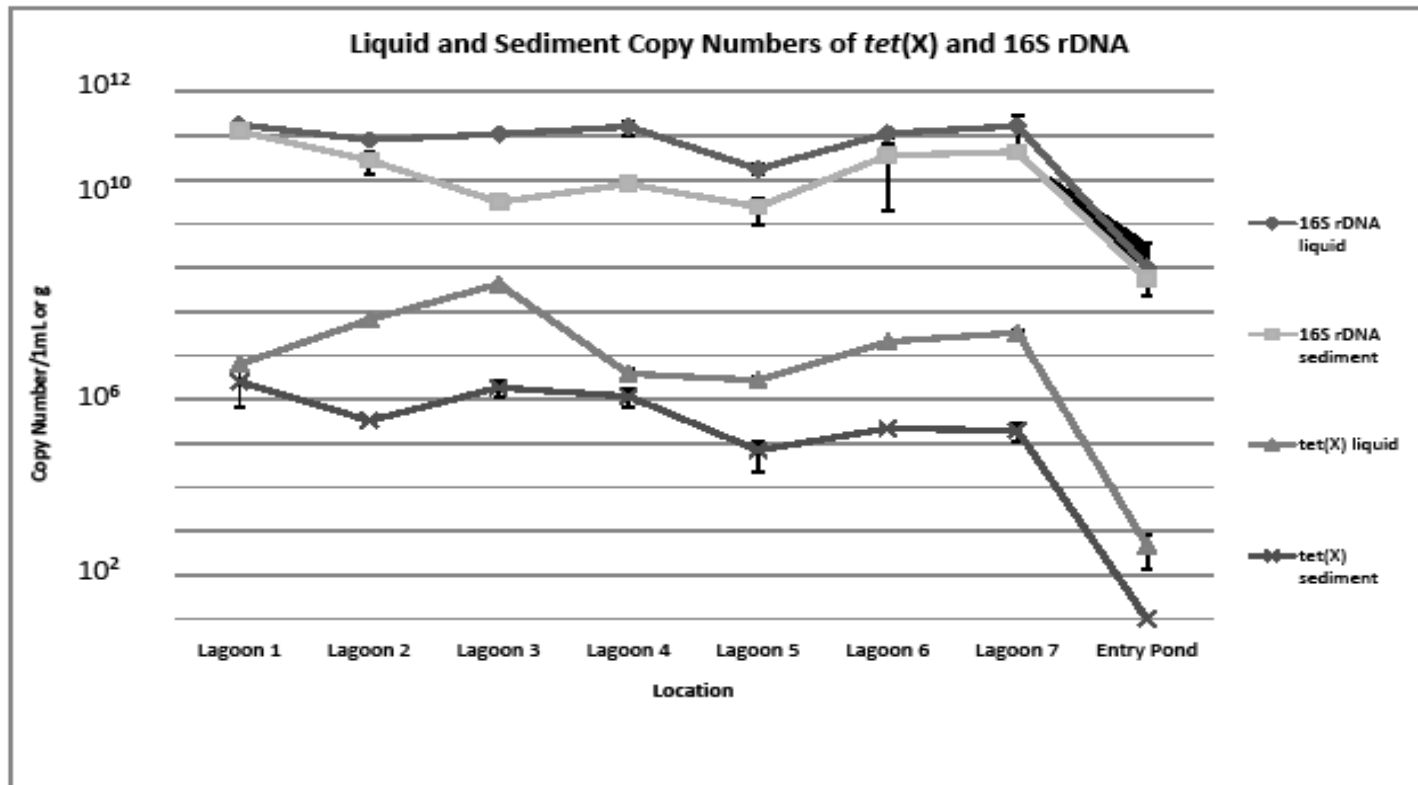


Fig. 2 The progression of *tet(X)* and 16S rRNA genes through the waste lagoons, and the discharge of *tet(X)* to a pond (Farm 3, Dec.'07).

What impacts the discharge of *tet* genes to the watershed?

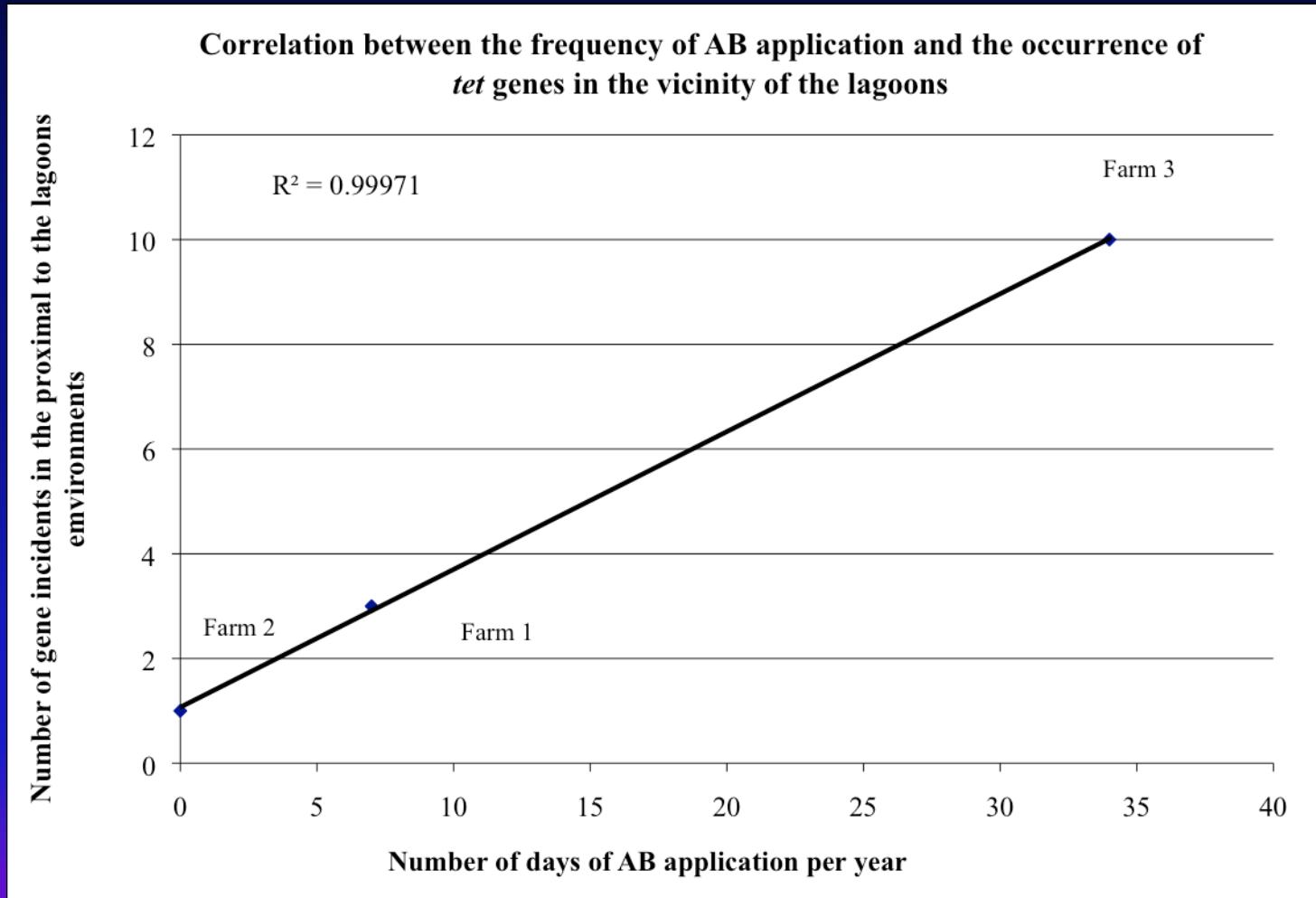


Fig. 3. Correlation between AB usage and the occurrence of *tet* genes in the watershed.

What impacts the discharge of *tet* genes to the watershed?

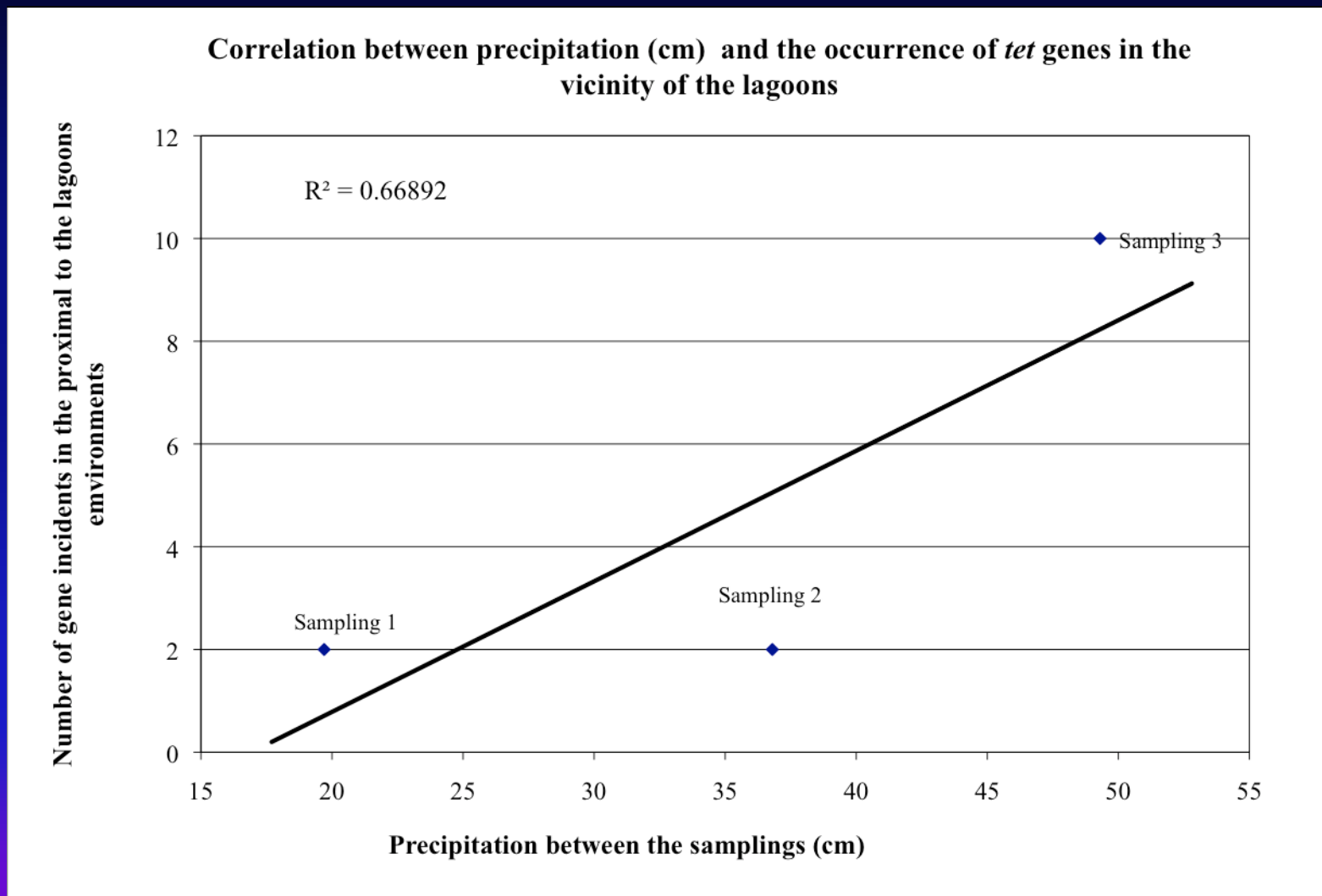


Fig. 4. Correlation between the rainfall and the occurrence of *tet* genes in the watershed.

What else helps them to get to the watershed?

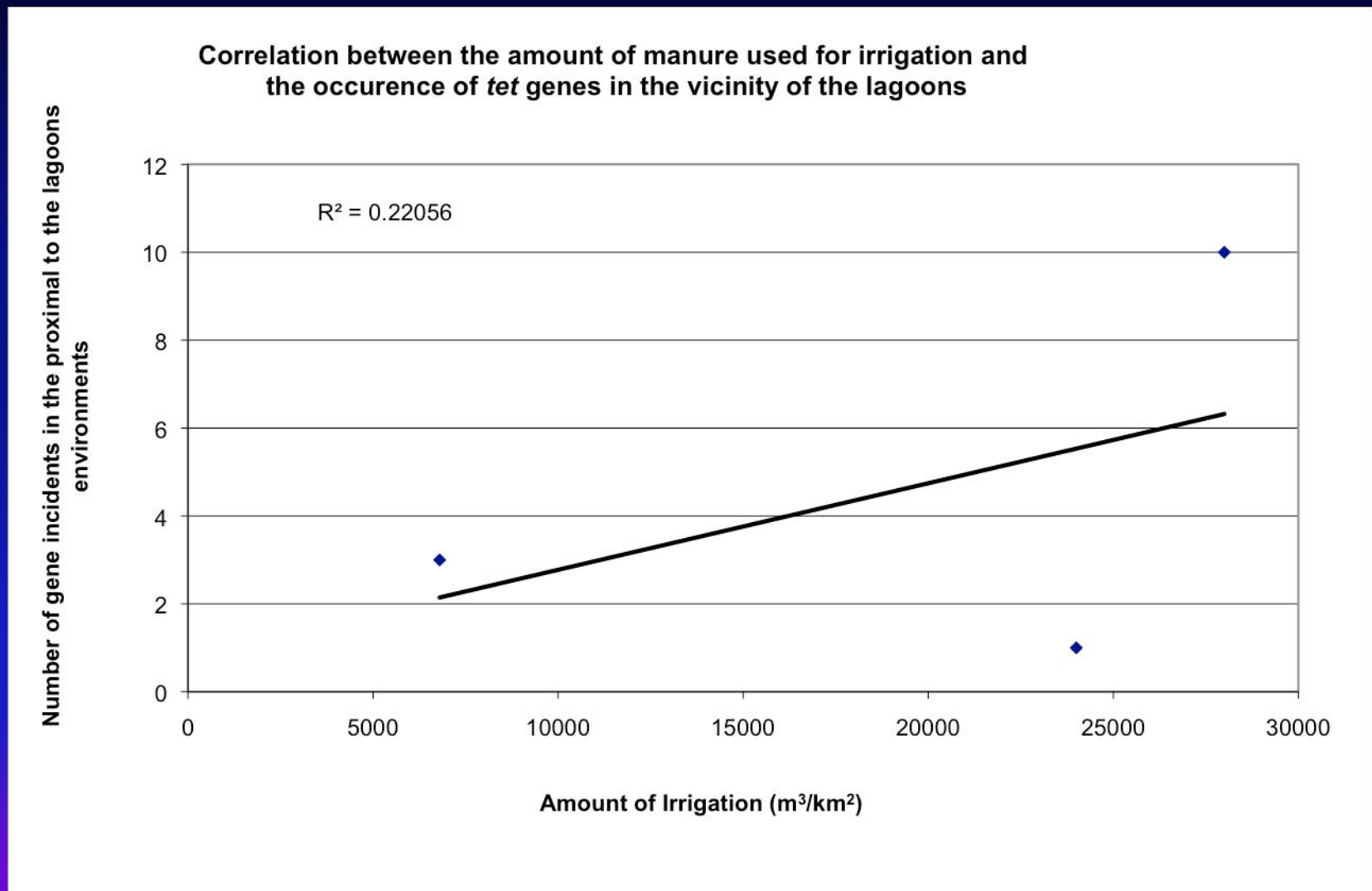


Fig. 5. Correlation between the manure irrigation and the occurrence of *tet* genes in the watershed.

Why Integrons?

- Integrons are mobile elements containing integrase (*intI*) genes and gene cassette integration sites (*attI*), into which the cassettes of antibiotic resistance genes could be inserted.
- There are about 90 distinctive integron specimens grouped into 6 classes, three of which, integron 1, 2, and 3, have been shown as primarily incorporating the antibiotic resistance gene cassettes.
- Class 1 integrons, being of environmental origin, are the most common and are primarily associated with clinical settings.
- Class 2 integrons are the second common, are primarily associated with transposon *Tn 7*, and are carried primarily by *Acinetobacter*, *Shigella*, and *Salmonella*.
- Class 3 integrons are the least common, have not been previously reported for environmental settings, and are associated with *Serratia marcescens*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Pseudomonas putida*, and *Alcaligenes xylosoxidans*.
- The profiles and abundance of integrons in oyster beds may serve as integrative tools for non-point source anthropogenic contamination.

Station/ Time	<u>Int1</u>		<u>Int2</u>		<u>Int3</u>	
	10/08	12/ 08	10/08	12/ 08	10/08	12/08
Station 1						
sediment			+			
water						
oysters			+		+	+
Station 2^a						
sediment						
water						
oysters						
Station 3						
sediment			+			
water						
oysters			+		+	+
Station 4						
sediment						
water						
oysters	+					
Station 5						
sediment			+			
water						
oysters					+	+
Station 6						
sediment						
water						
oysters	+				+	+
Station 7						
sediment					+	+
water			+			
oysters					+	+

a - no 10/08 data available

Table 6. The presence and profiles of integrons in oyster beds during 10/08-12/08 samplings

How many genes are there?

Station/ Sample	11/07 Conc	2/08 Conc	5/08 Conc	11/07 Ratio	2/08 Ratio	5/08 Ratio	StAve Conc	StAve Ratio
Station 1								
Sediment	2.0×10^9	3.1×10^9	4.7×10^9	7×10^{-4}	7×10^{-1}	5×10^{-3}	3.2×10^9	2×10^{-1}
Water	1.0×10^3	2.6×10^{20}	3.3×10^2	2×10^{-8}	8×10^{-4}	4×10^{-9}	8.6×10^9	3×10^{-4}
Oyst/fld	8.0×10^9	1.6×10^{20}	5.3×10^9	2×10^1	8×10^2	1×10^2	9.6×10^9	3×10^2
Oyst/tis	6.8×10^1	6.8×10^6	1.3×10^9	2×10^1	1×10^7	1×10^7	4.3×10^8	8×10^6
Station 2								
Sediment	1.7×10^{20}	1.5×10^{20}	1.9×10^{20}	4×10^{-2}	3×10^0	2×10^2	1.7×10^{20}	5×10^1
Water	1.5×10^{20}	2.0×10^{20}	2.3×10^{20}	2×10^{-3}	1×10^{-3}	9×10^{-2}	1.9×10^{20}	5×10^{-2}
Oyst/fld	8.3×10^9	8.4×10^9	8.5×10^9	3×10^2	9×10^1	3×10^1	8.4×10^9	1×10^2
Oyst/tis	3.0×10^8	1.0×10^8	5.2×10^8	2×10^3	8×10^5	5×10^6	3.1×10^8	1×10^5
Station 3								
Sediment	3.1×10^9	1.5×10^9	4.1×10^9	3×10^{-4}	1×10^0	6×10^1	2.9×10^9	7×10^{-1}
Water	2.9×10^{20}	1.6×10^{20}	1.7×10^{20}	5×10^3	2×10^{-3}	2×10^5	2.1×10^{20}	6×10^4
Oyst/fld	7.2×10^9	3.9×10^9	7.4×10^9	3×10^2	9×10^0	3×10^1	6.2×10^9	1×10^2
Oyst/tis	0	4.1×10^5	2.8×10^2	0	3×10^5	1×10^0	1.4×10^5	1×10^5
Station 4								
Sediment	N.D.*	9.6×10^9	5.0×10^8	N.D.	8×10^{-4}	9×10^{-5}	9.6×10^9	2×10^{-3}
Water	N.D.	1.8×10^{20}	2.1×10^{20}	N.D.	1×10^{-3}	2×10^{-1}	2.1×10^{20}	1×10^{-1}
Oyst/fld	N.D.	1.0×10^9	9.4×10^9	N.D.	4×10^1	2×10^1	6.4×10^9	5×10^1
Oyst/tis	N.D.	2.0×10^9	3.6×10^8	N.D.	2×10^8	7×10^8	2.1×10^9	1×10^9
Station 5								
Sediment	N.D.	1.5×10^{20}	5.1×10^9	N.D.	9×10^{-3}	1×10^{-2}	1.1×10^{20}	7×10^{-3}
Water	N.D.	1.2×10^{20}	9.3×10^9	N.D.	2×10^0	3×10^{-3}	1.4×10^{20}	2×10^{-3}
Oyst/fld	N.D.	1.3×10^9	4.8×10^8	N.D.	1×10^2	2×10^1	6.0×10^8	4×10^1
Oyst/tis	N.D.	1.6×10^2	7.7×10^8	N.D.	1×10^{-8}	8×10^{-2}	2.6×10^8	2×10^2

* No sampling was performed

Table 7. *Tet* (D) concentrations and *tet*(D)/16 S rDNA ratios in oyster beds

What happens to these genes in oysters?

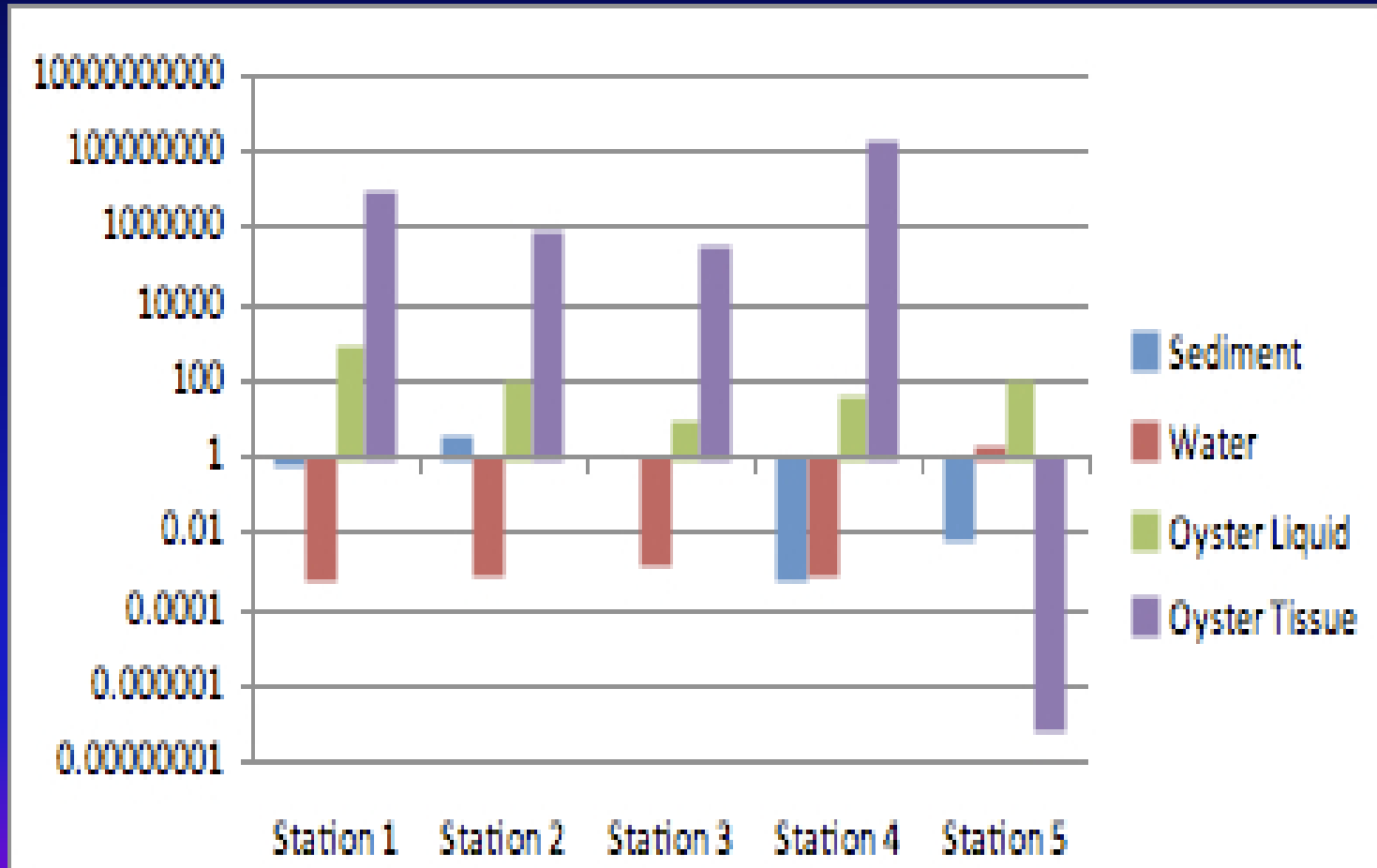


Fig. 6. Bioaccumulation of *tet(D)* in oyster tissues and liquids

Conclusions

- Tetracycline resistance genes are quite common in oyster beds indicating their heavy anthropogenic contamination.
- Their profiles vary temporally and spatially between the seasons and beds.
- Animal feeding operations are likely main sources of these genes.
- The discharge of these genes from farms to the watershed was found correlating to the frequency of antibiotic usage by animal farms, precipitation; and, to a lesser extent, to the amount of manure used for irrigation.
- Integrons were never detected in oyster beds before Fall of 2008, but become abundant thereafter.
- Integrons class 2 and 3 were most commonly observed in oyster beds.
- Oysters *Crassostrea virginica* bioaccumulated antibiotic resistance genes.
- The study suggests that tetracycline resistance genes and integrons could be used for estimation and tracking of fecal contamination in oyster beds, and for monitoring the mobility and fate of antibiotic

Acknowledgements

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