

## UK Harvest Area Study (circa 2001)

- 6 oyster harvesting areas chosen for study
  - to reflect a variety of faecal contamination levels (category A, A/B, B and prohibited)
  - including sites with known association with disease outbreaks (NLV)
- Monitored shellfish for E.coli, FRNA bacteriophage and NLVs for up to 2 years
- Characterised all NLV isolates
- Compared NLV occurrence with harvesting area pollution status (E.coli and FRNA bacteriophage) and with association with disease outbreaks

## Faecal pollution levels and illness associated with study sites

Site	EU Classification	E.coli MPN /100g in oysters			Association with illness
		Minimum	Maximum	Geometric mean	
1	A	<20	<20	0 (12)	No
2	A/B	<20	<18,000	30 (44)	No
3	B	<20	5,000	40 (16)	(Yes)
4	B	<20	>18,000	380 (52)	Yes
5	B	<20	>18,000	820 (63)	Yes
6	Prohibited	310	2,400,000	53,000 (35)	na

## Summary of E.coli, RNA phage and NLV PCR results from study sites

Site	E.coli geometric mean (n)	FRNA geometric mean (n)	NLV* % +ve (n)
1	0 (12)	0 (12)	0 (8)
2	30 (44)	12 (44)	0% (39)
3	40 (16)	130 (16)	6% (16)
4	380 (62)	3100 (62)	33% (39)
5	820 (63)	4000 (63)	23% (49)
6	53,000 (35)	42,000 (35)	47% (32)

\*amplicon confirmed as NLV by sequence analysis

## Correlation of log % positive NLV with mean log E.coli and mean log FRNA phage at 6 sites

	% NLV +ve (by sequencing)
E.coli (GM)	0.85
FRNA bacteriophage (GM)	0.96

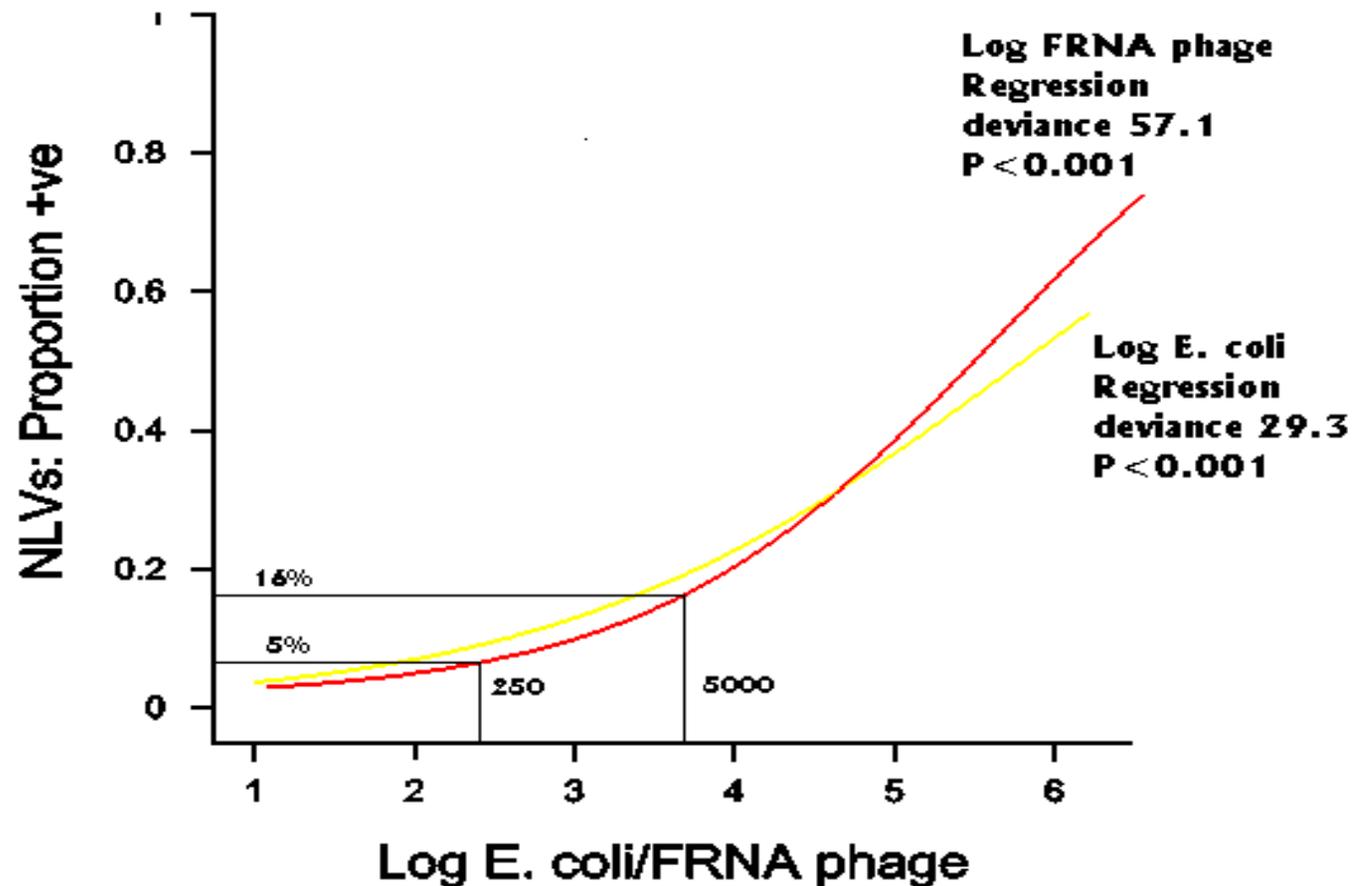
Values presented are Pearson correlation coefficients

## Risk of NLV contamination in shellfish harvesting areas

- EU wide study : 2000 – 2002 (Formiga-Cruz et al., 2003)
- range of shellfish species from commercial class A, B, C and prohibited harvesting areas
- analysis for E.coli, FRNA bacteriophage, and enteric viruses (including HAV and NLV)
- large data set : Spain (122), Greece (144), Sweden (54), UK (225), Eire (76) : total 621 samples
- all NLV positives confirmed by genome sequencing

# Risk of NLV contamination

**Logistic Regression**  
**Fitted Relationship: NLVs versus Log E.coli/FRNA phage**



# Comparison of MSC and norovirus by quantitative PCR in two commercial sites over 2 years

TABLE 3. Comparison of norovirus positivity and *E. coli* most-probable-number count

<i>E. coli</i> MPN (CFU/100 g shellfish flesh) <sup>a</sup>	No. of samples positive for noroviruses/total no. (%)	Avg no. of norovirus PCR units/ positive sample
<30	26/51 (51)	73
30–299	57/95 (60)	51
≥300	18/39 (46)	56

Lowther et al., 2008

} No significant difference

TABLE 4. Comparison of norovirus positivity and FRNA bacteriophage count

FRNA bacteriophage count (PFU/100 g shellfish flesh)	No. of samples positive for norovi- ruses/total no. (%)	Avg no. of norovirus PCR units/ positive sample		
		Total	Area A	Area B
<100	21/68 (31)	6	5	6
100–999	36/54 (67)	22	20	23
≥1,000	32/46 (70)	108	100	112

} Significant difference  
(P>0.001)

## But note of caution

Although high levels of norovirus contamination were associated with high FRNA bacteriophage counts, norovirus-positive results were also returned in some samples with bacteriophage levels below the assay limit of detection (30 PFU/100 g flesh).

Further work is required to understand the correlation, if any, between sample norovirus template titer, as judged by PCR, and human health risk. In the absence of such data, it is not possible to dismiss the significance of low-titer-norovirus results.

Nevertheless, the FRNA bacteriophage assay is considerably cheaper and less labor-intensive than are assays for noroviruses in shellfish, and has potential as a more effective risk management tool for noroviruses than does *E. coli*.

# EU regulatory proposal for MSC (not adopted)

- MSC research conducted over circa 15 years at Cefas (ending about 2006)
- Cefas designated EURL 1999 – part of remit to improve controls for enteric viruses
- Worked in parallel on MSC and PCR for viruses (1995 to date)
- Also worked extensively with other EU NRLs
- Revision of regulations for Microbiological Criteria for Food (circa 2002) gave opportunity for discussion on possible use of MSC (but only in 2 contexts – i.e. not harvest area application)
  - End product standard
  - Food processing criterion (depuration)

## 1. MICROBIOLOGICAL CRITERIA DEFINING THE SAFETY OF FOODSTUFFS

Food category	Micro-organisms	Sampling plan <sup>1</sup>		Limits		Analytical reference method <sup>2</sup>	Stage where the criterion apply	Action in case of unsatisfactory results
		n	c	m	M			
Live bivalve molluscs and live echinoderms, tunicates and gastropods	<i>E. coli</i> <sup>12</sup>	1 (13)	0	<230 / 100g of flesh and intra-valvular liquid		Donovan <i>et al.</i> , 1998, Communicable Disease and Public Health, 1, 188-196	Products ready to be placed on the market and products on the market	The batch shall not be placed on the market or it shall be withdrawn from the market <sup>4</sup>

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## 2. CRITERIA INDICATING THE ACCEPTABILITY OF THE PROCESS

### 2.4. Live bivalve molluscs and live echinoderms, tunicates and gastropods

Food category	Micro-organisms	Sampling plan <sup>1</sup>	Limit	Analytical reference method <sup>2</sup>	Stage where the criterion apply	Action in case of unsatisfactory results
		n	m			
Live bivalve molluscs, which must be purified	FRNA bacteriophages	1 <sup>3</sup>	≥95% removal or removal to ≤ 100 pfu/100g during the process	ISO 10705-1	Before and after the purification process	Modification of temperature and/or duration of the purification process

<sup>1</sup> n= number of units comprising the sample; c= number of samples units giving values between m and M

<sup>2</sup> The most recent edition of the standard shall be used

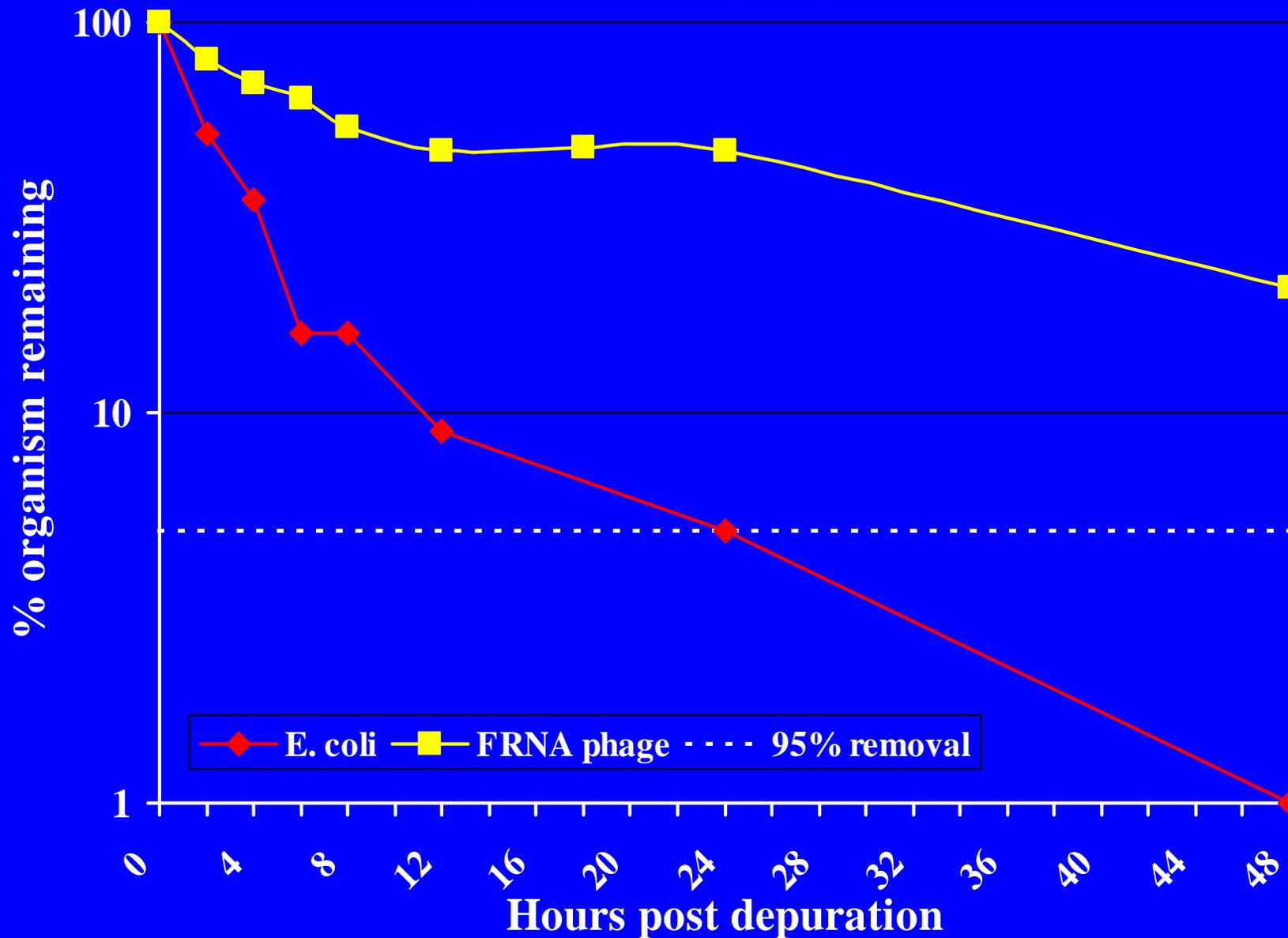
<sup>3</sup> A pooled sample comprising a minimum of 10 individual animals

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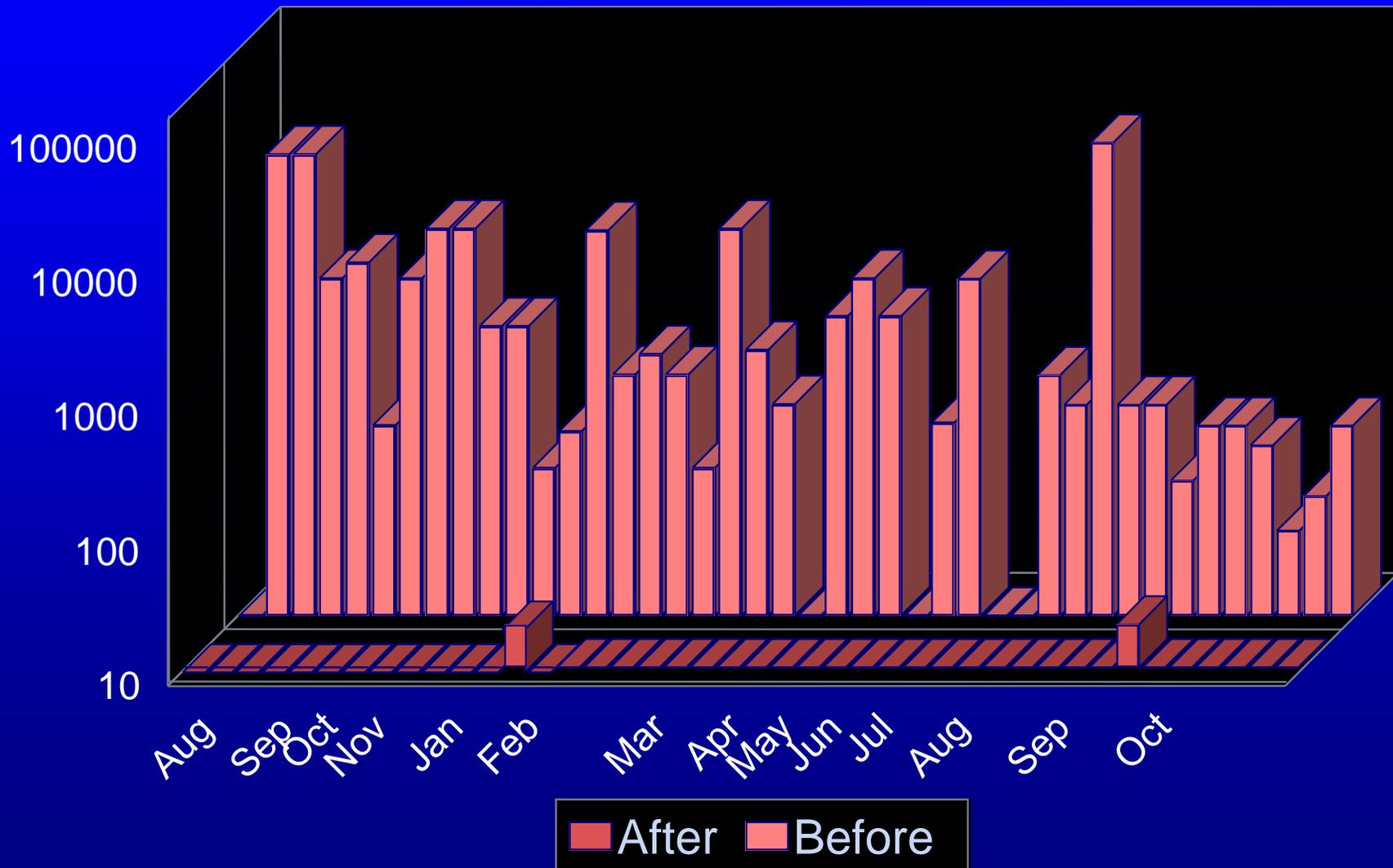
### Status of classifications in EU Member States, 2009

Member State <sup>1</sup>	Number of classified areas <sup>2</sup>	% class A	% class B	% class C	% prohibited <sup>3</sup>
Belgium <sup>4</sup>	1	100	0	0	0
Denmark <sup>5</sup>	49	76	24	0	0
Eire <sup>6</sup>	58	19	76	5	0
France <sup>7</sup>	785	24	57	5	15
Germany <sup>8</sup>	10	100	0	0	0
Greece <sup>9</sup>	43	44	9	2	0
Italy <sup>10</sup>	397	53	46	<1	N/A
Netherlands <sup>11</sup>	6	100	0	0	0
Portugal <sup>12</sup>	44	18	59	16	7
Slovenia <sup>13</sup>	3	0	100	0	0
Spain <sup>14</sup>	951	58	37	5	0
Sweden <sup>15</sup>	33	36	64	0	0
United Kingdom <sup>16</sup>	688	27	64	7	1
Overall	3068	40%	50%	5%	4% <sup>17</sup>

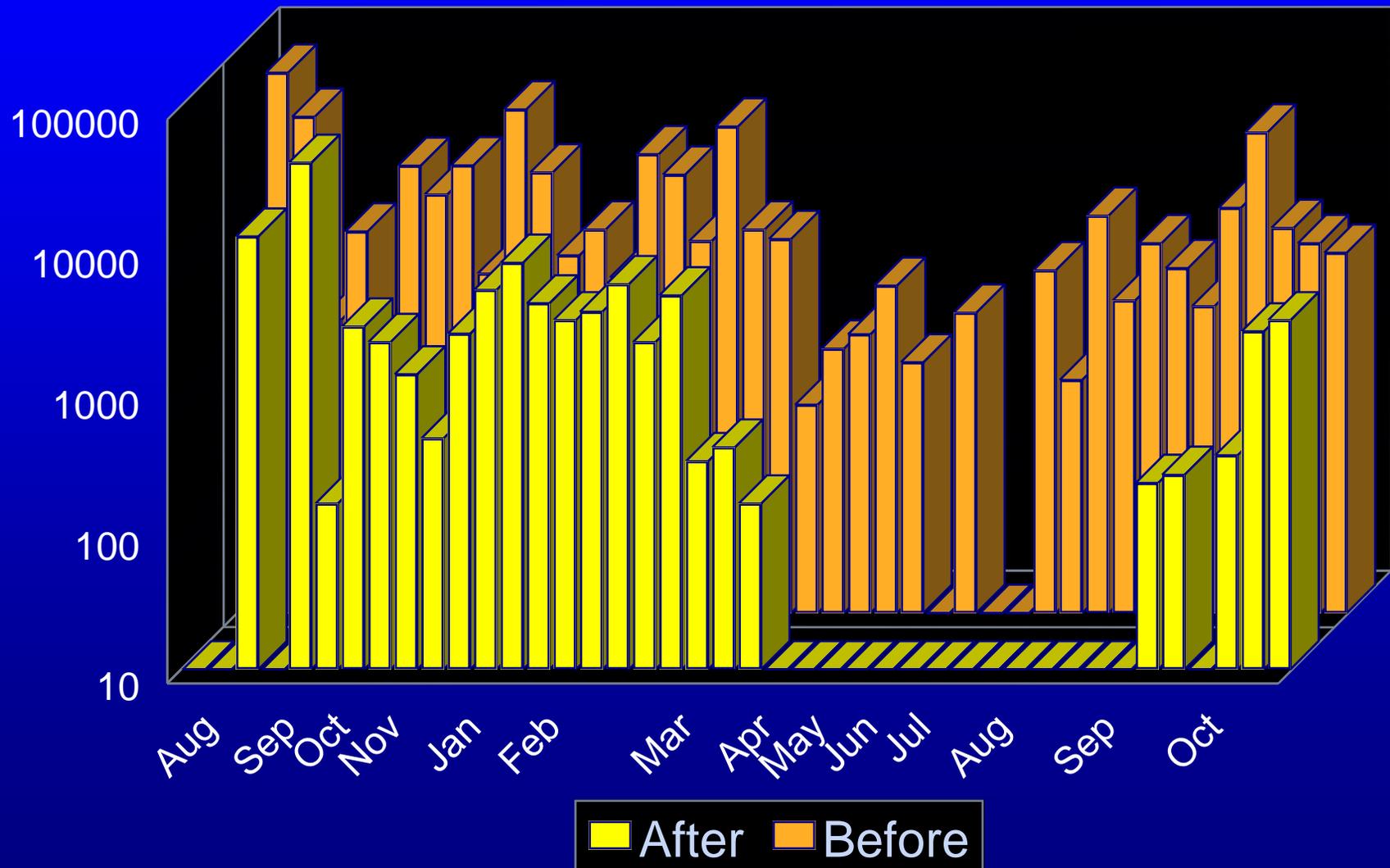
# Elimination of *E. coli* and FRNA bacteriophage from oysters during depuration



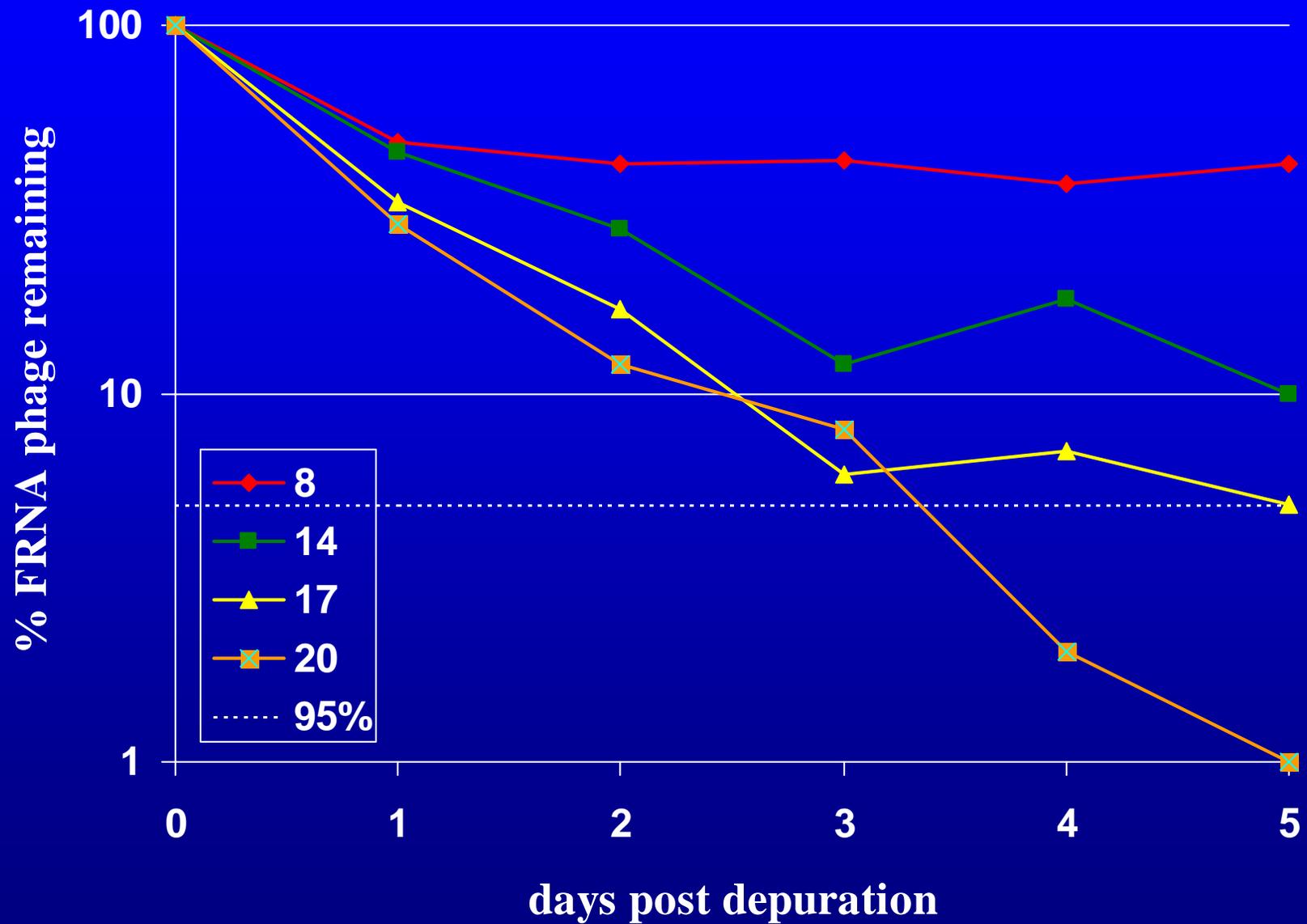
# E.coli removal during purification



# F+ phage removal during purification



# Elimination of FRNA phage with varying temperature



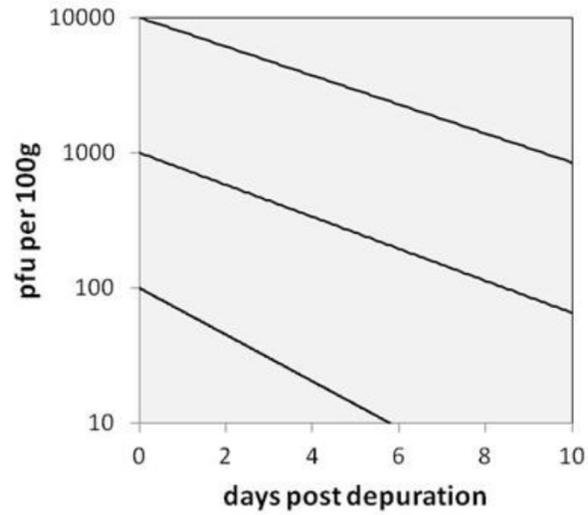
# Extensive MSC depuration studies at Cefas 2000 – 2003

- Predictable removal of MSC by time/temperature
- Some variation by species
- Not improved by feeding (limited studies)
- Not impacted by harvest month or harvest area temperature
- Little impact of higher temperature on mortality
- Similar results in commercial operations

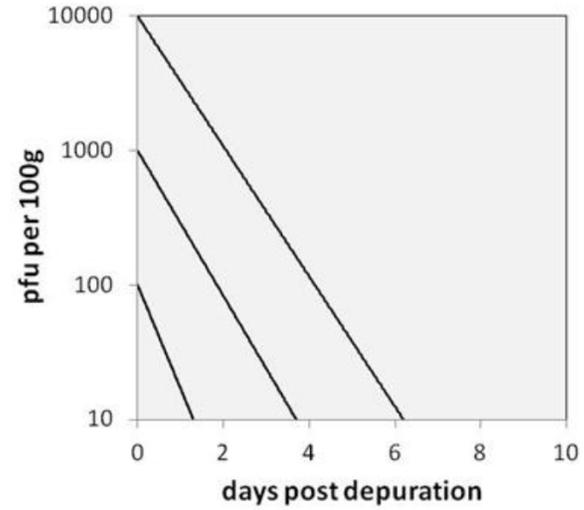


<b>FSA Project Code:</b>	<b>B04002</b>
<b>FSA Project Title:</b>	<b>Development of procedures for improved viral reduction in oysters during commercial depuration.</b>
<b>Start Date:</b>	<b>1<sup>st</sup> May 2000</b>
<b>End Date:</b>	<b>30<sup>th</sup> April 2003</b>
<b>Project Leader's Name:</b>	<b>William Dore</b>
<b>Institution:</b>	<b>CEFAS</b>
<b>Date of submission:</b>	<b>31<sup>st</sup> July 2003</b>

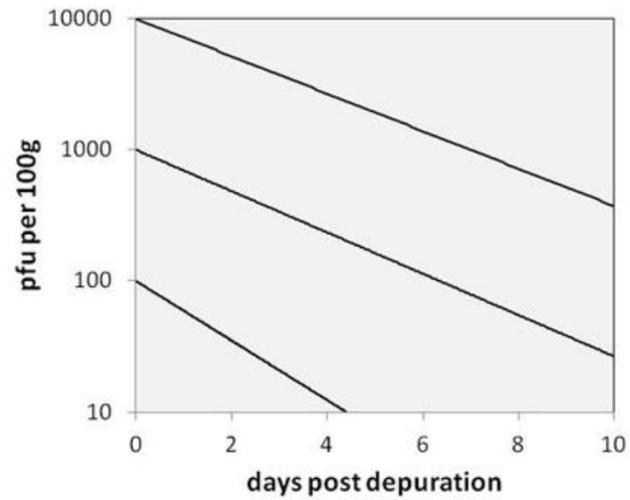
*C. gigas*  
8°C



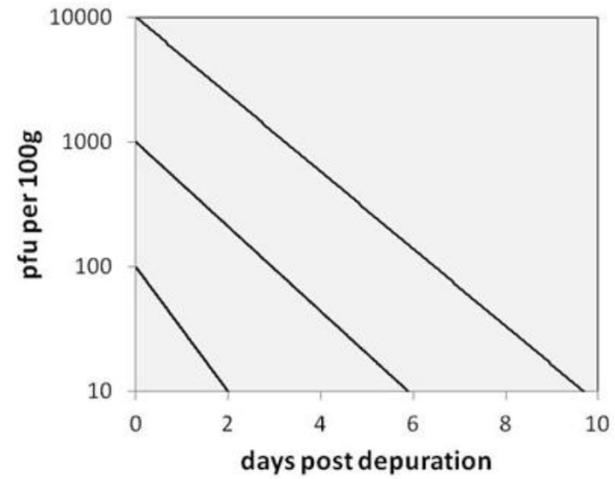
*C. gigas*  
20°C

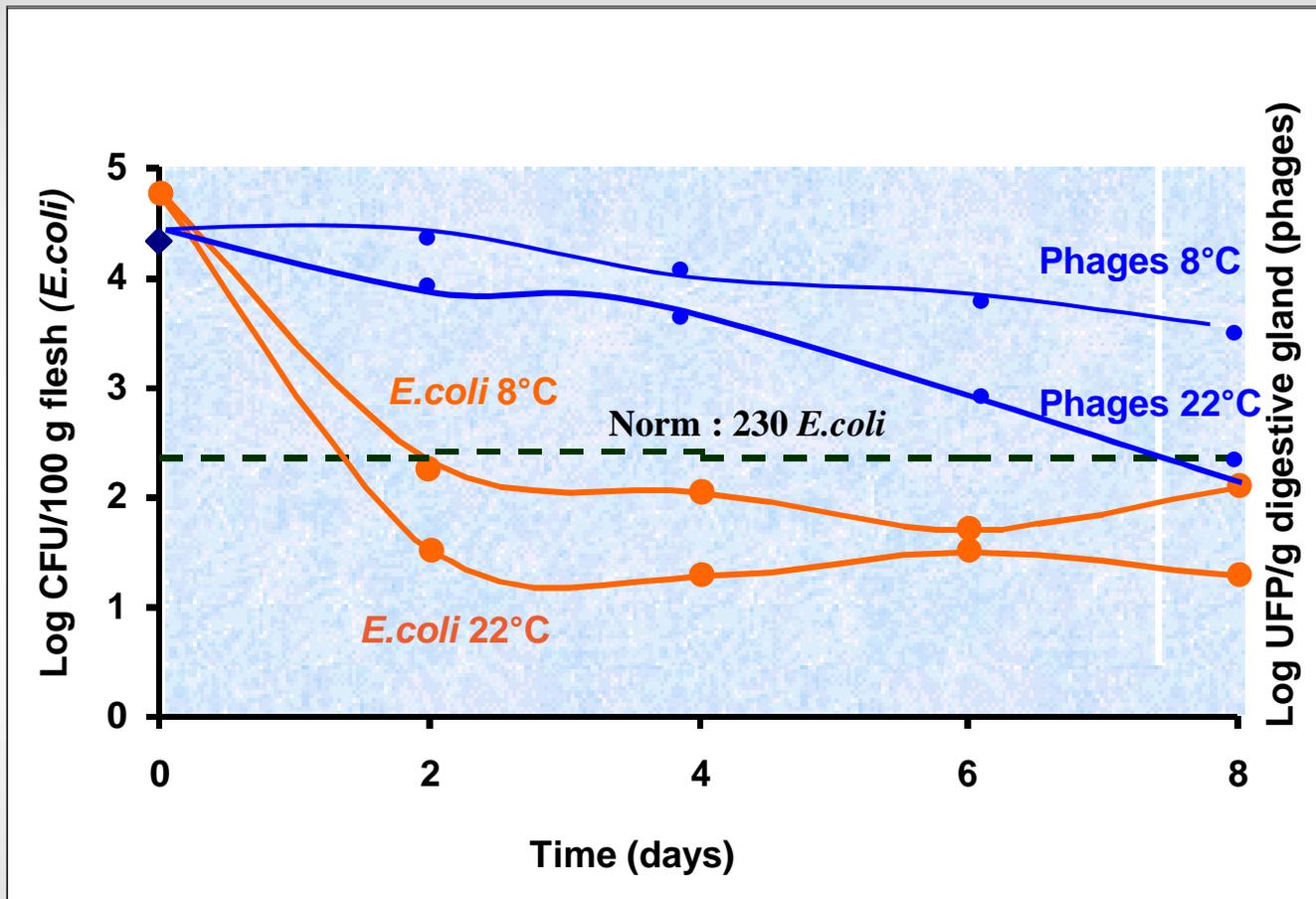


*O. edulis*  
8°C



*O. edulis*  
20°C





**Elimination of *E. coli* : > 99% in 2 days**  
**MS2: > 99% in 8 days**

## 2. CRITERIA INDICATING THE ACCEPTABILITY OF THE PROCESS

### 2.4. Live bivalve molluscs and live echinoderms, tunicates and gastropods

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<sup>1</sup> n= number of units comprising the sample; c= number of samples units giving values between m and M

<sup>2</sup> The most recent edition of the standard shall be used

<sup>3</sup> A pooled sample comprising a minimum of 10 individual animals

# EU survey on impact (EU funded)

## COMMERCIAL DEPURATION SITES (9 centres, 182 cycles)

- Recirculation and flow-through
- UV, Chlorination, Ozone, Chlorine Dioxide
- Depuration periods
  - Italy 24 h
  - Spain 42 – 48 h
  - UK 42 – 95 h



**Table 1.1** reduction of *E. coli* and FRNA bacteriophage levels during commercial depuration and assessment of compliance with existing and proposed microbiological standards

Centre	Country	No. of cycles tested	Percent reduction of <i>E. coli</i>	Percent current failing standard (230 <i>E.coli</i> /100g)	Percent reduction of bacteriophage	Percent failing proposed standard (>95% removal)
1	Italy	21	83	10	60	33
2	Italy	20	88	15	28	50
3	Italy	21	100	0	46	33
4	Spain	21	50	10	29	48
5	Spain	21	43	5	34	29
6	Spain	24	59	8	60	42
7	England	16	88	0	59	31
8	England	35	93	3	36	94
9	England	24	79	0	41	29
<b>TOTAL</b>		<b>182</b>	<b>75</b>	<b>6</b>	<b>43</b>	<b>47</b>

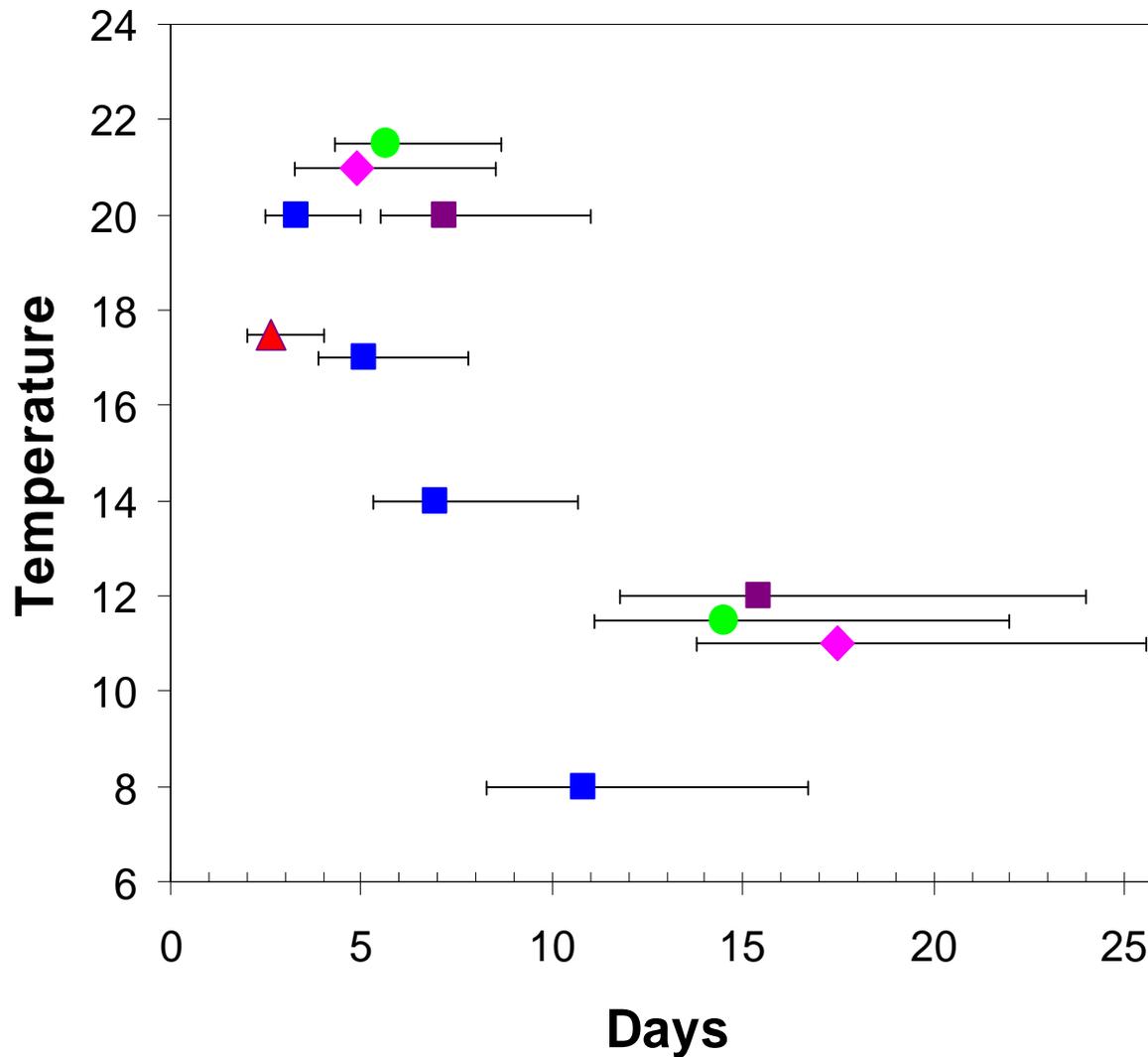
<sup>1</sup> Only using data from cycles with levels of *E. coli* or FRNA bacteriophage in pre-depuration samples

<sup>2</sup> post-depuration samples below the limit of detection treated as zero

<sup>3</sup> Proposed standard of 95% reduction of FRNA bacteriophage or to below 100 pfu 100g<sup>-1</sup> shellfish flesh

<sup>4</sup> current standard of <230 *E. coli* 100g<sup>-1</sup> of shellfish flesh

## Depuration Times for 95% (90%-99%) Removal of FRNA phage

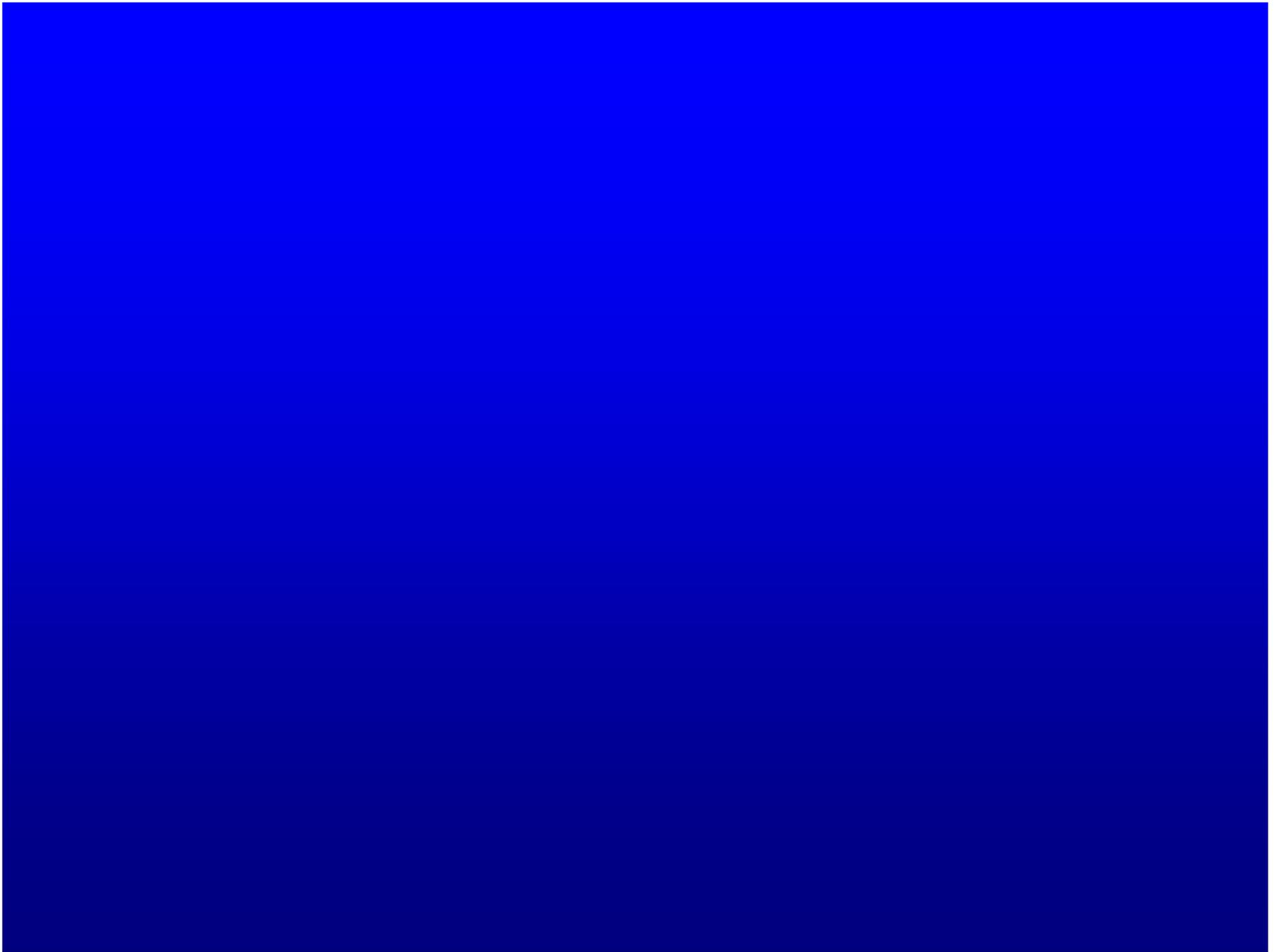


- C. gigas (UK)
- C. gigas (France)
- ▲ mussels (Spain)
- ◆ C. virginica (USA)
- M. mercenaria (USA)

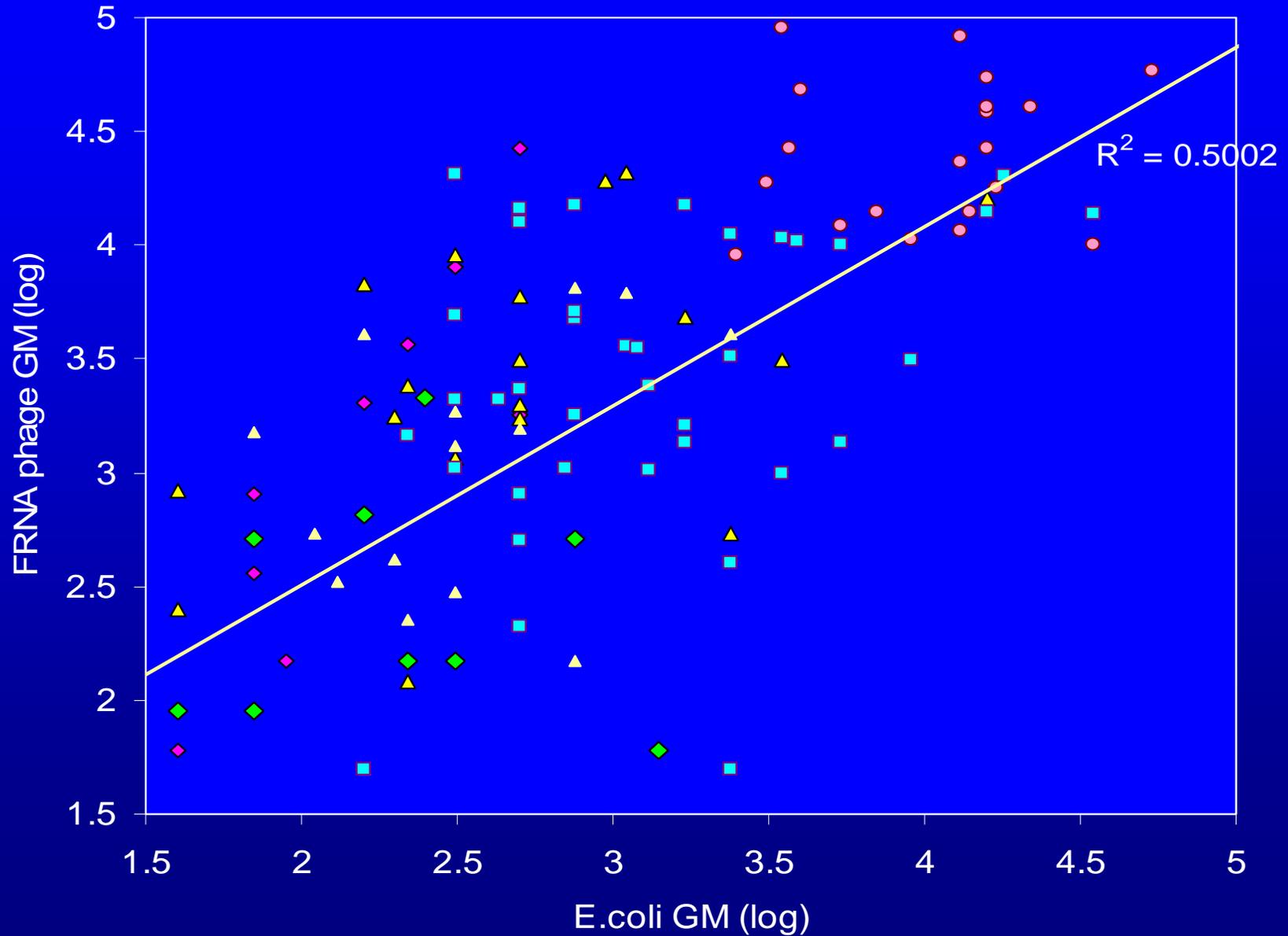
1. UK data : Dore et al., 1995, Appl. Environ. Microbiol., 61,p2830 and unpublished data (funded by UK Food Standards Agency)
2. French data : IFREMER, personal communication Dr S LeGuyader
3. Spanish data : Muniain-Mujika et al., 2002, Int.J.Food Microbiol., 77, p125
4. USA data : Burkhardt et al.,1995, Proceedings Second Int Conf Molluscan Shellfish Safety, Renne, p217

# Conclusion

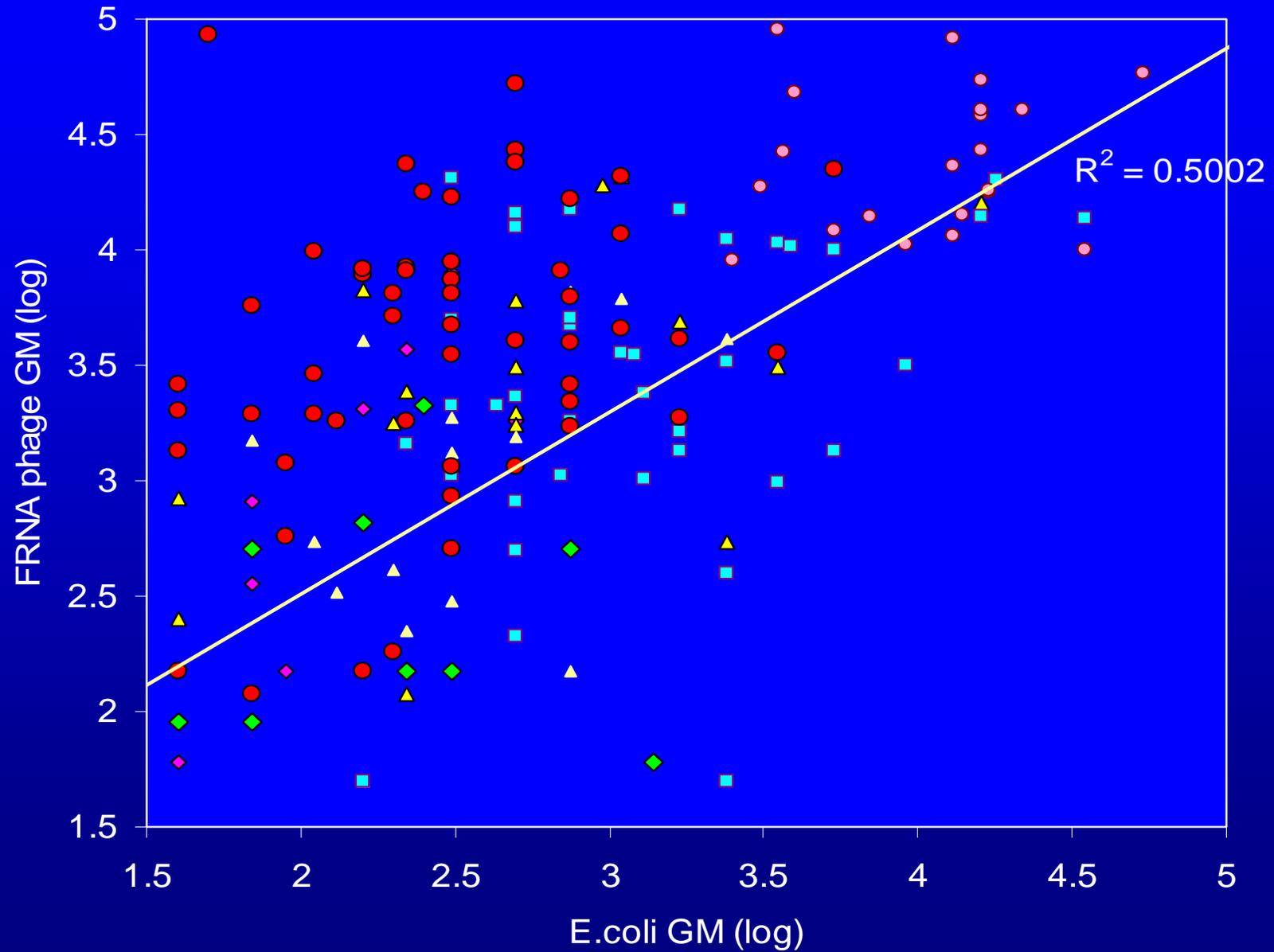
- MSC standard strongly opposed by EU industry
- A number of influential scientists argued that there was not a correlation between MSC and viral pathogens
- Scientific worries about possible vibrio proliferation with depuration at high temperatures
- Policy makers concluded introduction of MSC standard for depuration was impractical (since no clear way forward for processors)
- Consensus was to focus on pathogen (norovirus/HAV) detection



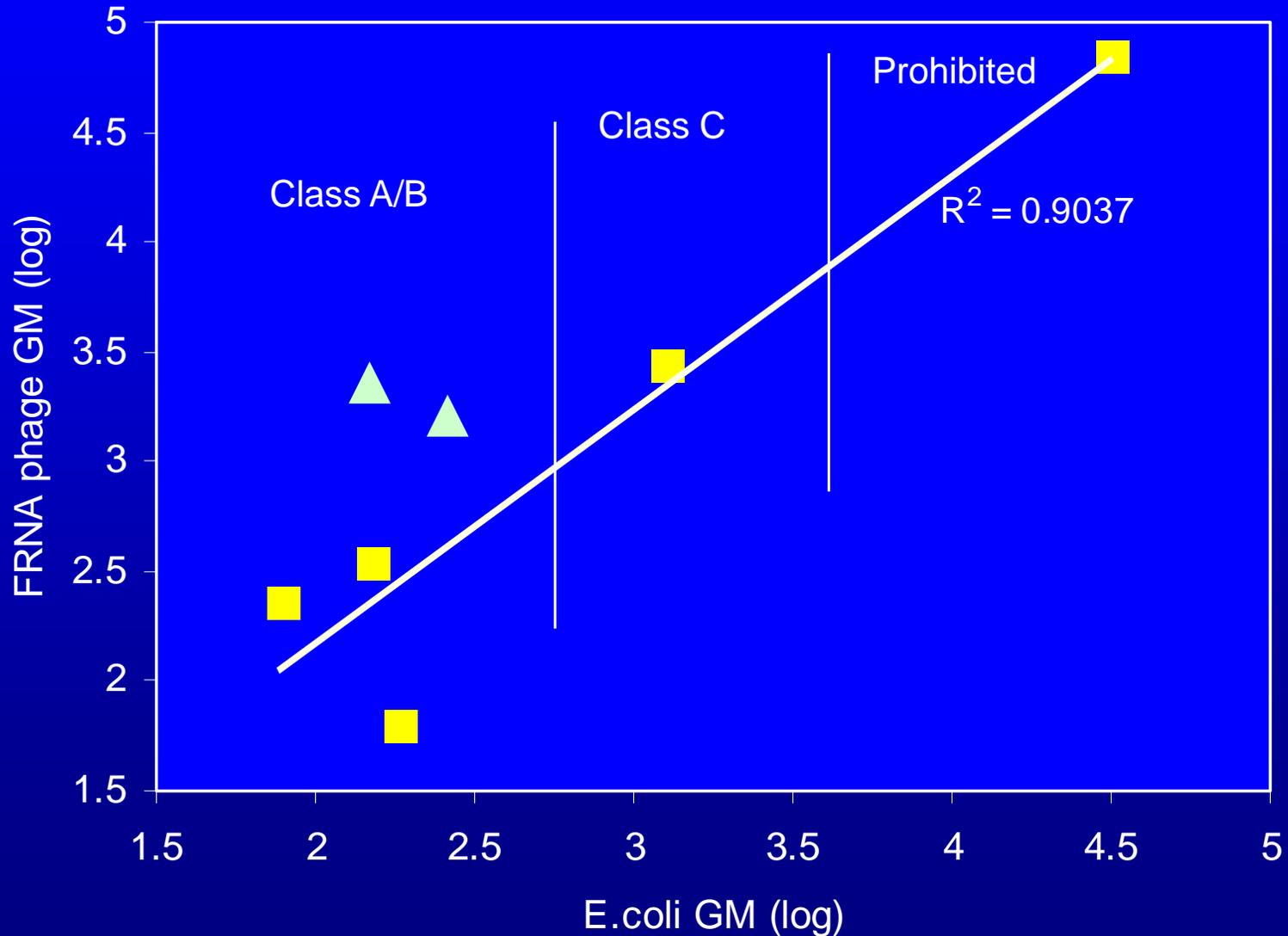
# E.coli vs FRNA phage in harvesting sites

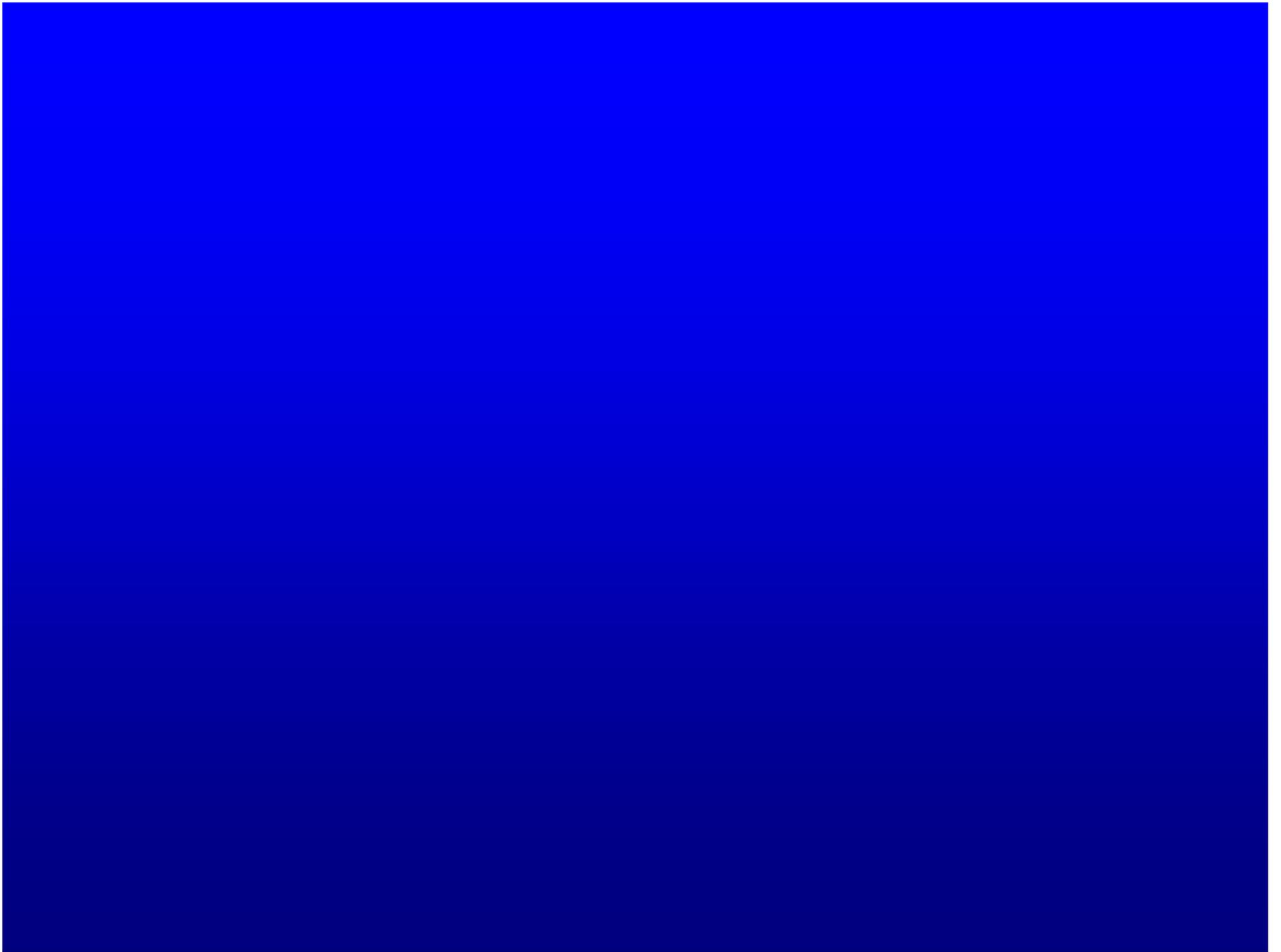


# E.coli vs FRNA phage in harvesting sites

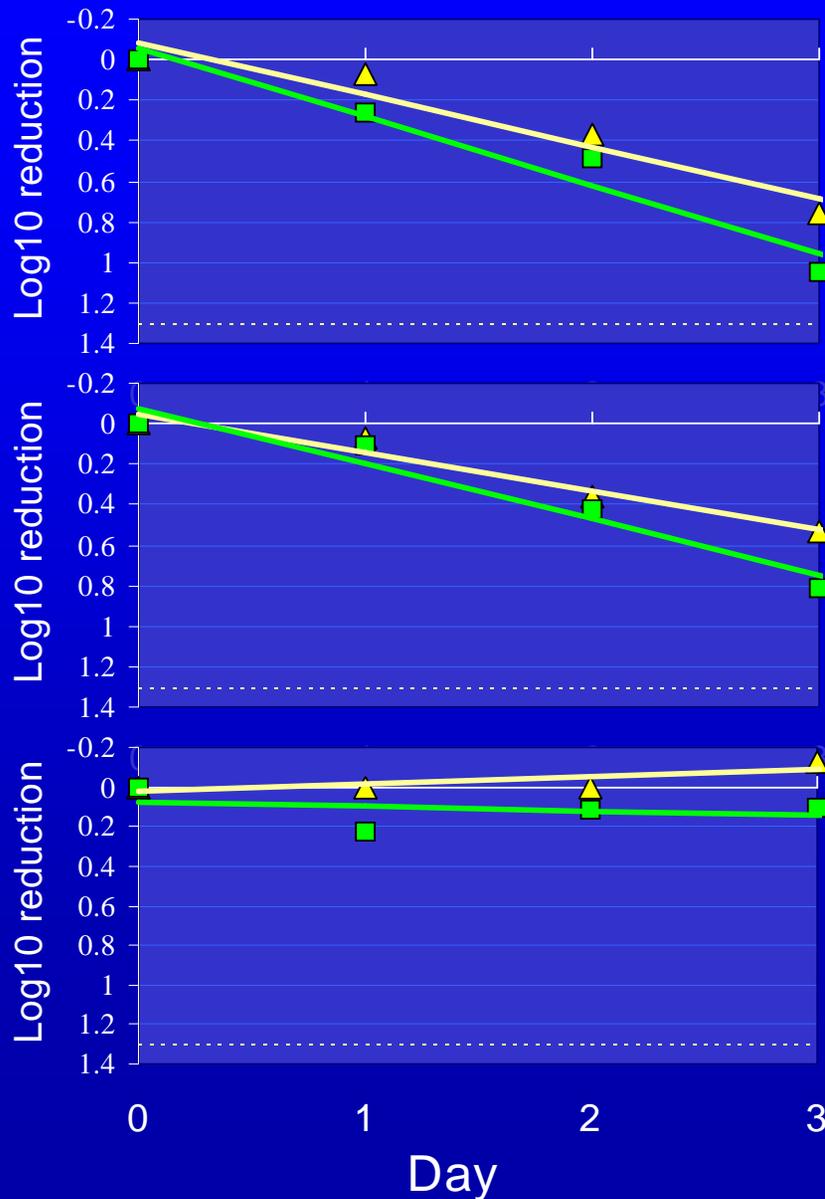


# Comparison of E.coli geomean vs FRNA bacteriophage geomean by site





# Depuration of FRNA phage ( $\blacktriangle$ ) vs NV ( $\blacksquare$ )



20°C

FRNA phage:  $y = 0.258x - 0.087$

NLV:  $y = 0.338x - 0.057$

17°C

FRNA phage:  $y = 0.1883x - 0.0432$

NLV:  $y = 0.274x - 0.076$

9°C

FRNA phage:  $y = -0.09x - 0.025$

NLV:  $y = 0.0197x + 0.0802$

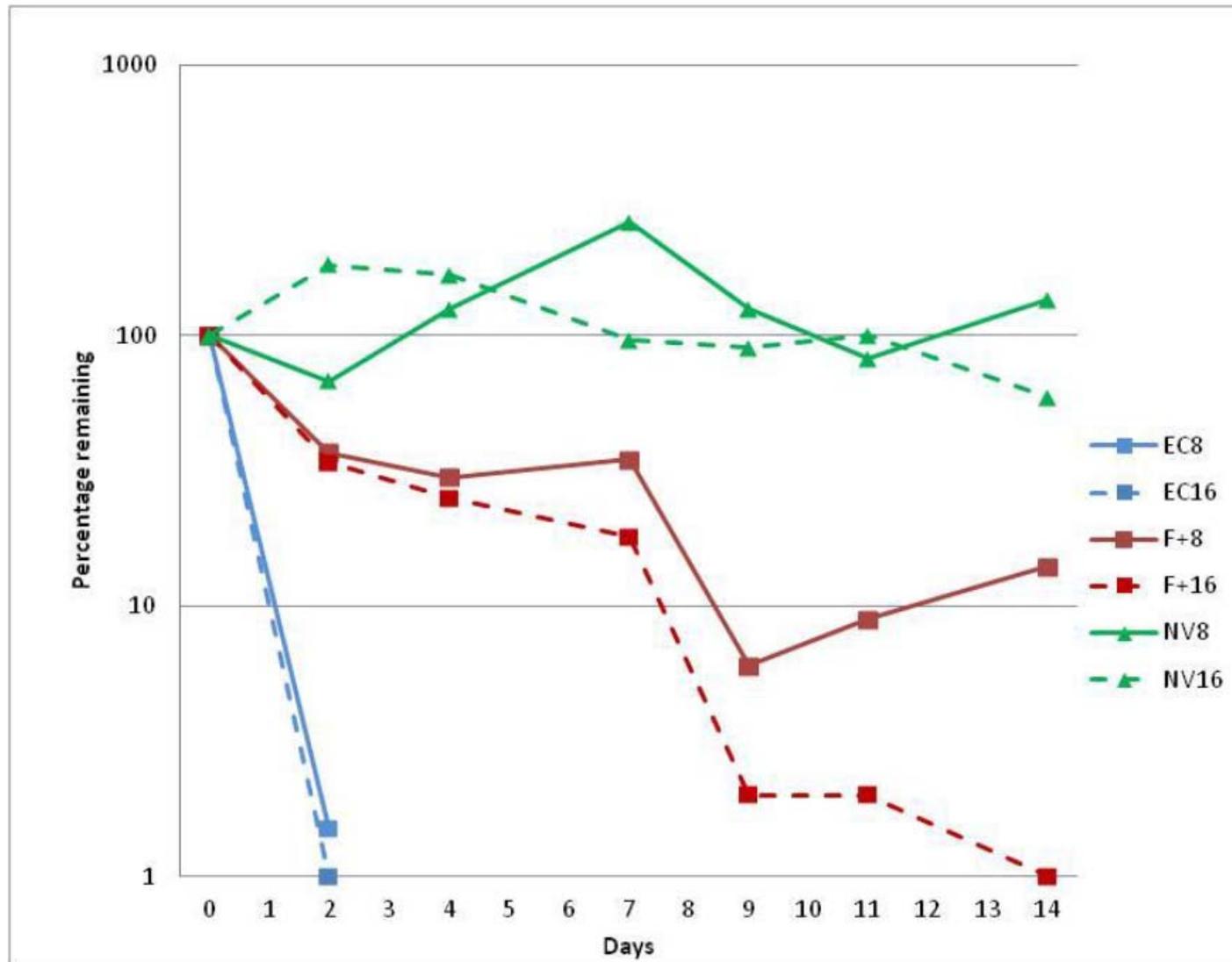


Figure 7. Comparison of *E.coli*, FRNA bacteriophage and norovirus reductions during depuration. Depuration conducted at 8°C (solid line) and 16°C (dashed line). EC = *E. coli*, F+ FRNA Bacteriophage, NV = norovirus. (Each data point calculated from the geometric mean of four replicates).

Neish 2013 (unpublished)

Cefas contract report C5224

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# Investigative trials on the purification of oysters to identify ways of reducing norovirus

Authors: Anna Neish

Issue date: April 22<sup>nd</sup> 2013

# E.coli clearance

## 1. SURVEY RESULTS

### SITE 1. ITALY, RECIRCULATION SYSTEM WITH OZONE DISINFECTION

Date	Pre/post	Species	Harvest Area		Depuration conditions			Results 100g-1		
			Temp (°C)	Salinity (ppt)	Time h	Temp (°C)	Salinity (ppt)	E. coli	phage	HAV (5-8g)
04:06:03	Pre	<i>T. philipinarum</i>	24	32				200	< 30	-
04:06:03	Post	<i>T. philipinarum</i>			24	16	30	< 180	< 30	
11:06:03	Pre	<i>T. philipinarum</i>	25	32				200	< 30	-
11:06:03	Post	<i>T. philipinarum</i>			24	15	30	< 180	< 30	
18:06:03	Pre	<i>M. galloprovincialis</i>	26	32				< 180	< 30	-
18:06:03	Post	<i>M. galloprovincialis</i>			24	16	29	< 180	< 30	
25:06:03	Pre	<i>M. galloprovincialis</i>	27	33				< 180	< 30	-
25:06:03	Post	<i>M. galloprovincialis</i>			24	16	30	< 180	< 30	
09:07:03	Pre	<i>T. philipinarum</i>	28	32				< 180	< 30	+
09:07:03	Post	<i>T. philipinarum</i>			24	16	30	< 180	< 30	+
16:07:03	Pre	<i>M. galloprovincialis</i>	27	30				< 180	< 30	-
16:07:03	Post	<i>M. galloprovincialis</i>			24	16	29	< 180	< 30	
10:09:03	Pre	<i>T. philipinarum</i>	21	27				1700	< 30	-
10:09:03	Post	<i>T. philipinarum</i>			24	15	30	< 180	< 30	
17:09:03	Pre	<i>T. philipinarum</i>	21	27				35000	1350	+
17:09:03	Post	<i>T. philipinarum</i>			24	15	30	680	960	+
24:09:03	Pre	<i>T. philipinarum</i>	24	26				< 180	< 30	-
24:09:03	Post	<i>T. philipinarum</i>			24	16	29	< 180	< 30	
01:10:03	Pre	<i>T. philipinarum</i>	20.5	31				< 180	540	-
01:10:03	Post	<i>T. philipinarum</i>			24	16.5	30	< 180	180	
08:10:03	Pre	<i>T. philipinarum</i>	19	27.5				< 180	60	-
08:10:03	Post	<i>T. philipinarum</i>			24	17	29	< 180	< 30	
15:10:03	Pre	<i>T. philipinarum</i>	19	29				200	60	-
15:10:03	Post	<i>T. philipinarum</i>			24	16.2	27	< 180	< 30	
22:10:03	Pre	<i>T. philipinarum</i>	15.5	31				< 180	90	-
22:10:03	Post	<i>T. philipinarum</i>			24	16.2	29.5	< 180	120	
29.10.03	Pre	<i>T. philipinarum</i>	13	31				780	2040	-
29.10.03	Post	<i>T. philipinarum</i>			24	16.1	28	< 180	30	
5.11.03	Pre	<i>T. philipinarum</i>	13.5	30				450	3090	-
5.11.03	Post	<i>T. philipinarum</i>			24	16	28	< 180	< 30	
12.11.03	Pre	<i>T. philipinarum</i>	13	30				1200	1890	-
12.11.03	Post	<i>T. philipinarum</i>			24	16	28	< 180	30	
19.11.03	Pre	<i>T. philippinarum</i>	12.5	22				< 180	2430	-
19.11.03	Post	<i>T. philippinarum</i>			24	16	29	< 180	1410	
26.11.03	Pre	<i>T. philippinarum</i>	13	21.5				2700	125280	-
26.11.03	Post	<i>T. philippinarum</i>			24	16	27	< 180	51420	
3.12.03	Pre	<i>T. philippinarum</i>	12.5	27				< 180	4380	-
3.12.03	Post	<i>T. philippinarum</i>			24	16	27	< 180	6030	
10.12.03	Pre	<i>T. philippinarum</i>	9	21				14000	15450	+
10.12.03	Post	<i>T. philippinarum</i>			24	16	26	2300	2280	-
17.12.03	Pre	<i>T. philippinarum</i>	9.5	27.5				780	73200	-
17.12.03	Post	<i>T. philippinarum</i>			24	16	29	< 180	436320	

# UK site 8

## SITE 8. CONTINUED

Date Tested	Pre/Post	Species	Harvest Area Temp (°C)	Depuration conditions			Results 100g <sup>-1</sup>	
				Time (h)	Temp (°C)	Salinity ppt	<i>E. coli</i>	phage
23.10.03	Pre	<i>M. edulis</i>	16		10	35	1850	660
24.10.03	Post	<i>M. edulis</i>		44	10	35	20	500
23.10.03	Pre	<i>C. gigas</i>	16		10	35	310	2805
24.10.03	Post	<i>C. gigas</i>		46	11	35	20	1767
29.10.03	Pre	<i>M. edulis</i>	14		10	34	1500	900
31.10.03	Post	<i>M. edulis</i>		43	10	34	20	540
29.10.03	Pre	<i>C. gigas</i>	14		10	35	625	1110
31.10.03	Post	<i>C. gigas</i>		43	12	35	20	645
12:11:03	Pre	<i>M. edulis</i>	16		14	35	1850	7095
14:11:03	Post	<i>M. edulis</i>		48	15	35	20	4020
12:11:03	Pre	<i>C. gigas</i>	16		14	35	405	5235
14:11:03	Post	<i>C. gigas</i>		53	15	35	<20	2175
26.11.03	Pre	<i>M. edulis</i>	12		13	35	7250	4500
02.12.03	Post	<i>M. edulis</i>		95	11	35	20	660
26.11.03	Pre	<i>S. solida</i>	12		13	35	<20	2700
02.12.03	Post	<i>S. solida</i>		95	12	35	45	930
11.12.03	Pre	<i>M. edulis</i>	12		12	29	>18000	3150
12.12.03	Post	<i>M. edulis</i>		45	13.5	29	<20	1069
10.12.03	Pre	<i>S. solida</i>	12		11	30	1415	2355
11.12.03	Post	<i>S. solida</i>		48	13	30	<20	1767

**Table 1.2** Elimination of FRNA bacteriophage during temperature controlled depuration experiments.

Trial	Species	Initial conc <sup>n</sup> of FRNA phage pfu 100g <sup>-1</sup>	Temp ±1°C	Time to 95% reduction or percent reduction after 5 days	Days to 10% mortality
1 <sup>1</sup>	<i>M. edulis</i>	3184	10	5 days	8
			20	4 days	8
2 <sup>2</sup>	<i>M. edulis</i>	2205	10	83%	3
			20	4 days	3
3	Clams	57,690	10	77%	9
			20	4 days	7
4	Clams	72,400	10	72%	12
			15	81%	12
5	<i>M. edulis</i>	6,300	10	74%	4
			15	88%	3
6 <sup>3</sup>	Clams	18,525	10	65%	ND
			18	61%	ND

<sup>1</sup> approximately 15% of mussels had died by day 5 of depuration at 20°C.

<sup>2</sup> approximately 10% of mussels had died by day 5 of depuration at 20°C

<sup>3</sup> approximately 33% of clams had died by day 5 of depuration at 18°C

## Estimated reduction in FRNA phage (pfu/100g) per day of depuration

### Days depuration at 20°C

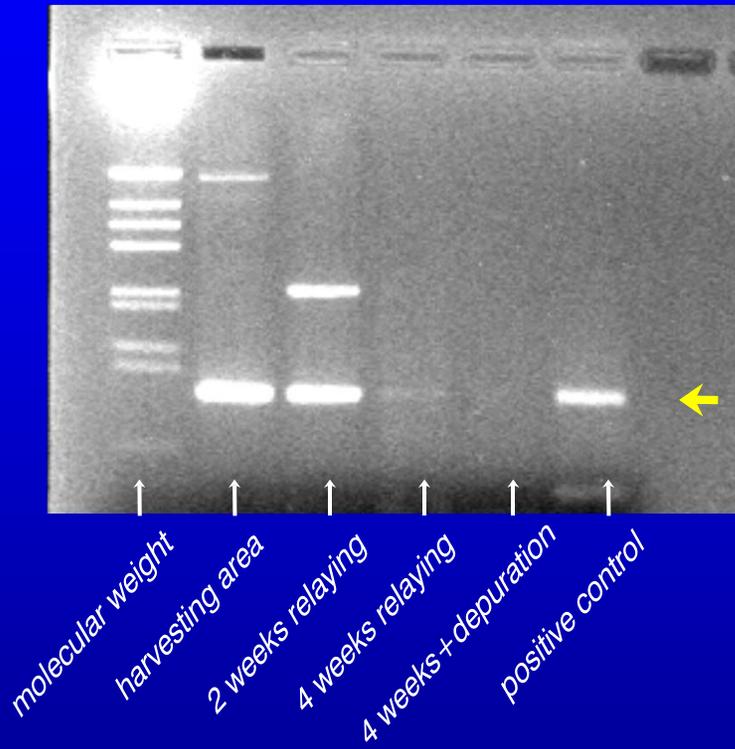
0	1	2	3	4	5	6	7	8	9	10	Days to <30pfu/100g
100	40	16	6	3							1.3
1000	398	158	63	25	10	4	1.6				3.8
10000	3981	1580	630	251	100	40	16	6			6.3
20000	7943	3162	1258	501	200	79	32	13	5		7.1
50000	19952	7943	3162	1258	501	200	79	32	13	5	8.1

### Days depuration at 8°C

0	1	2	3	4	5	6	7	8	9	10	Days to <30pfu/100g
100	72	53	38	28	20	15	11				3.7
1000	720	525	380	275	200	145	105	76	55	40	10.9
10000	7200	5250	3800	2750	2000	1450	1050	760	550	400	18.0
20000	14454	10471	7585	5495	3981	2884	2089	1513	1096	794	20.2
50000	36307	26302	19054	13804	10000	7244	5248	3801	2754	1995	23.0

# Removal of Norovirus in commercial depuration and relaying (UK)

## Relaying



## Depuration

