

# When things go wrong

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September 18, 2017

- Purpose of bioassays
- Bioassay Test Acceptability Criteria
- Case Study 1: Reference Toxicant Test Failure
- Case Study 2: Control Failure
- Case Study 3: Toxicant in sample?!

# Purpose of bioassays



- Assess toxicity of test sample as a whole on live organisms
- Required by discharge permits
  - Frequency
  - Sampling location
  - Specific species
  - Method
  - Additional supporting analysis during course of bioassay

# Test Acceptability Criteria



- General Criteria
  - Concurrent reference toxicant test
    - Reference toxicant is of known toxicity
    - Expose organism to concentration series of reference toxicant in control water matrix
    - Determine LC50, concentration of toxicant that kills half of sample population, statistically from concentration series exposure data
    - Determine sensitivity of the test organisms
    - Demonstrates laboratory capability
    - Assess inter and intra laboratory comparability

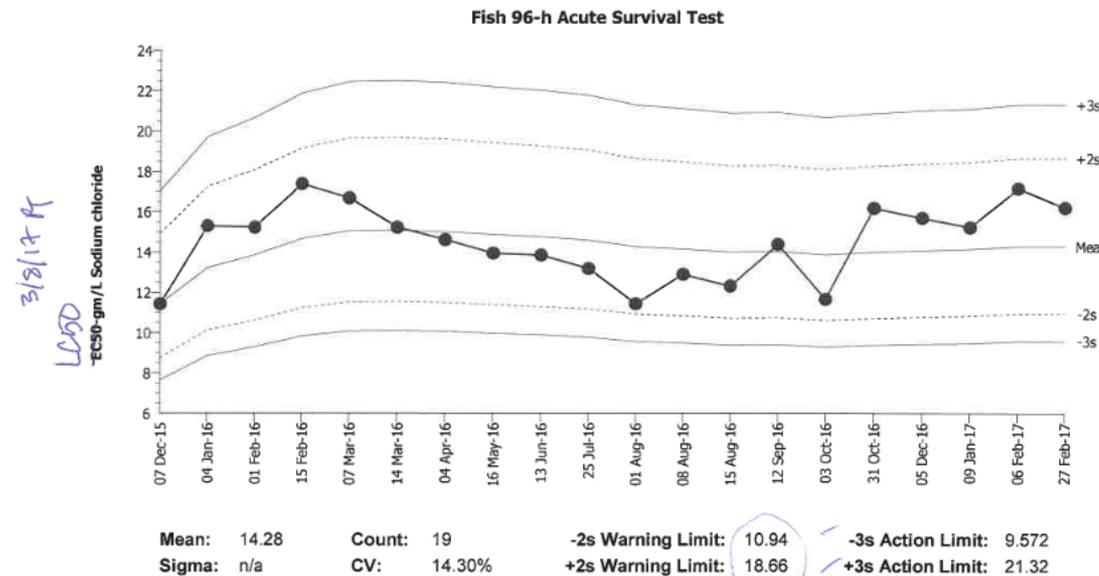
# Test Acceptability Criteria



- General Criteria

- Maintain control chart of the most recent 20 LC50 with mean and control limits (defined as  $\pm 2SD$  from mean of the 20 data points)

Fish 96-h Acute Survival Test		East Bay Municipal Utility District	
Test Type: Survival (96h)	Organism: <i>Oncorhynchus mykiss</i> (Rainbow Tro)	Material: Sodium chloride	
Protocol: EPA/600/4-90/027F (1991)	Endpoint: 96h Survival Rate	Source: Reference Toxicant-REF	



# Test Acceptability Criteria



- Test Specific Criteria
  - Acceptable survival/ growth in Control
  - Precision among replicates
    - Percent Minimum Significant Difference (PMSD)

# Case Study 1: Ref. Tox. Test Failure



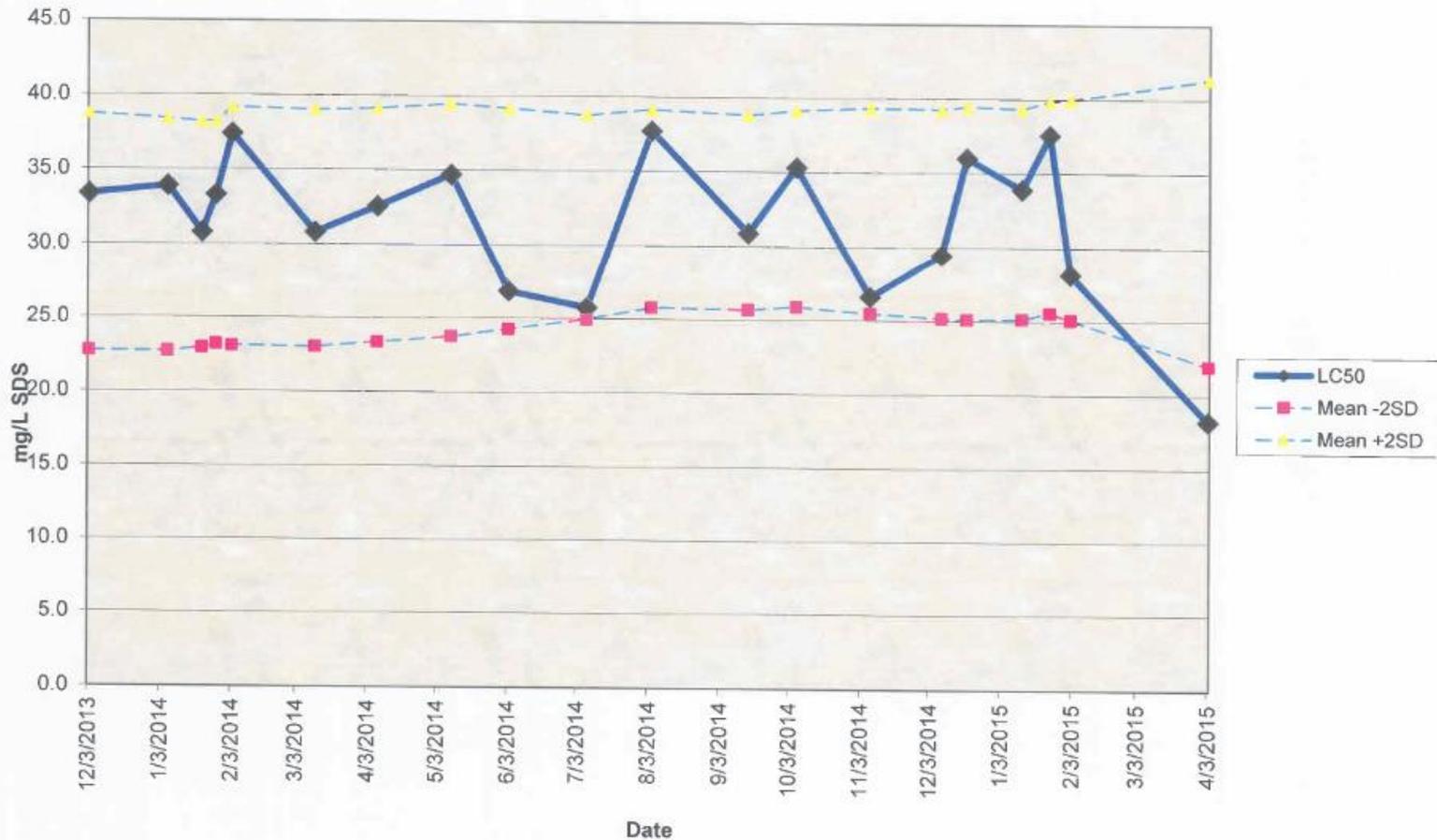
## Acute Toxicity Bioassay using Rainbow Trout

- Issue -
  - Reference Toxicant test resulted in LC50 below lower control limit
  - Test organism exhibited hyper sensitivity, poor health?
  - Control demonstrated 100% survival!
  - Samples also demonstrated 100% survival!!

# Case Study 1: Ref. Tox. Test Failure



96 Hour Control Chart -- Rainbow Trout  
Toxicant: Sodium Dodecylsulfate



# Case Study 1: Ref. Tox. Test Failure



REFERENCE TOXICANT TEST FOR FISH CULTURES																			
					TEST DURATION (HOURS)					TEST ID NUMBER					WG/197953/WG/18024				
					24 48 72 96					REFERENCE TOXICANT LOT #					M9655				
START -- DATE / TIME					3/30/15 @ 1105					DILUTION WATER *					01				
END -- DATE / TIME					4/3/15 @ 1105					*Dilution Water Code					water bath # 2				
TOXICANT					SODIUM DODECYL SULFATE					01- DeCl <sub>2</sub> Tap					02 - 80% DeCl <sub>2</sub> Tap & 20% Evian				
SPECIES					RAINBOW TROUT					03 - Arrowhead & 2ppt forty fathoms					04 - DeCl <sub>2</sub> Tap & metabisulfite				
BATCH #					444 O.M.					CONDITIONS									
HATCH DATE					3/10/15					STATIC									
SUPPLIED BY					THOMAS														

CONC (ppm)	# LIVE					DO (mg/l)					pH					TEMP (C)				
	0	24	48	72	96	0	24	48	72	96	0	24	48	72	96	0	24	48	72	96
0	10	10	10	10	10	9.09	9.46	9.71	9.75	10.28	7.62	7.38	7.38	7.28	7.36	12.0	11.7	11.3	11.3	11.3
15	10	10	10	10	9	9.95	8.98	9.31	8.63	10.56	7.62	7.29	7.27	7.07	6.82	12.0	11.6	11.3	11.3	11.2
20	10	10	10	10	3	9.00	9.39	9.35	8.92	10.64	7.65	7.27	7.21	7.12	6.65	11.8	11.5	11.3	11.3	11.2
25	10	10	10	10	0	9.29	9.54	9.45	9.25	11.60	7.65	7.24	7.20	7.20	6.62	11.6	11.5	11.3	11.1	11.2
30	10	10	9	7	0	9.06	9.19	9.52	9.14	8.26	7.66	7.25	7.16	7.17	6.67	11.7	11.5	11.3	11.2	11.0
35	10	10	9	2	0	9.06	9.27	9.50	8.80	7.28	7.67	7.27	7.16	7.21	6.54	11.7	11.5	11.3	11.2	11.0
40	10	10	3	2	0	9.14	9.30	9.61	9.35	8.93	7.67	7.28	7.16	7.22	6.67	11.7	11.5	11.3	11.2	11.1

\* Continuous Chart Recorder :

ANALYST INITIALS	cat																		
------------------	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----

TOXICANT			CONTROL			ANALYST		LC50 = 18.25 mg/l SDS			
ALK	HARD	COND	ALK	HARD	COND	cat					
18	24	114	17	22	101						

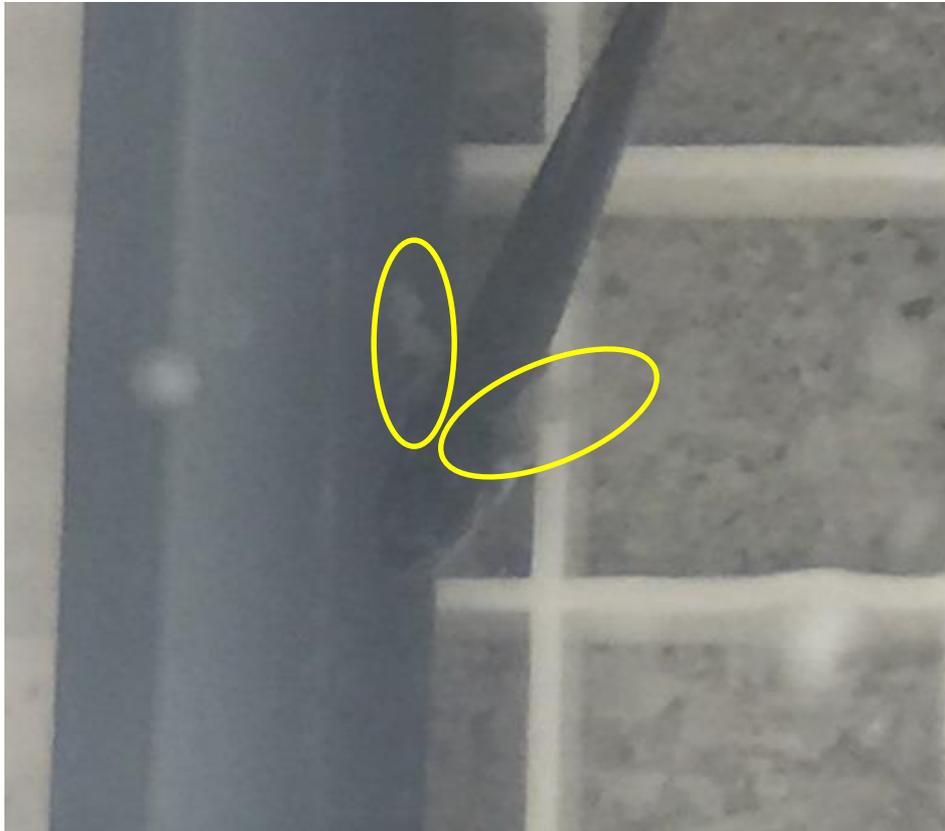
Note: Aeration to all tanks for duration of the test @ < 100 bubbles/min.      Note: Generate LC 50 from ToxCalc & Control Chart from Excel

Add 12, 16, 20, 24, 28, and 32mL of 10,000ppm (5g in 500mL) SDS stock solution respectively into each tank containing ~8L of Dilution H<sub>2</sub>O.

W:\LAB\wppublic\MICRO\Worksheets\Toxicology\SD-1 BIORef Tox Trout\3/24/2015 \* Started O<sub>2</sub> @ 1500 isolated pump Aeration because O<sub>2</sub> trend suggested DO may go below 6.0 overnight. cat 4/2/15

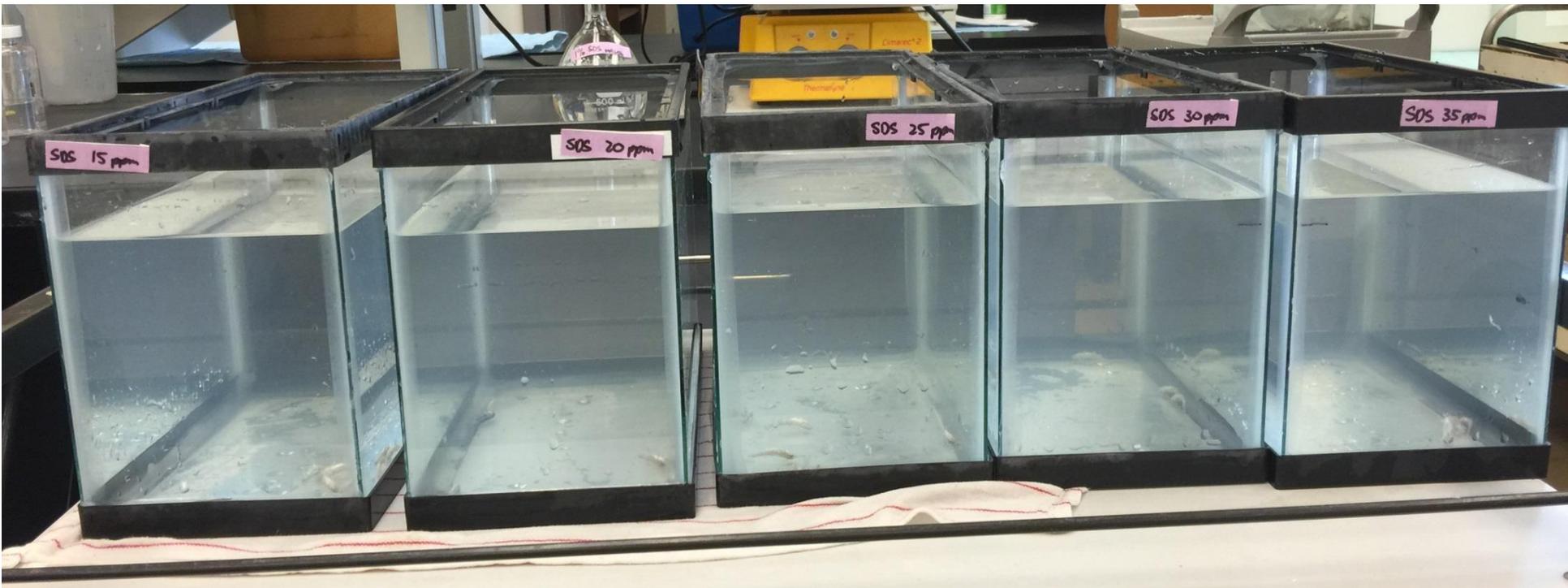
# Case Study 1: Ref. Tox. Test Failure

Growth surrounding the gill is evident



# Case Study 1: Ref. Tox. Test Failure

Turbidity observed in ALL tanks



# Case Study 1: Ref. Tox. Test Failure

- Identifying the bacteria
  - Took aliquots of 35 ppm SDS fish tank
  - Filtered through 0.45 $\mu$ m filter grow on mEndo
  - Picked colonies of different morphology for identification by Analytical Profile Index



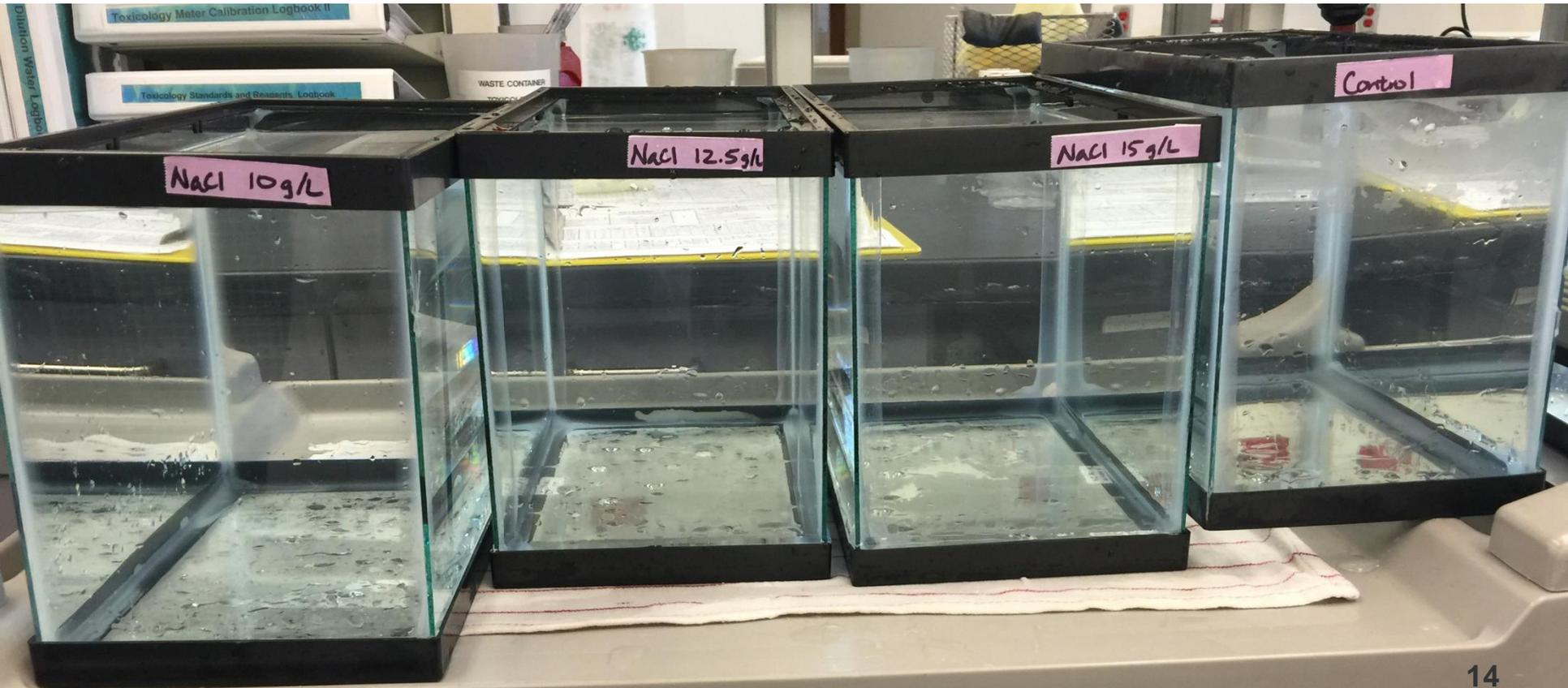
# Case Study 1: Ref. Tox. Test Failure



- All identified to be *Aeromonas hydrophila*
  - Can be eliminated using sodium hypochlorite
- Unable to completely eradicate *Aeromonas hydrophila* as it is commonly found in fish
- Use of alternative toxicant
  - NaCl
  - Concentration series: 10, 12.5, 15, 17.5 & 20 g/L

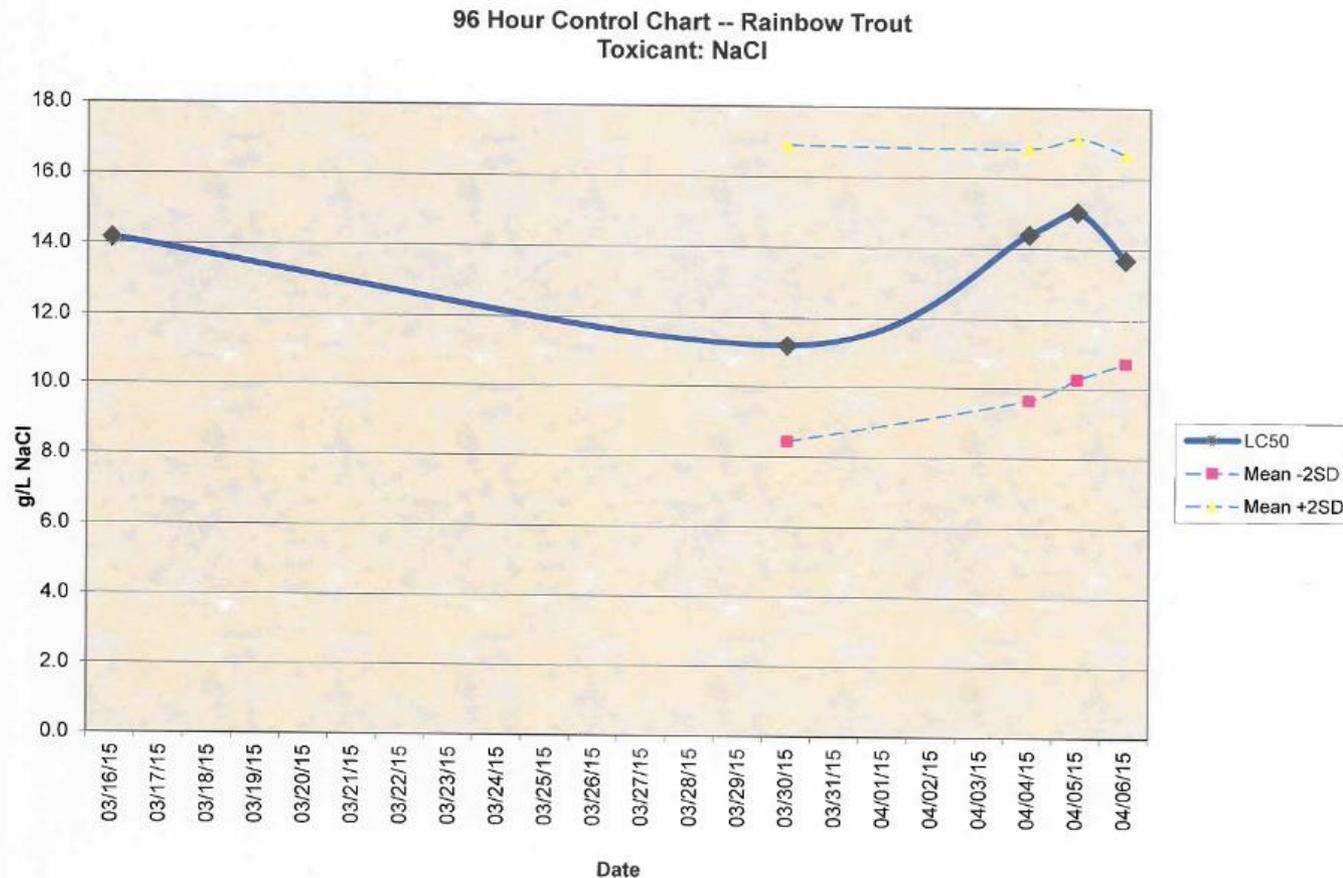
# Case Study 1: Ref. Tox. Test Failure

- Turbidity and DO-drop not observed in NaCl Ref Tox Test



# Case Study 1: Ref. Tox. Test Failure

- Control chart established



# Case Study 1: Ref. Tox. Test Failure



- What we learned
  - Review data
    - Keep notes/ record observations
  - Reach out to those with relevant expertise
  - Prepare for changes

# Case Study 2: When control failed

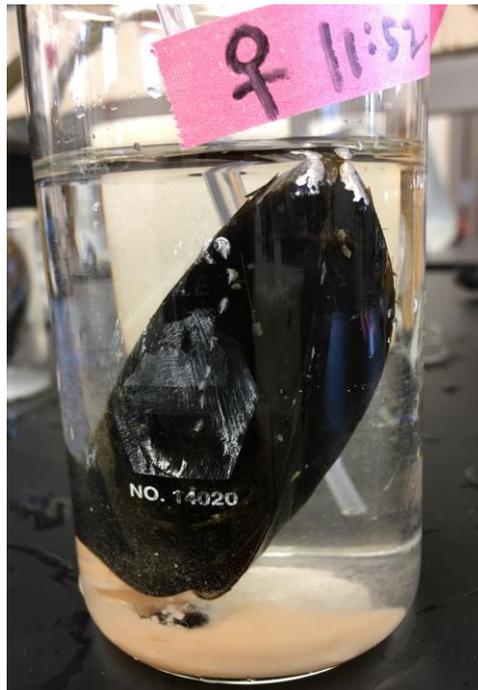
## Chronic Toxicity Bioassay using *Mytilus* mussels



# Case Study 2: When control failed

## Chronic Toxicity Bioassay using Mytilus mussels

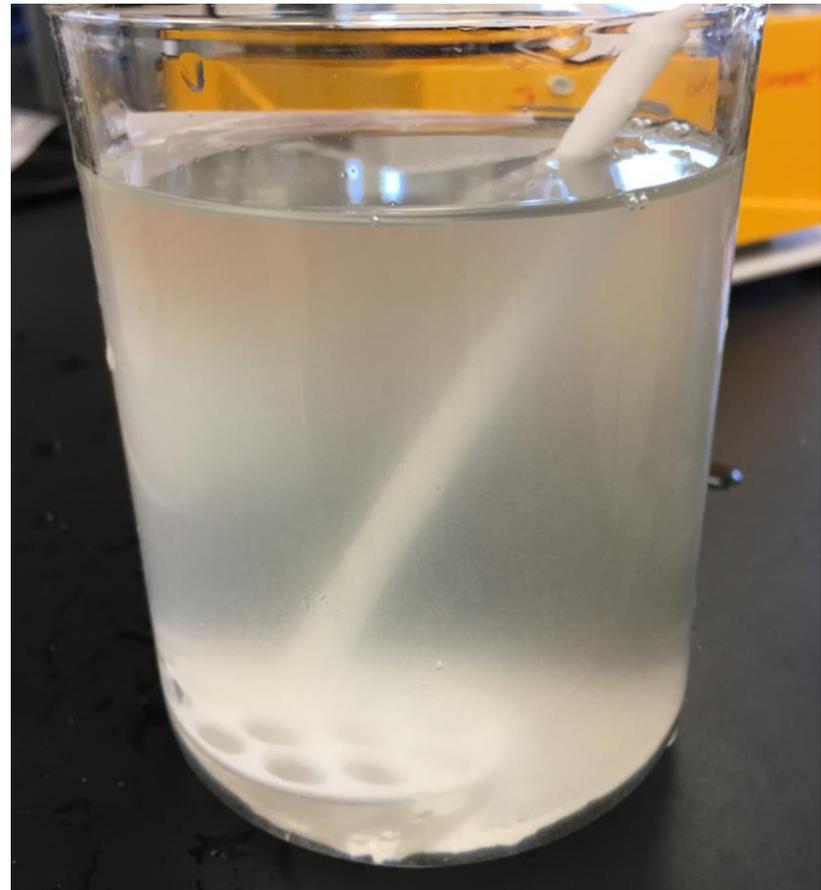
- Induce spawning of Mytilus mussels



# Case Study 2: When control failed

## Chronic Toxicity Bioassay using *Mytilus* mussels

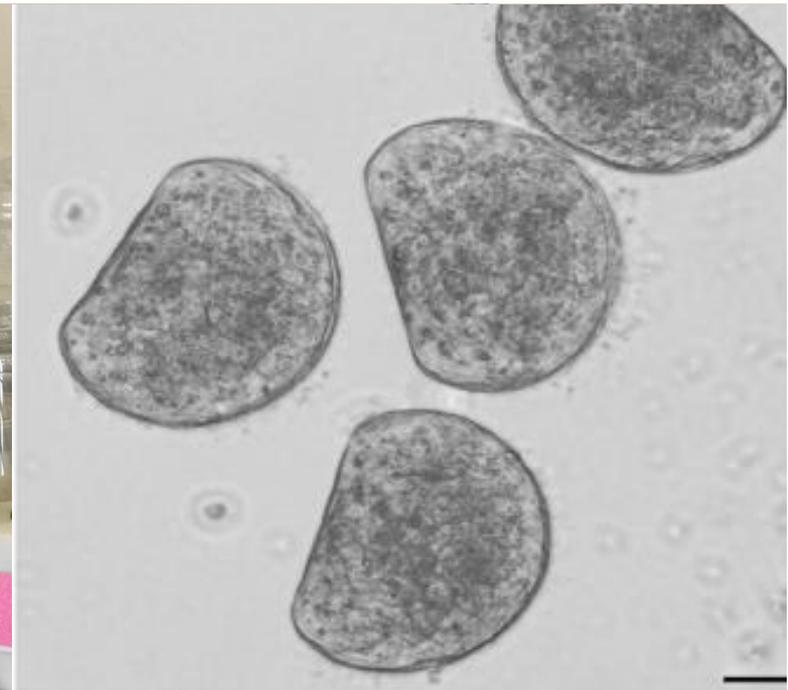
- Fertilization



# Case Study 2: When control failed

## Chronic Toxicity Bioassay using *Mytilus* mussels

- Endpoint: survival and shell development

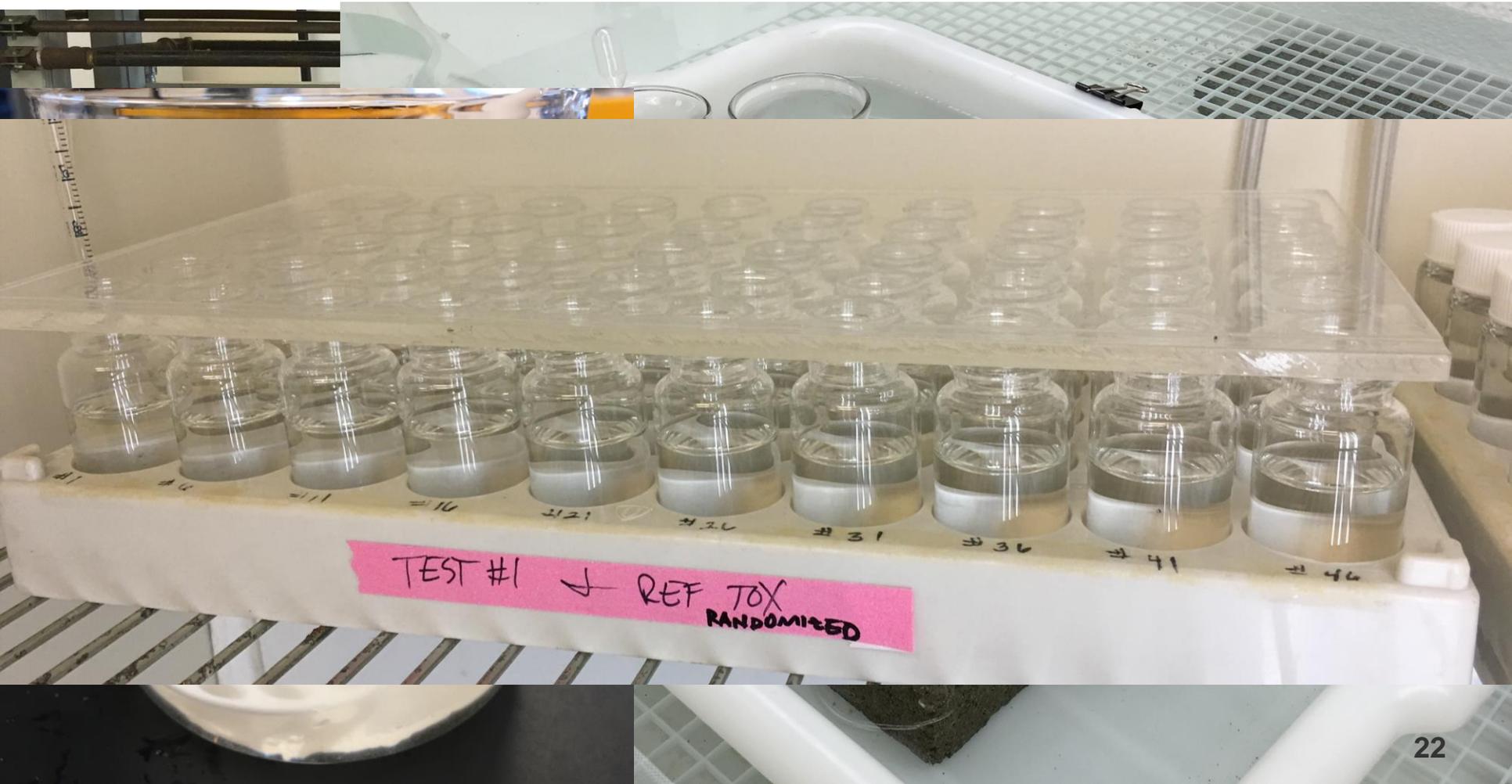


## Chronic Toxicity Bioassay using *Mytilus* mussels

- Issue -
  - Reference toxicant test yielded EC50 within control limits ✓
  - Control failed to meet acceptance criteria of 90% normal shell development in surviving organisms ✗

# Case Study 2: When control failed

All effort wasted!!



# Case Study 2: When control failed



## Root Cause Analysis -

### – Embryos condition?

- Reference toxicant test yielded EC50 within control limits

### – Sea water?

- Met annual control water quality criteria
  - No detectable organics
    - » organochlorine pesticides & PCBs
  - No detectable metals
    - » Ag, Al, As, Cd, Co, Cr, Cu, Fe, Hg, Pb, Ni & Zn

# Case Study 2: When control failed



## Root Cause Analysis -

### – Filter?

- Charcoal filter and a 1.0  $\mu\text{m}$  filter
- Compare before and after filter for all the parameters monitored in annual water check

# Case Study 2: When control failed



- Unfiltered seawater from Bodega Marine Laboratory

Sample Comments: Unfiltered seawater from Bodega Marine Laboratory.

## Method Reference

Parameter	Qualifier	Result	Units	Dilution	MDL
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Method: EPA 200.7 - Rev. 4.4, ICP Scan

### TARGET ANALYTES

SILVER	U	27.5	ug/L	5.5	27.5
ALUMINUM	U	71.5	ug/L	5.5	71.5
CADMIUM	U	11.0	ug/L	5.5	11
COBALT	U	3.30	ug/L	5.5	3.3
CHROMIUM	U	16.5	ug/L	5.5	16.5
COPPER	U	38.5	ug/L	5.5	38.5
IRON	U	22.0	ug/L	5.5	22
NICKEL	U	16.5	ug/L	5.5	16.5
LEAD	U	27.5	ug/L	5.5	27.5
ZINC	U	16.5	ug/L	5.5	16.5

Run ID: R255808 / Work Group No.: WG195305

# Case Study 2: When control failed



- Filtered seawater from Bodega Marine Laboratory

Sample Comments: Filtered seawater from Bodega Marine Laboratory.

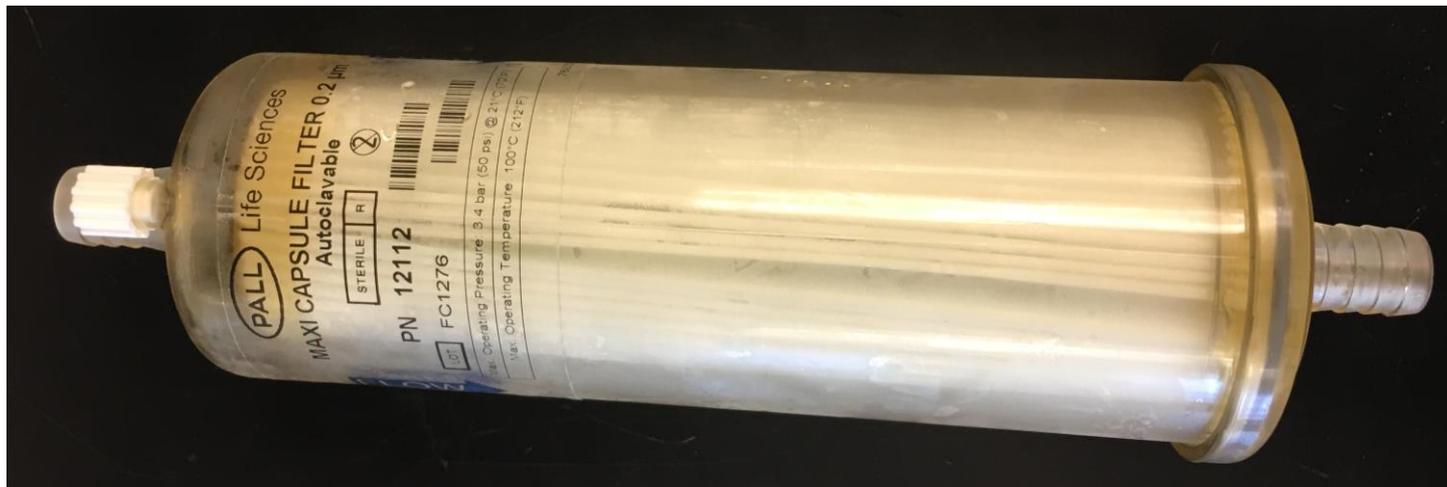
## Method Reference

Parameter	Qualifier	Result	Units	Dilution	MDL
Method: EPA 200.7 - Rev. 4.4, ICP Scan					
TARGET ANALYTES					
SILVER	U	27.5	ug/L	5.5	27.5
ALUMINUM	U	71.5	ug/L	5.5	71.5
CADMIUM	U	11.0	ug/L	5.5	11
COBALT	U	3.30	ug/L	5.5	3.3
CHROMIUM	U	16.5	ug/L	5.5	16.5
COPPER	U	38.5	ug/L	5.5	38.5
IRON		65.5	ug/L	5.5	22
NICKEL	U	16.5	ug/L	5.5	16.5
LEAD	U	27.5	ug/L	5.5	27.5
ZINC	U	16.5	ug/L	5.5	16.5

Run ID: R255808 / Work Group No.: WG195305

# Case Study 2: When control failed

- What we learned
  - Filtration apparatus contributed to detectable amount of iron to filtered seawater
  - Switched to PALL Maxi Capsule for filtered seawater preparation



# Case Study 2: When control failed



- Corrective Action
  - Monitor quality of control water in addition to the source of control water
- With *new* filtration apparatus, control of subsequent tests consistently met Test Acceptance Criteria of 90% normal development:  
93.5%, 94.5% and 91.3% in subsequent tests

# Case 3: Toxicant in Sample?



## Acute Toxicity Test with Rainbow Trout

- Issue
  - Reference Toxicant Test exhibited acceptable LC50 within control limits ✓
  - Control 100% survival ✓
  - Sample 22% survival ✗

# Case 3: Toxicant in Sample?



## What went wrong? Review data

### Daily Measurements

#### Test Effluent Tanks

Time	Survival	Observations	Temp	pH	DO	Conductivity	Initials
	(# alive)	(code)	(°C)	(units)	(ppm)	(uS/cm)	
	Tank1 / Tank2						
Day 0 1250	20 / 20	1 / 1	12.3 / 12.3	6.92 / 6.91	10.34 / 10.27	1450 / 1451	CCT
	1	1	1	1	1	1	

Comments \_\_\_\_\_ Fish Fed? (Y/N)

Day 1 0900	20 / 20	1 / 1	11.9 / 12.0	6.88 / 6.86	9.25 / 8.91	1785 / 1783	CCT
1500	20 / 20	1 / 1	12.1 / 12.1	6.94 / 6.93	9.01 / 8.93	1870 / 1873	CCT

Comments Removed fish waste Fish Fed? (Y/N)

Day 2 0810	20 / 20	1 / 1	11.9 / 12.0	6.74 / 6.73	8.96 / 8.92	1768 / 1777	CCT
1500	20 / 20	1 / 1	11.9 / 12.0	6.75 / 6.74	8.57 / 8.74	1080 / 1682	CCT

Comments \_\_\_\_\_ Fish Fed? (Y/N)

Day 3 1040	15 / 17	11,13,4,1 / 11,13,4,1	12.1 / 12.2	6.73 / 6.70	7.95 / 7.75	1812 / 2025	JL
1530	12 / 13	2,4,10,11 / 13,11,10,6,4,2	12.0 / 12.3	6.80 / 6.81	7.86 / 7.73	1860 / 1876	JL

Comments significant amt of settleable solids in water Fish Fed? (Y/N)

Day 4 0845	5 / 7	13,4,2,6,11 / 12,4,2,6	12.1 / 12.3	6.80 / 6.84	7.36 / 7.47	1965 / 1982	JL
1335	3 / 6	1	1	1	1	1	

Comments significant amt of settleable solids in water Fish Fed? (Y/N)

# Case 3: Toxicant in Sample?



## What went wrong? Review data

### Daily Measurements

Effluent Mixing Tank - Dissolved CO<sub>2</sub>

Day	Time	Alkalinity (mg/L)	pH (Mix Tank)	DO (mg/L)	Flow Rate (mL/min)	pH Set Point	pH (pH Adjust Chamber)	Initials
Day 0	1300	132	6.95	10.27	44	6.005	6.05	CET
Comments								
Day 1	0815	148	7.00	10.68	44	6.005	6.08	CET
	1512	157	6.97	10.51	44	6.005	6.06	CET
Comments								
Day 2	0820	145	6.79	10.44	43/47*	6.005	6.04	CET
	1520	154	6.76	10.17	47	6.005	6.06	CET
Comments * increased slightly to 47 @ 0825 cor								
Day 3	1030	165	6.97	10.06	47/45	6.005	6.00	Jew
	1510	110	6.84	10.59	44	5.800	5.81	Jew
Comments lowered pH setp to 5.800 to enhance CO <sub>2</sub> removal. Jew 2/4/16 lowered pump rate for effluent. Jew 2/4/16								
Day 4	0828	115	6.82	11.02	44	5.800	5.82	Jew
Comments								

# Case 3: Toxicant in Sample?



- Dissolved CO<sub>2</sub> resulting in mortality?

## Appendix C

Table 2: Dissolved carbon dioxide concentration (mg/L) as a function of pH and alkalinity (mg/L as CaCO<sub>3</sub>) at 12 °C

ALK pH	75	80	85	90	95	100	105	110	115	120	125	130	135	140	145	150	155	160
6.00	182	194	206	218	230	242	254	267	279	291	303	315	327	339	351	364	376	388
6.05	162	173	184	194	205	216	227	238	248	259	270	281	292	302	313	324	335	346
6.10	144	154	164	173	183	193	202	212	221	231	241	250	260	270	279	289	298	308
6.15	129	137	146	154	163	172	180	189	197	206	214	223	232	240	249	257	266	275
6.20	115	122	130	138	145	153	161	168	176	184	191	199	206	214	222	229	237	245
6.25	102	109	116	123	129	136	143	150	157	164	170	177	184	191	198	204	211	218
6.30	91	97	103	109	115	121	128	134	140	146	152	158	164	170	176	182	188	194
6.35	81	87	92	97	103	108	114	119	125	130	135	141	146	152	157	162	168	173
6.40	72	77	82	87	92	96	101	106	111	116	121	125	130	135	140	145	150	154
6.45	64	69	73	77	82	86	90	95	99	103	107	112	116	120	125	129	133	138
6.50	57	61	65	69	73	77	80	84	88	92	96	100	103	107	111	115	119	123
6.55	51	55	58	61	65	68	72	75	79	82	85	89	92	96	99	102	106	109
6.60	46	49	52	55	58	61	64	67	70	73	76	79	82	85	88	91	94	97
6.65	41	43	46	49	52	54	57	60	62	65	68	71	73	76	79	81	84	87
6.70	36	39	41	44	46	48	51	53	56	58	60	63	65	68	70	73	75	77
6.75	32	34	37	39	41	43	45	47	50	52	54	56	58	60	62	65	67	69
6.80	29	31	33	35	36	38	40	42	44	46	48	50	52	54	56	58	60	61
6.85	26	27	29	31	33	34	36	38	39	41	43	45	46	48	50	51	53	55
6.90	23	24	26	27	29	31	32	34	35	37	38	40	41	43	44	46	47	49
6.95	20	22	23	24	26	27	29	30	31	33	34	35	37	38	39	41	42	44
7.00	18	19	21	22	23	24	25	27	28	29	30	32	33	34	35	36	38	39
7.05	16	17	18	19	21	22	23	24	25	26	27	28	29	30	31	32	33	35
7.10	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
7.15	13	14	15	15	16	17	18	19	20	21	21	22	23	24	25	26	27	27
7.20	11	12	13	14	15	15	16	17	18	18	19	20	21	21	22	23	24	24
7.25	10	11	12	12	13	14	14	15	16	16	17	18	18	19	20	20	21	22
7.30	9	10	10	11	12	12	13	13	14	15	15	16	16	17	18	18	19	19
7.35	8	9	9	10	10	11	11	12	12	13	14	14	15	15	16	16	17	17
7.40	7	8	8	9	9	10	10	11	11	12	12	13	13	14	14	14	15	15
7.45	6	7	7	8	8	9	9	9	10	10	11	11	12	12	12	13	13	14
7.50	6	6	7	7	7	8	8	8	9	9	10	10	10	11	11	11	11	12

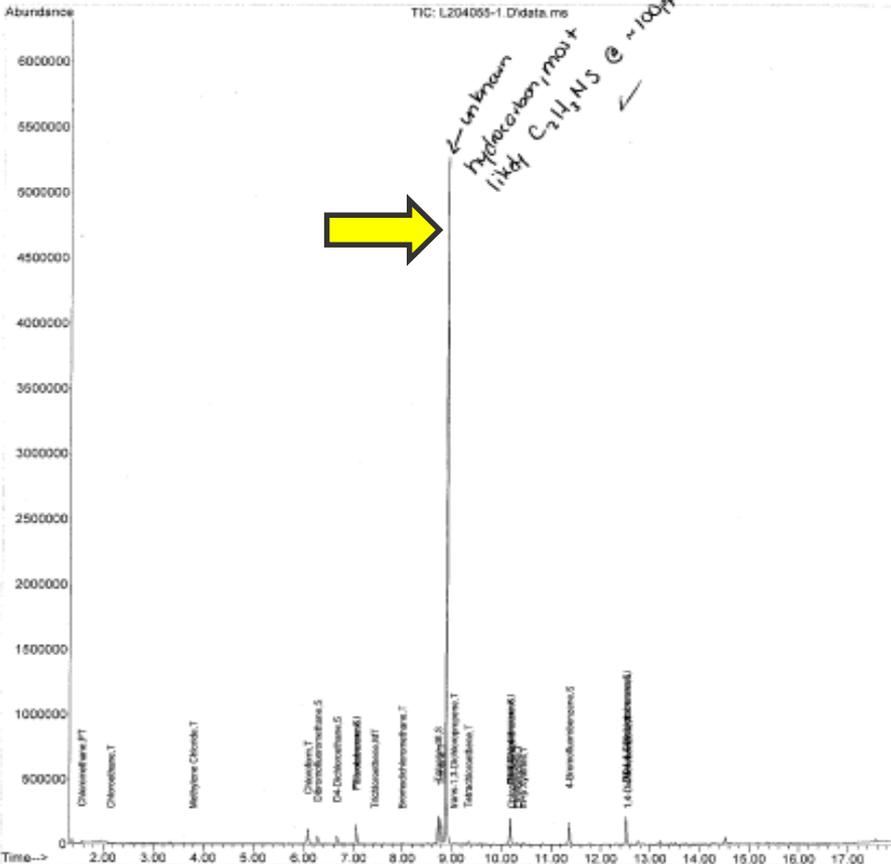
# Case 3: Toxicant in Sample?



Data File : L204055-1.D  
 Acq On : 04 Feb 2016 01:13 pm  
 Operator : jallard  
 Sample : L204055-1 5 ml eff  
 Misc : wg204915;gcms voa 1;624  
 ALS Vial : 11 Sample Multiplier: 1

Collected on  
 Day 2, before  
 mortality

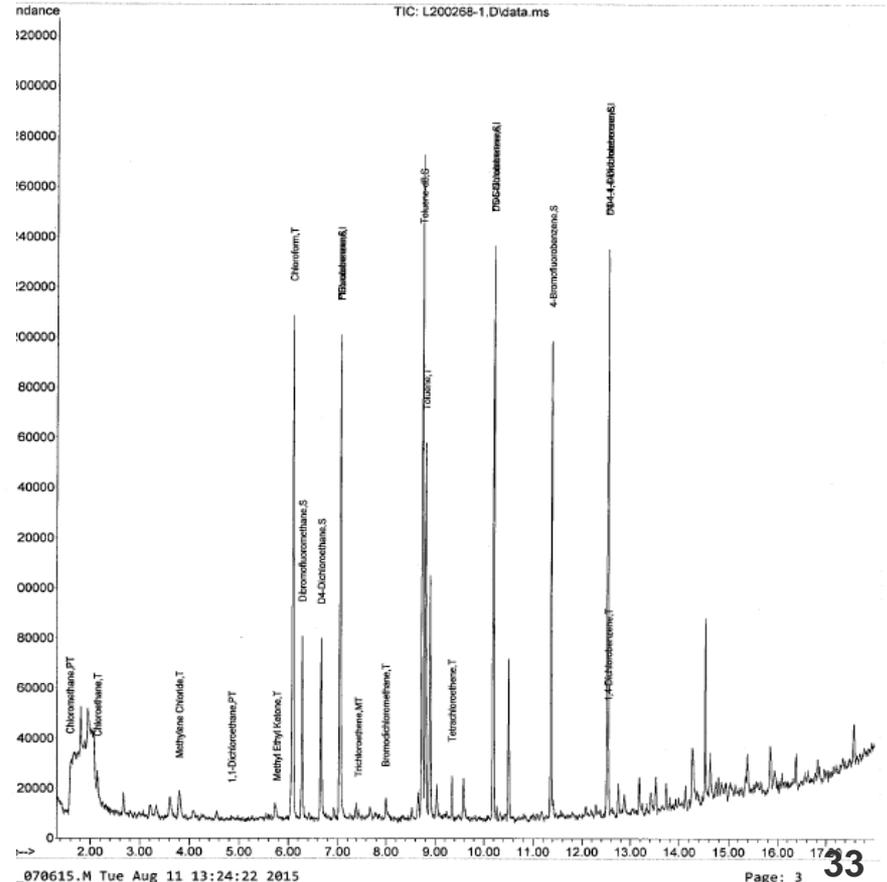
Quant Time: Feb 08 16:38:39 2016  
 Quant Method: D:\MassHunter\GCMS\1\methods\624R2\_010616.M  
 Quant Title: Method 8260B  
 Qlast Update: Mon Feb 08 16:13:25 2016  
 Response via: Initial Calibration



Data File : L200268-1.D  
 Acq On : 06 Aug 2015 12:53 pm  
 Operator : jallard  
 Sample : L200268-1 5 ml eff  
 Misc : wg201145;gcms voa 1;624  
 ALS Vial : 10 Sample Multiplier: 1

Typical effluent  
 chromatogram

Quant Time: Aug 11 13:20:56 2015  
 Quant Method: D:\MassHunter\GCMS\1\methods\624R2\_070615.M  
 Quant Title: Method 8260B  
 Qlast Update: Mon Jul 13 15:08:51 2015  
 Response via: Initial Calibration



# Case 3: Toxicant in Sample?

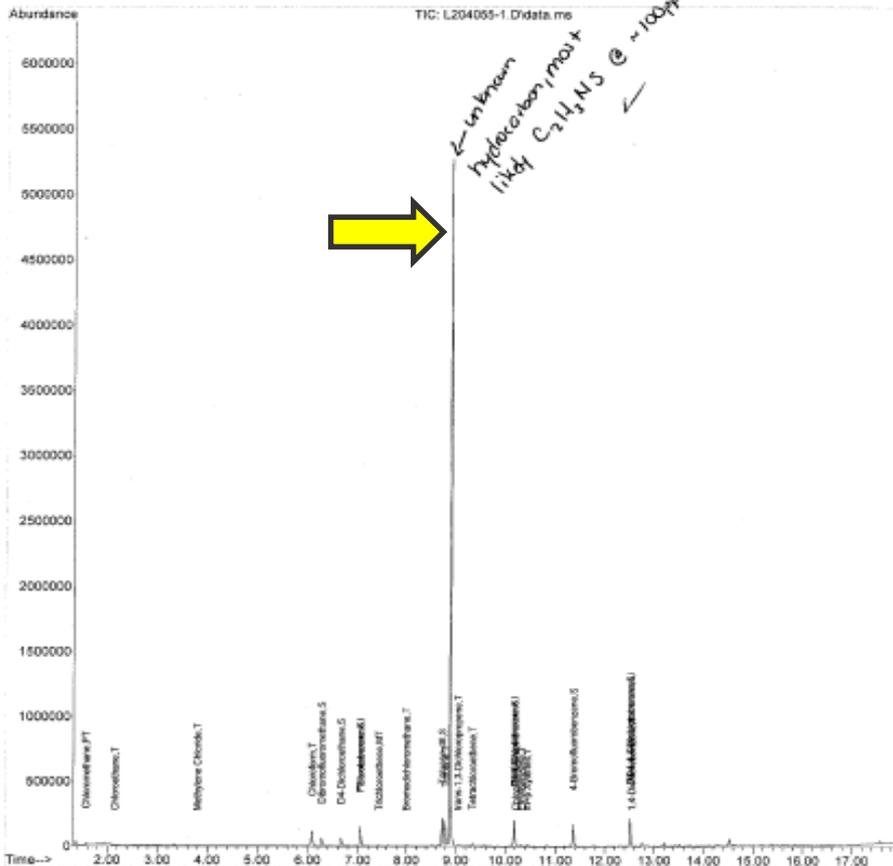
Data File : L204055-1.D  
Acq On : 04 Feb 2016 01:13 pm  
Operator : jallard  
Sample : L204055-1 5 ml eff  
Misc : wg204915;gcms von 1;624  
ALS Vial : 11 Sample Multiplier: 1

Quant Time: Feb 08 16:38:39 2016  
Quant Method: D:\MassHunter\GCMS\1\methods\624R2\_010616.M  
Quant Title: Method 8268B  
Last Update: Mon Feb 08 16:13:25 2016  
Response via: Initial Calibration

Collected on  
Day 2, before  
mortality

Methyl Isothiocyanate (MITC)

- Identified by library spectrum search
- Breakdown product of metam sodium, a common soil fumigant used for fungi and weeds
- SDS listed it as “very toxic to aquatic organisms”
- Rainbow trout: LC50 = 0.37 mg/L
- After obtaining standards, the *estimated* concentrated of this sample is 330 ug/L



# Case 3: Toxicant in Sample?



- Investigation continued with archived samples collected during acute toxicity bioassay
- Detection of MITC confirmed from Day 2, Day 3 and Day 4 samples

## Confirmed Methylisothiocyanate (MITC) values:

Rainbow trout LC50 of 370 ug/L

## Semi-Annual Organics sample collected ahead of bioassay mortality event:

Sample #	Collect Date	Locator	Result
L204055-1	2/3/2016 12:10	EFF EPS 04	MITC concentration 330 ug/L

## Bioassay Archive Samples From the Test Initiated 2/1/2016 (22% Survival) Sample collected during mortality event

Sample #	Collect Date	Locator	Result
L204118-1	2/4/2016 2:00	EFF EPS 04	MITC concentration 44 ug/L

# Case 3: Toxicant in Sample?



- Bioassay repeated with 100% survival
- MITC was not detected throughout the duration of bioassay

## **Bioassay Archive Samples From the Test Initiated 2/15/2016 (100% Survival)**

Sample #	Collect Date	Locator	Result
L204282-1	2/15/2016 20:00	EFF EPS 04	MITC concentration < 1 ug/L
L204284-1	2/16/2016 8:00	EFF EPS 04	MITC concentration < 1 ug/L
L204315-1	2/17/2016 2:00	EFF EPS 04	MITC concentration < 1 ug/L
L204383-1	2/18/2016 14:03	EFF EPS 04	MITC concentration < 1 ug/L

# Case 3: Toxicant in Sample?



- What we learned
  - Lab collaboration is crucial
  - Keep up with archive sampling
    - Volatile organics
    - Semi-volatile organics
    - No headspace sampling without preservation
  - Bioassay is a useful tool to detect presence of toxicant

# Summary



- Root Cause Analysis
- Identify Corrective Action
- Toxicology requires analytical support
  - Microbiological analysis
  - Metal analysis
  - Wet chemistry analysis: alkalinity, hardness, etc.
  - Organic analysis

# Acknowledgements



- EBMUD Laboratory Team:
  - Biology, Inorganics, Organics, QAO, Project Managers, sample control staff
- Experts from environmental laboratory community:
  - who generously shared their knowledge



Questions?