Test of Significant Toxicity

A deep dive into the Test of Significant Toxicity (TST) approach for analyzing whole effluent toxicity (WET) test data in the NPDES WET Program.

We will discuss the TST approach, its benefits, and how to evaluate real world WET test data using this method.

Aquatic Toxicology
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Introduction

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For additional information, please refer to:
EPA 833-R-10-004: National Pollutant Discharge Elimination System Test of Significant Toxicity Technical Document (June 2010)
https://www3.epa.gov/npdes/pubs/tst-techdoc.pdf

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TST Approach: Background and Overview

What is the TST?

- The Test of Significant Toxicity (TST)
- Statistical method used to analyze WET test results to determine toxicity
- It uses hypothesis testing techniques based on research and peer-reviewed publications

How it Works

- The TST approach analyzes two-concentration data
- Compares the IWC (Influent Water Concentration) or RWC (Receiving Water Concentration) to a control
- Examines whether the effluent differs from the control by an unacceptable amount, which could have a detrimental effect on aquatic organisms.

Potential Test Outcomes: Correct Decisions and Errors

1. **Correct Decision 1**

   The IWC is truly toxic and is declared toxic.

2. **Correct Decision 2**

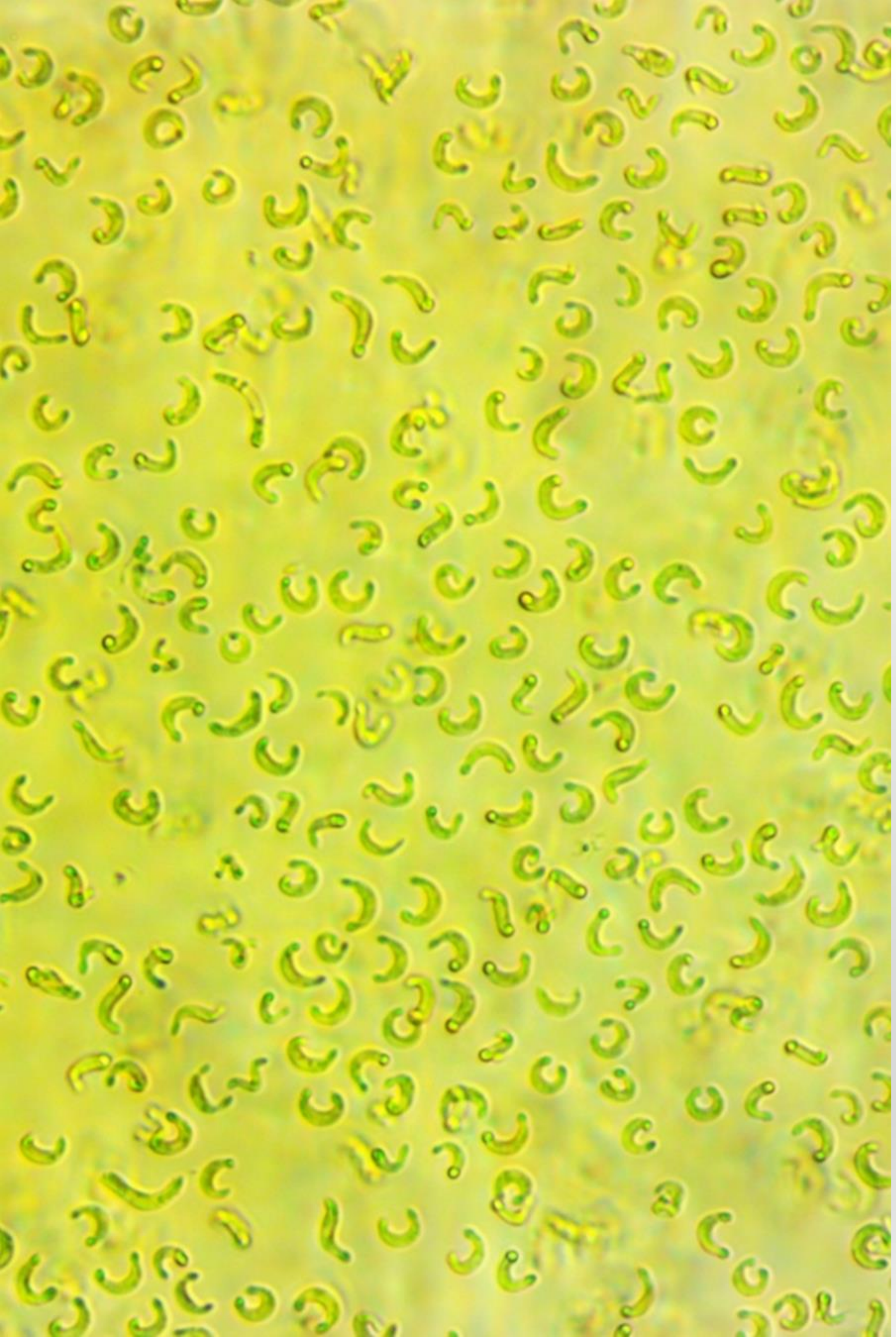
   The IWC is truly non-toxic and is declared non-toxic.

3. **Decision Error 1**

   The IWC is truly toxic but is declared non-toxic. This can occur due to high within-test variability.

4. **Decision Error 2**

   The IWC is truly non-toxic but is declared toxic. This usually occurs due to low within-test variability.



The TST Approach: Adapting the Null Hypothesis

Instead of simply asking if there is a difference between the effluent and the control, we can ask: "Is the mean response in the effluent less than a defined biological amount?“

In the TST approach, the null hypothesis is stated as: "The organism response in the effluent is less than or equal to a fixed fraction (b) of the control response.“

For example: "Treatment mean ≤ 0.75 × Control mean."

Rejecting this null hypothesis means the effluent is considered non-toxic. Accepting the null hypothesis means the effluent is toxic.

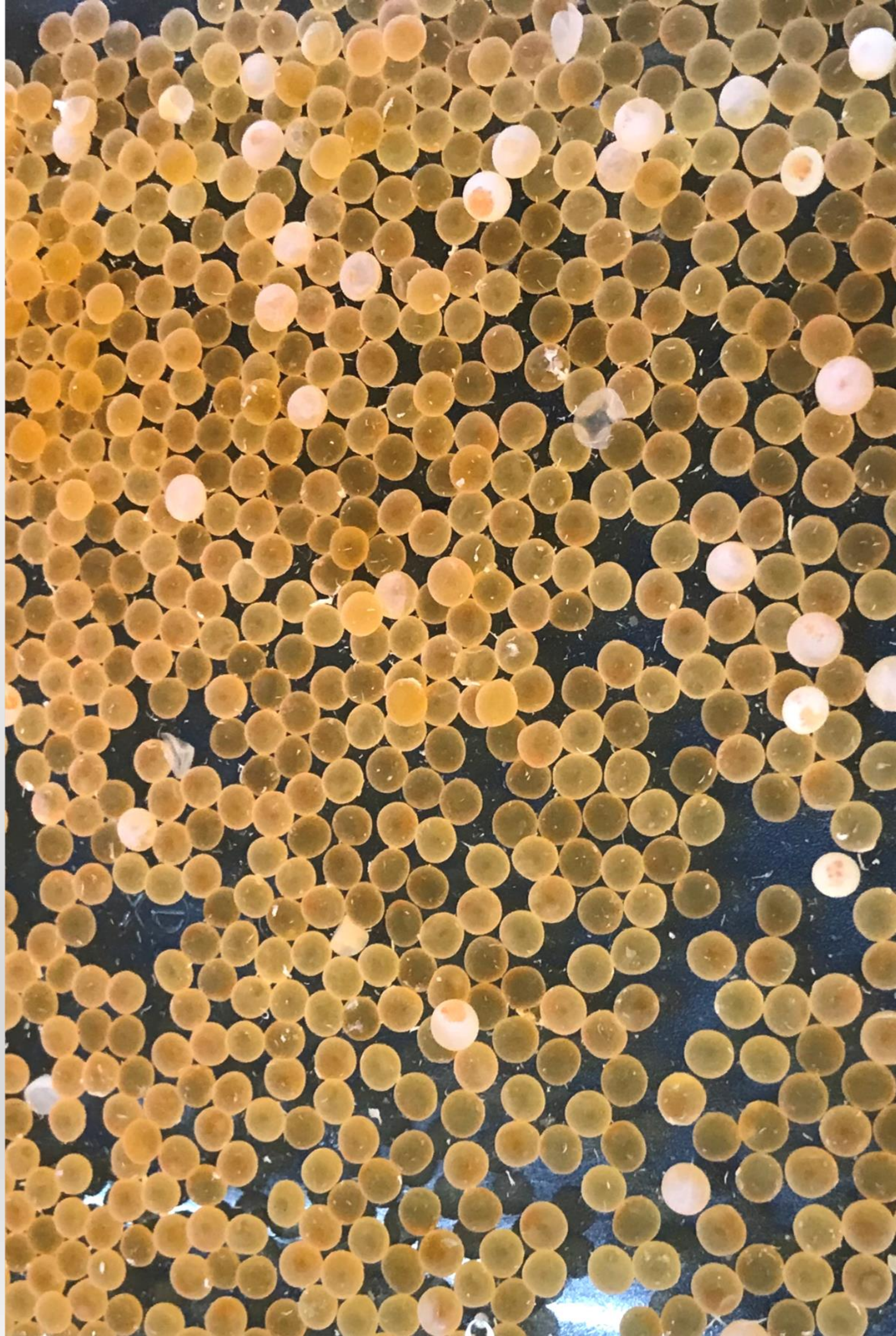
This approach is similar to the one used by the Food and Drug Administration to evaluate drugs.

Regulatory Management Decisions (RMDs) and the TST Approach

- The b value in the null hypothesis represents the threshold for unacceptable toxicity
- Chronic testing
  - b = 0.75
  - 25% effect or more at the IWC is considered toxic
- Acute testing:
  - b = 0.80
  - 20% effect or more at the IWC is considered toxic

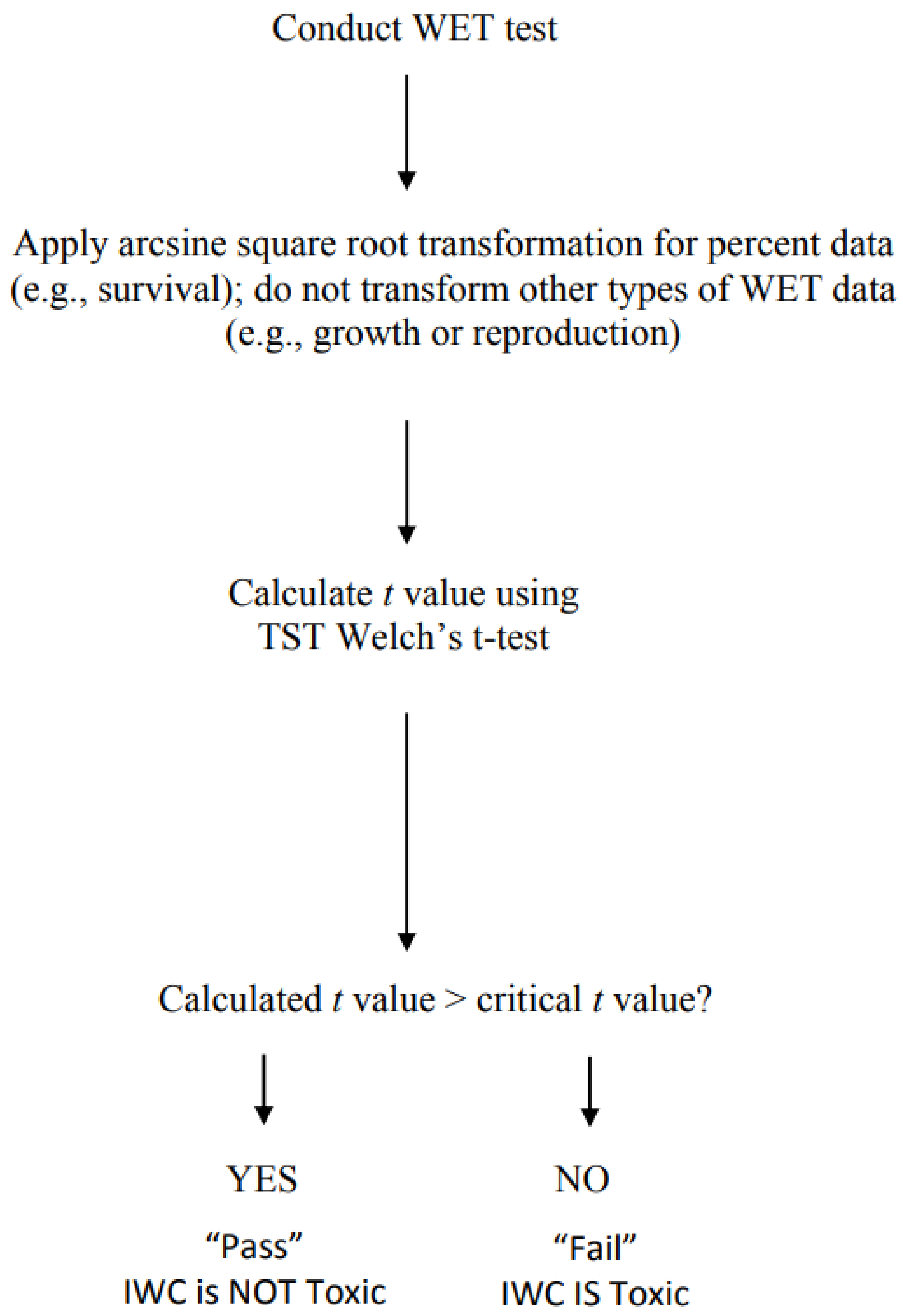
Benefits of the TST Approach: Increased Precision and Power

- The TST approach has been validated through extensive testing and simulations.
- Results indicate that TST is a suitable alternative to the traditional hypothesis approach for analyzing two-concentration WET data.
- A key benefit: increasing the precision and power of the test increases the chances of declaring an effluent non-toxic.



Improving Test Data Quality: Decreasing Variability and Increasing Replication

- Using the TST, a permittee can demonstrate that its effluent is acceptable by improving the quality of the test data
- Achieved by decreasing within-test variability and/or increasing replicates
- By improving the quality of data, permittees can more accurately assess the toxicity of their effluent and demonstrate compliance with regulatory requirements.



Guide to Evaluating WET Test Data

This section outlines a step-by-step guide for evaluating WET test data using the TST approach, ensuring accurate and reliable interpretation of results.

Guide to Evaluating Valid WET Test Data Using the TST Approach

Step 1

Conduct WET test following procedures in the appropriate EPA WET test method manual. This includes following all test requirements specified in the method.

Step 2

If there is zero variance in the endpoint in both concentrations being compared, skip the remaining steps and compute the percent difference between the control and the other concentration. Compare this difference against the RMD values of 25% for chronic and 20% for acute endpoints.

Step 3

For data consisting of proportions from a binomial (response/no response; live/dead) response variable, the variance within the ith treatment is proportional to Pi (1 – Pi), where Pi is the expected proportion for the treatment. That violates the homogeneity of variance assumption required by parametric procedures such as the TST procedure because the existence of a treatment effect implies different values of Pi for different treatments, i. Also, when the observed proportions are based on small samples, or when Pi is close to zero or one, the normality assumption might be invalid. The arcsine square root transformation is used for such data to stabilize the variance and satisfy the normality requirement. The square root of percent data (e.g., percent survival, percent fertilization), expressed as a decimal fraction (where 1.00 = 100 percent) for each treatment, is first calculated. The square root value is then arcsine transformed before analysis in Step 4. Note: Excel and most statistical software packages can calculate arcsine values.

Step 4

Conduct Welch's t-test (Zar 1996) using Equation 1.

Step 5

Adjust the degrees of freedom (df) using Equation 2.

Step 6

Using the calculated t value from Step 4, compare that t value with the critical t value table in Appendix C using the test method-specific alpha values shown in Table 4-1. To obtain the correct t value, look across the table for the alpha value that corresponds to the WET test method and then look down the table for the appropriate df.

Step 7

If the calculated t value is less than the critical t value, the IWC is declared toxic, and the test result is Fail. If the calculated t value is greater than the critical t value, the IWC is not declared toxic and the test result is Pass.

Step 1: Conduct WET Test

The first step involves conducting the WET test according to the procedures outlined in the appropriate EPA WET test method manual. This includes adhering to all test requirements specified in the method, ensuring a standardized and reliable test process.

Example Chronic Fathead Minnow survival test data:

7d Survival Rate Binomials					
Conc-%	Code	Rep 1	Rep 2	Rep 3	Rep 4
0	LW	10/10	10/10	9/10	10/10
100		10/10	10/10	10/10	10/10

Example Chronic Fathead Minnow growth test data:

Mean Dry Biomass-mg Detail					
Conc-%	Code	Rep 1	Rep 2	Rep 3	Rep 4
0	LW	0.282	0.246	0.252	0.323
100		0.278	0.257	0.272	0.398

Step 2: Analyze Test Endpoints

For each test endpoint specified in the WET test method manual, follow Steps 3–7. If there is zero variance in the endpoint in both concentrations being compared, skip the remaining steps and compute the percent difference between the control and the other concentration. Compare this difference against the RMD values of 25% for chronic and 20% for acute endpoints.

$$\%\ \text{Effect at IWC} = \frac{\text{Mean Control Response} - \text{Mean Response at IWC}}{\text{Mean Control Response}} \times 100$$

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Toxic

If the percent mean response is greater than the RMD, the sample is declared toxic and the test is "Fail".

Non-Toxic

If the percent mean response is less than the RMD, the sample is declared non-toxic and the test is "Pass".


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Step 3: Data Transformation

Data consisting of proportions from a binomial response variable (survival) violates the homogeneity of variance assumption required by parametric procedures such as the TST procedure. Thus, the arcsine square root transformation is used to stabilize the variance and satisfy the normality requirement.

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y' = arcsin(sqrt(y)) or y' = arcsin(sqrt((y + 1/4n) / (n + 1/2))) (corrected)
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The square root of percent data (e.g., percent survival, percent fertilization), expressed as a decimal fraction (where 1.00 = 100 percent) for each treatment, is first calculated. The square root value is then arcsine transformed before analysis in Step 4.

Example Chronic Fathead Minnow survival test data once transformed:
(growth data is not transformed)

Angular (Corrected) Transformed Detail

Conc-%	Code	Rep 1	Rep 2	Rep 3	Rep 4
0	LW	1.41	1.41	1.25	1.41
100		1.41	1.41	1.41	1.41

Step 4: Welch's t-test

Conduct Welch's t-test using Equation 1 to determine if there is a statistically significant difference between the control and the other concentration.

Welch's t-test is appropriate to use when there are an unequal number of replicates between control and the IWC. When sample sizes of the control and treatment are the same (i.e., nt = nc), Welch's t-test is equivalent to the usual Student's t-test.

S² = (∑(x_i - x̄)²) / (n - 1)
x_i = each data point
x̄ = average

Equation 1

$$t = \frac{\overline{Y}_t - b \times \overline{Y}_c}{\sqrt{\frac{S_t^{\,2}}{n_t} + \frac{b^2 S_c^{\,2}}{n_c}}}$$

where

$\overline{Y}_c$ = Mean for the control

$\overline{Y}_t$ = Mean for the IWC

s_c^2 = Estimate of the variance for the control

s_t^2 = Estimate of the variance for the IWC

n_c = Number of replicates for the control

n_t = Number of replicates for the IWC

b = 0.75 for chronic tests; 0.80 for acute tests

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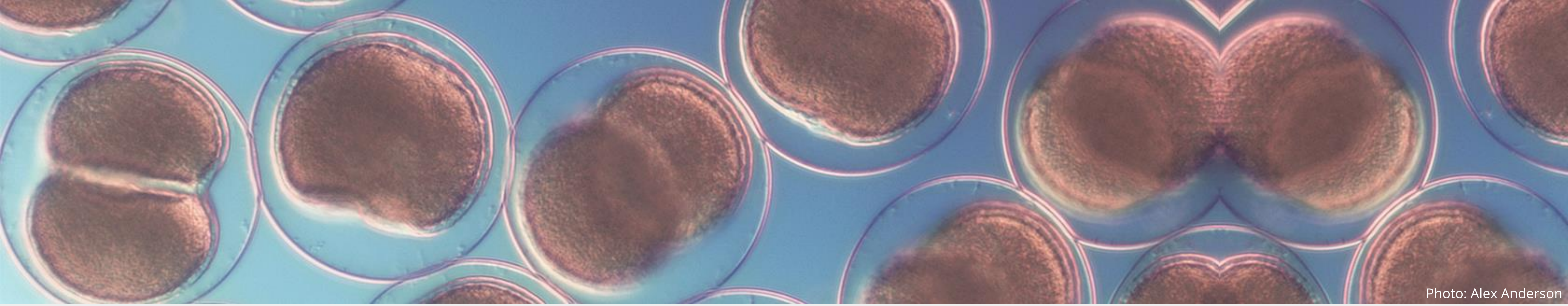
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Step 5: Adjust Degrees of Freedom

Adjust the degrees of freedom (df) using Equation 2 to account for unequal sample sizes.

Equation 2

$$\upsilon = \frac{\left(\frac{S_t^2}{n_t} + \frac{b^2 S_c^2}{n_c}\right)^2}{\frac{\left(\frac{S_t^2}{n_t}\right)^2}{n_t - 1} + \frac{\left(\frac{b^2 S_c^2}{n_c}\right)^2}{n_c - 1}}$$

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Using Welch's t-test, df is the value obtained for v in Equation 2 above. Because v is most likely a non-integer, round v to the next smallest integer, and that number is the df.

Table ES-2. Summary of alpha (α) levels or false negative rates recommended for different EPA WET test methods using the TST approach.

EPA WET test method	*b* value	Probability of declaring a toxic effluent non-toxic False negative (α) error[a]
Chronic Freshwater and East Coast Methods		
Ceriodaphnia dubia (water flea) survival and reproduction	0.75	0.20
Pimephales promelas (fathead minnow) survival and growth	0.75	0.25
Selenastrum capricornutum (green algae) growth	0.75	0.25
Americamysis bahia (mysid shrimp) survival and growth	0.75	0.15
Arbacia punctulata (Echinoderm) fertilization	0.75	0.05
Cyprinodon variegatus (Sheepshead minnow) and *Menidia beryllina* (inland silverside) survival and growth	0.75	0.25
Chronic West Coast Marine Methods		
Dendraster excentricus and *Strongylocentrotus purpuratus* (Echinoderm) fertilization	0.75	0.05
Atherinops affinis (topsmelt) survival and growth	0.75	0.25
Haliotis rufescens (red abalone), *Crassostrea gigas (oyster), Dendraster excentricus, Strongylocentrotus purpuratus* (Echinoderm) and *Mytilus sp* (mussel) larval development methods	0.75	0.05
Macrocystis pyrifera (giant kelp) germination and germ-tube length	0.75	0.05
Acute Methods		
Pimephales promelas (fathead minnow), *Cyprinodon variegatus* (Sheepshead minnow), *Atherinops affinis* (topsmelt), *Menidia beryllina* (inland silverside) acute survival[b]	0.80	0.10
Ceriodaphnia dubia, *Daphnia magna*, *Daphnia pulex*, *Americamysis bahia* acute survival[b]	0.80	0.10

Notes:
a. α levels shown are the probability of declaring an effluent toxic when the mean effluent effect = 25% for chronic tests or 20% for acute tests and the false positive rate (β) is ≤ 0.05 (5%) when mean effluent effect = 10%.
b. Based on a four replicate test design

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Step 6: Compare Calculated t Value

Using the calculated t value from Step 4, compare that t value with the critical t value table (C-1) using the test method-specific alpha values shown in Table 4-1. To obtain the correct t value, look across the table for the alpha value that corresponds to the WET test method and then look down the table for the appropriate df.

Table C-1. Critical values of the *t* distribution. One tail probability is assumed.

	Alpha				
Degrees of freedom	**0.25**	**0.20**	**0.15**	**0.10**	**0.05**
1	1	1.3764	1.9626	3.0777	6.3138
2	0.8165	1.0607	1.3862	1.8856	2.92
3	0.7649	0.9785	1.2498	1.6377	2.3534
4	0.7407	0.941	1.1896	1.5332	2.1318
5	0.7267	0.9195	1.1558	1.4759	2.015
6	0.7176	0.9057	1.1342	1.4398	1.9432
7	0.7111	0.896	1.1192	1.4149	1.8946
8	0.7064	0.8889	1.1081	1.3968	1.8595
9	0.7027	0.8834	1.0997	1.383	1.8331
10	0.6998	0.8791	1.0931	1.3722	1.8125
11	0.6974	0.8755	1.0877	1.3634	1.7959
12	0.6955	0.8726	1.0832	1.3562	1.7823
13	0.6938	0.8702	1.0795	1.3502	1.7709
14	0.6924	0.8681	1.0763	1.345	1.7613
15	0.6912	0.8662	1.0735	1.3406	1.7531
16	0.6901	0.8647	1.0711	1.3368	1.7459
17	0.6892	0.8633	1.069	1.3334	1.7396
18	0.6884	0.862	1.0672	1.3304	1.7341
19	0.6876	0.861	1.0655	1.3277	1.7291
20	0.687	0.86	1.064	1.3253	1.7247
21	0.6864	0.8591	1.0627	1.3232	1.7207
22	0.6858	0.8583	1.0614	1.3212	1.7171
23	0.6853	0.8575	1.0603	1.3195	1.7139
24	0.6849	0.8569	1.0593	1.3178	1.7109
25	0.6844	0.8562	1.0584	1.3163	1.7081
26	0.684	0.8557	1.0575	1.315	1.7056
27	0.6837	0.8551	1.0567	1.3137	1.7033
28	0.6834	0.8546	1.056	1.3125	1.7011
29	0.683	0.8542	1.0553	1.3114	1.6991
30	0.6828	0.8538	1.0547	1.3104	1.6973
inf	0.6745	0.8416	1.0364	1.2816	1.6449

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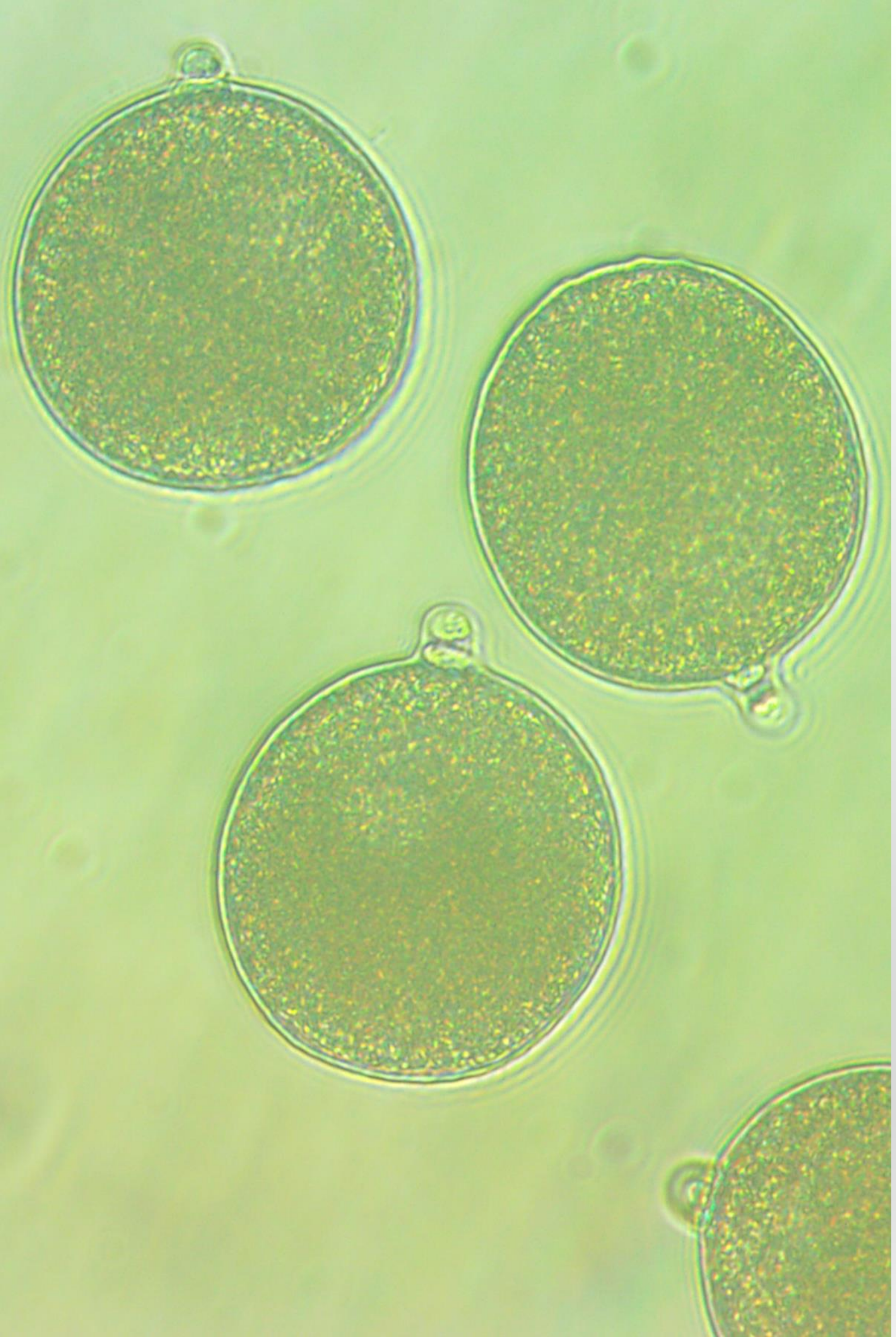

Step 7: Interpret Results

If the calculated t value is less than the critical t value, the IWC is declared toxic, and the test result is Fail. If the calculated t value is greater than the critical t value, the IWC is not declared toxic and the test result is Pass.

Example Chronic Fathead Minnow survival test data (Pass):

TST-Welch's t Test

Control	vs	Control II	Test Stat	Critical	DF	P-Type	P-Value	Decision(α:25%)
Lab Water		100*	12.6	0.765	3	CDF	5.5E-04	Non-Significant Effect

Example Chronic Fathead Minnow growth test data (Pass):

TST-Welch's t Test

Control	vs	Control II	Test Stat	Critical	DF	P-Type	P-Value	Decision(α:25%)
Lab Water		100*	2.69	0.765	3	CDF	0.0373	Non-Significant Effect

Example Chronic Topsmelt growth test data (Fail):

TST-Welch's t Test

Control	vs	Conc-%	Test Stat	Critical	DF	P-Type	P-Value	Decision(α:25%)
Lab Water		100	0.407	0.741	4	CDF	0.3523	Significant Effect

Table 2. Test Results Summary for Species Screening

Q1

Species	Endpoint	Percent Effect		
		10% Effluent	50% Effluent	100% Effluent
Selenastrum[a]	Final Cell Density	-55.49%	-5.32%	-1.81%
Mussel	Larval Development	1.32%	**44.27%***	**100%**
Topsmelt	Percent Survival	0.00%	24.00%	24.00%
	Growth	-21.90%	**22.93%***	18.77%

Q2

Species	Endpoint	Percent Effect		
		10% Effluent	50% Effluent	100% Effluent
Selenastrum	Final Cell Density	20.69%	-19.03%	-16.83%
Mussel	Larval Development	2.32%	-0.22%	**27.72%***
Topsmelt	Percent Survival	0.00%	4.00%	4.00%
	Growth	15.68%	-0.54%	19.01%

Q3

Species	Endpoint	Percent Effect		
		10% Effluent	50% Effluent	100% Effluent
Selenastrum	Final Cell Density	-40.76%	-73.42%	-40.92%
Mussel	Larval Development	2.11%	9.52%	**100%***
Topsmelt	Percent Survival	8.33%	**62.50%***	**54.17%***
	Growth	-0.86%	**58.96%***	**66.81%***

Q4

Species	Endpoint	Percent Effect		
		10% Effluent	50% Effluent	100% Effluent
Selenastrum	Final Cell Density	33.16%	-27.83%	-2.17%
Mussel	Larval Development	-0.14%	**88.06%***	**100%***
Topsmelt	Percent Survival	4.17%	12.50%	20.83%
	Growth	4.41%	7.52%	**25.15%***

* - Test treatment resulted in a "Fail" under the TST Welch's t Test.

Conclusion: TST as a Powerful Tool for Evaluating WET Test Data

- Provides a robust and reliable method for evaluating WET test data
- Helps to identify potential toxic effects of environmental samples
- Aims to ensure accurate and consistent interpretation of WET results
- Understanding the principles of TST allows permittees to:
  - Effectively analyze their data
  - Make informed decisions about effluent discharge
  - Ensure compliance with regulatory requirements
- The TST is a valuable tool for protecting aquatic ecosystems and promoting sustainable environmental practices

Credits

- This presentation followed EPA 833-R-10-004: National Pollutant Discharge Elimination System Test of Significant Toxicity Technical Document (June 2010)
- All photos used in this presentation are property of MAI, unless otherwise stated.
- Comprehensive Environmental Toxicology Information System (CETIS)

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