Southern California Stormwater Monitoring Coalition

Potential Impacts of Toxicity Testing Variability on Waterbody Impairment Status

DRAFT White Paper

Prepared by:
J. Michael Trapp, PhD and Karin Peternel
Michael Baker International
Carlsbad, California

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Abstract

A recent SMC funded intercalibration study for toxicity revealed significant variability in *C. dubia* reproduction toxicity results in water quality samples tested.[1] While a goal of the study (Schiff and Greenstein, 2016) was to quantify and make recommendations to minimize inter-laboratory variability, the variability observed created concerns about the potential to affect toxicity results in real world environmental samples, and thus the possible toxic impairment listing status of waterbodies on the federal 303(d) list. This paper uses data from eight sites within SMC member agency jurisdictions to see if the original toxicity test outcomes from these sites would change based on the variability seen in the SMC intercalibration, and if these outcomes would result in changes to their impairment status.

Results show that under the lower variability treatment (+/- 22%) at least 50% of tests would change results from pass to fail or fail to pass, and under the higher variability treatment (+/- 46%) as many as 76-91% of samples could change results. When these potential changes were applied to 303(d) listing criteria, 7 of the 8 sites could change their listing status. These revelations present significant management implications. As a result, it is suggested that the use of the *C. dubia* reproduction toxicity be reconsidered or efforts undertaken to improve the consistency of its results and understand the roots of the observed variability that was seen in the SMC interlaboratory intercalibration testing. Use of other approved species, results of which were shown to have improvements after the intercalibration study, could be used as alternatives.

[1] Only water quality samples were analyzed; sediment toxicity was not included in the intercalibration study or this analysis.

Introduction

One of the primary goals of the Southern California Stormwater Monitoring Coalition (SMC) is to conduct the science to support stormwater management. SMC's research efforts include developing regional stormwater monitoring infrastructure that is expected to lead to increased comparability among the numerous monitoring programs and laboratories in Southern California. Stormwater monitoring samples collected from water bodies throughout California are analyzed at various laboratories using standard EPA test methods (EPA 2002). Results from these analyses have been used for management activities and to drive regulatory actions. EPA (2000) and Bay et al. (2003) conducted studies to quantify method variability within laboratories (repeatability) to enable NPDES programs to distinguish between variability caused by the testing method and variability associated with toxicity of multiple effluent samples taken from the same facility. To help ensure full confidence in the data produced by these labs, the SMC has funded intercalibration studies; the most recent of these studies was a toxicity intercalibration. The SMC intercalibration study identified poor comparability in *Ceriodaphnia dubia* reproduction results among laboratories, as well as between samples within individual laboratories.

Stormwater management agencies in Southern California spend nearly $1 million per year on toxicity sampling alone. As a result of the variability observed in the intercalibration study, significant concerns were raised among SMC member organizations about ramifications of assessment errors in toxicity test results; consequences of which could result in the incorrect status of waterbodies on the Clean Water Act (CWA) 303(d) listings or the initiation of unnecessary toxicity reduction evaluation (TRE) investigations. While the SMC intercalibration study recommended *C. dubia* tests continue as part of the SMC monitoring program, additional intercalibrations were recommended specifically for the *Ceriodaphnia* reproduction test to assess sources of variability in both stormwater and laboratory dilution water. This paper aims to determine if the variability observed in the laboratory intercalibration results could impact changes to the impairment status of real world samples in a non-biased investigation (from fail to pass, or pass to fail). Further, the variability on individual tests are explored in regulatory samples to determine if changes in results could potentially cause changes to 303(d) impairment status.

Background

Regulations and Policy

The goal of the CWA, passed in 1972, is "to restore and maintain the chemical, physical, and biological integrity of the Nation's waters" (33 U.S.C. Sec. 1251(a)). CWA Section 303(d) requires each state to develop, update, and submit to the U.S. Environmental Protection Agency (EPA) for approval a list of water bodies not meeting water quality standards or "beneficial uses." This List of Impaired Waters, submitted biennially, is referred to as the 303(d) list. The guidance by which the California Water Boards comply with the listing requirements of Section 303(d) is the Water Quality Control Policy (2015), a standardized approach used to meet the overall goals of achieving water quality standards and maintaining beneficial uses.

Regulatory protection of beneficial uses is carried out through water quality objectives established in each of California's nine Regional Water Quality Control Board's (RWQCB) Basin Plans. These Basin Plans contain narrative toxicity objectives that require all water to be maintained free of toxic substances in concentrations that produce detrimental physiological responses in humans, plants, terrestrial animals, and aquatic organisms. In 2005, the Policy for Implementation of Toxics Standards for Inland Surface Waters, Enclosed Bays, and Estuaries of California (SIP) was adopted to provide a mechanism to implement the water quality criteria established in the California Toxics Rule. The SIP requires that the Regional Water Boards determine compliance with narrative chronic toxicity objectives using EPA methodology to estimate the potential effects on the survival, growth, and reproduction of species, where compliance is determined by conducting tests on at least one species of aquatic plant, one invertebrate, and one vertebrate (EPA 2002). According to the EPA testing methods and 303(d) policy for toxicity, if any of three tested species exhibit toxicity over two sampling events, the site will be placed on the 303(d) list. The 2012 EPA 303(d) list includes 260 impaired water bodies for toxicity in California, of which 85 fall within the watersheds of the SMC member organizations located in RWQCBs Los Angeles, Santa Ana, and San Diego.

Intercalibration

A successful chemistry intercalibration study led by SMC (Gossett et al. 2004) brought attention to the need for a similar study and steps to be taken toward comparability for aquatic toxicity testing in water quality samples, as they are a standard measurement in stormwater management. Species are held in test chambers and exposed to receiving waters and known toxic or nontoxic aqueous samples, and observations are made that test the response of the test organisms to estimate the effects of the toxicant (EPA 2000). Because of standardized procedures for laboratories to follow, there is an expectation of repeatability between independent test results from the same laboratory, and reproducibility between test results obtained from different laboratories. However, in response to different outcomes from laboratories and known uncertainties in testing protocols, SMC conducted an interlaboratory intercalibration study to assess comparability among and between labs that conduct toxicity testing, resulting in the Stormwater Monitoring Coalition Toxicity Testing Laboratory Guidance Document (Schiff and Greenstein, 2016). This interlaboratory

intercalibration study conducted two rounds of testing using eight toxicity laboratories, finding a significant level of variability in the results. Each testing round was comprised of four samples, delivered blind to each laboratory: lab dilution water, lab dilution water spiked with copper, runoff sample created with artificial rainfall, and a duplicate. Reasonable comparability was found with the two marine species, yet poor comparability was found for the two freshwater species, *Ceriodaphnia dubia* and *Hyalella azteca*. Table 1 reveals that Round 1 results showed poor comparability in *H. azteca* survival and *C. dubia* survival and reproduction among laboratories, while Round 2 showed improved comparability for *H. azteca* survival and *C. dubia* survival, but poor comparability for *C. dubia* reproduction. Additionally, a blind sample of laboratory dilution water, prepared in accordance with standardized guidance and expected to be nontoxic, was found to be toxic by some of the participating labs. This prompted a review of test methods and standardization of protocols prior to the second round of interlaboratory testing. The results of the second round of testing also identified toxicity in the dilution water sample and relatively poor comparability of the laboratories between testing events. Intercalibration exercises have found that although specific guidance and procedures exist, there is still variability in results within and between laboratories as evidenced by the outcome of the intercalibration study (Table 1). Additional intercalibrations have been recommended, specifically for the *C. dubia* reproduction test, to assess sources of variability in both stormwater samples and lab dilution water.

Table 1. Summary of laboratory comparability scoring for Ceriodaphnia dubia (6-8 day) survival and reproduction and Hyalella survival from SMC Toxicity Testing Laboratory Guidance Document for water quality samples (Schiff and Greenstein, 2016), Table ES-1. Comparability was scored based on test acceptability, intra-laboratory precision, and interlaboratory precision.

Lab	**Ceriodaphnia Survival**		**Ceriodaphnia Reproduction**		**Hyalella Survival**	
	Round 1	Round 2	Round 1	Round 2	Round 1	Round 2
A	Moderate	High	Very High	Low	Low	High
B	Very High	High	Moderate	High	Low	High
C	Low	High	Low	High	Low	Very High
E	Moderate	-	Moderate	-	-	
F	Moderate	High	Moderate	Low	Low	Very High
G	High	-	High	-	-	-
H	Low	-	Low	-	-	-
I	High	Moderate	High	Low	Moderate	Very High
J	Low	High	Low	Low	High	Very High

Variability

EPA test method 1002.0 (EPA 2002) details the potential for increased variability in results due to potential interferences in sampling and analysis, as well as other laboratory factors. Such factors that have been identified to influence toxicity test results include failure to initiate tests due to unsuccessful cultures (DeGraeve et al., 1992, EPA, 2001) along with water renewal and feeding regime (Cooney et

al., 1992); LaRocca et al. (1994) and Belanger et al. (1989) also identified feeding regime as an influence. While the SMC intercalibration study did not find a clear relationship between feeding, water renewal, and test variability, they are both included as part of the standardized guidance recommendations, and have the potential to contribute to the uncertainty of using such biological testing methods. This may be due in part to the variability inherent in the test (i.e., live organisms), or it may be the variability inherent to the laboratories. Such research, in addition to the variability seen from laboratories in the toxicity intercalibration study, brings into question the laboratory results used for water bodies placed on the 303(d) list for toxicity as the pollutant, as assessment error could affect the resulting regulatory action or inaction. Laboratory procedures, cultures, and QAQC especially should be evaluated in light of the significant variability seen in the laboratory dilution water. EPA (2001) conducted a thorough toxicity intercalibration for the *Ceriodaphnia* reproduction endpoint as part of its whole effluent testing (WET) program. The EPA intercalibration observed a mean response of 3% effect amongst 27 labs that tested laboratory dilution water. However, laboratory dilution water samples were prepared differently for the EPA intercalibration compared to the SMC intercalibration study. Enough uncertainty has been shown between and among laboratories (where specific method options or interpretations are left to each laboratory) to increase concerns about toxicity testing's inherent variability. These uncertainties can have a profound effect on test results, and therefore, on the regulatory actions placed on a water body.

Definition of Evaluation

The variability seen in the interlaboratory intercalibration exercises indicates that there is possible uncertainty in the accuracy of results from historical NPDES toxicity data. This generates a concern for the potential impact on the existing and future listings of the 303(d) list of impaired water bodies. The interlaboratory intercalibration exercises, as well as some of the standardizations to address variability, were conducted and reported in Schiff and Greenstein (2016). The majority of sites placed on the 303(d) list for toxicity resulted from laboratory testing that occurred significantly earlier than their study; therefore, some listings are suspected to be a result of potential inaccuracies.

In an effort to see if the level of variability observed in the SMC intercalibration study could have impacted real-world data, we used the original datasets that resulted in the eight water bodies' listing for toxicity to see if the variability could have caused an incorrect listing for toxicity. This would help determine that if similar conditions of variability existed during the original laboratory testing event, such variability could have led to assessment error that could have affected the site's listing status.

A review of the data for sites in the San Diego region that were 303(d) listed for toxicity shows that most were listed based on a 2002-2003 ecological condition assessment of water bodies across the region. Results show that there were positive toxicity tests because of *C. dubia,* as well as a significant number of sites toxic to the algae species *Selenastrum capricornutum*. Thus, it is possible that even if *C. dubia* tests resulted in assessment error, algae toxicity would still result in 303(d) inclusion. This uncertainty in the reliance on toxicity test methods overall are of concern due to the historical implications resulting from the variability and uncertainty created by inconsistent lab approaches and responses.

The need to minimize the variability in toxicity testing, and ensure laboratory testing of waters from sites placed on the 303(d) list be consistent as possible, is being recognized by the broader water quality community. This recognition extends to the State Board, where plans exist to revise its toxicity policy to that of 'provisions' within the next year. These revisions include statewide standards for sampling, a focus on quality of laboratories, and additionally the application of the test for significant toxicity (TST) statistical test (pers. comm., Z. Paulsen SWRCB). This study offers an opportunity to view variability in the context of real world regulatory outcomes and will help decision makers understand its ramifications.

To achieve these ends, this study sought representative sites on the 2010-2012[2] California (303)d list for which the original data was available to conduct an evaluation of potential impacts of assessment error. These data that resulted in a toxicity listing were exposed to the variability seen in the intercalibration study to determine if the listing may have been incorrect. The level of variability estimated in the intercalibration study was used as a threshold for the amount of potential variability.

Representative Site Selection

A full evaluation of every impaired water body in the state would not be realistic, so a criterion was developed to identify candidate sites for careful inspection. The 2012 CWA 303(d) list of 4,852 sites was reviewed for water bodies listed for toxicity and inclusion in this assessment using the following set of criteria:

- Included a chronic toxic impairment (260 water bodies).
- Contained within SMC member regions of Los Angeles (Region 4), Santa Ana (Region 8), and San Diego (Region 9). The resulting list consisted of 19 water bodies listed for toxicity in the Los Angeles Region; two listed for unknown toxicity in Santa Ana; and 29 listed for toxicity in San Diego. (Sediment Toxicity was not considered)
- The SMC-associated reaches were then sorted by the total number of impairments to focus on sites with lower chances of complications resulting from multiple stressors. This resulted in toxicity alone at four sites, toxicity plus one other pollutant at seven sites, and toxicity with two to three other pollutants at four sites.

The initial list of water bodies selected for potential reassessment of data is shown in Table 2.

[2] Region 9 data was not included in the 2012 Integrated Report so all decisions are carried over from the 2010 listing cycle.

Table 2. Fifteen water bodies listed in 303(d) for toxicity and limited other constituents.

Region	Water Body	Listing 1	Listing 2	Listing 3	Listing 4
4	Santa Clara River Reach 1 (Estuary to Hwy 101 Bridge)	Toxicity			
9	Jamul Creek	Toxicity			
9	Poggi Canyon Creek	Toxicity			
9	Santa Ysabel Creek (above Sutherland Reservoir)	Toxicity			
9	Encinitas Creek	Toxicity	Selenium		
9	Loma Alta Creek	Toxicity	Selenium		
9	Moro Canyon Creek	Toxicity	Selenium		
9	Oso Creek (lower)	Toxicity	Selenium		
9	Poway Creek	Toxicity	Selenium		
9	Rose Creek	Toxicity	Selenium		
9	Santa Margarita River (Upper)	Toxicity	Phosphorus		
9	Dana Point Harbor	Toxicity	Copper	Zinc	
9	Segunda Deshecha Creek	Toxicity	Turbidity	Phosphorus	
8	San Diego Creek Reach 2	Unknown Toxicity	Sedimentation/ Siltation	Nutrients	Indicator Bacteria
8	Lake Elsinore	Unknown Toxicity	Sediment Toxicity	Nutrients	Organic Enrichment

Site Descriptions

Monitoring data was obtained for the initial list of sites from the CEDEN database (www.ceden.org) and reviewed for the species type and the frequency at which each species or endpoint resulted in a positive test for toxicity. This list was narrowed down to 11 sites for which *C. dubia* was tested, and further reduced to 8 sites for which *C. dubia* reproduction resulted in a failed toxicity test. A brief description of these sites follows.

- Jamul Creek and Poggi Canyon Creek (toxicity) in the San Diego region underwent an ecological assessment in 2003 that indicated that both sites exceeded aquatic life thresholds for several water chemistry constituents, but there were more exceedances at Poggi Creek (4) than Jamul (3), and more pesticides were detected at Poggi (5) than at Jamul (2).
- The 3-mile segment of Encinitas Creek (toxicity, selenium) in Encinitas, California was delisted for phosphorus due to flaws in the original listing (2004). Although analyzed for Benthic Community Effects, diazinon, nitrogen, turbidity, and total dissolved solids, it was not listed for any of those pollutants.
- Santa Ysabel Creek (toxicity) is located above the Sutherland Reservoir and is the largest tributary to the San Dieguito Creek, which drains to the Pacific Ocean. It is considered a reference site for monitoring the rest of the watershed, and generally has the fewest number of aquatic life threshold exceedances in the monitoring program.

- Loma Alta Creek (selenium, toxicity) in Oceanside, California is the natural drainage for about 6,400 acres of heavily urbanized land that runs along Oceanside Boulevard, including residential, commercial, and industrial uses. Its waters feed into the Loma Alta Slough, or estuary, which is the final 1,600 feet of the watershed before it reaches the ocean. Loma Alta Creek is known to have persistent algal blooms, and there is an active TMDL for eutrophication/algae blooms.
- Oso Creek (toxicity, selenium) is an approximately 13.5-mile (21.7 km) tributary of Trabuco Creek in southern Orange County. Draining about 20 square miles (52 km^2) in a region north of the San Joaquin Hills and south of the Santa Ana Mountains, the creek is Trabuco Creek's largest tributary and is part of the San Juan Creek drainage basin. The creek is channelized and polluted along much of its length.
- Santa Margarita River (toxicity, phosphorus) in San Diego and Riverside Counties underwent an ecological assessment using data collected from 1998 to 2005. Toxicity was moderate, although samples from all sites were toxic to the freshwater algae *Selenastrum capricornutum* on at least one sampling date. Fish tissues from the downstream Santa Margarita River site showed no evidence of impact. Bioassessment samples indicated that large areas of the watershed are in poor ecological condition, yet other areas of the watershed are in fair or good condition.
- Rose Creek (toxicity, selenium) in San Diego drains to Mission Bay. It was diverted and channelized in the first half of the 20th century and now enters Mission Bay through an artificial channel further east. Although diazinon was measured in the creek, water quality standards were not exceeded.
- Poway Creek (toxicity, selenium) in San Diego County, is a tributary to the Los Penasquitos Creek. It is the most upstream site of the SWAMP sites in the Los Penasquitos Creek watershed.

Data Analysis

In order to assess whether the variability seen in the SMC intercalibration study could be quantified and compared to the results observed in the data from the eight sites that have been placed on the 303(d) list, the level of variability seen in the intercalibration study was assessed. In the intercalibration study, *C. dubia* reproduction results were evaluated for toxicity on the basis of percent effect (Equation 1). The percent effect (closer to zero = nontoxic, closer to 100 = toxic) is as follows, with results from Round 1 included in Table 1:

$$Percent\ Effect = \frac{(Mean\ Control - Mean\ Sample)}{Mean\ Control}(100\%) \qquad (1)$$

$$Threshold\ Value = 100 - Percent\ Effect \qquad (2)$$

Therefore, as sample mean values get closer to control means, the closer to zero the Percent Effect becomes and the sample is considered nontoxic, as control and treatment reproduction rates do not significantly differ. When sample means are smaller than control means, the percent effect becomes closer to 100, and the sample is considered toxic. Higher *C. dubia* reproduction sample test results therefore result in lower percent effect, or a sample that calculates closer to nontoxic.

The CEDEN and SWAMP datasets used to calculate toxicity for 303(d) listings applied a two-tiered approach to evaluate toxicity. The first is a hypothesis-testing approach comparing the organisms in the samples to the responses from the controls using a t-test statistical comparison. The second tier is a comparison to an evaluation threshold value (Equation 2) that is 20% less than the control response (where the control response is assumed to be 100%). An analysis of the CEDEN data shows that the calculated threshold value is 100 minus the calculated percent effect value (referred to as Percent Control) with an evaluation threshold value of 80. Therefore, any Percent Control less than 20 (resulting in a calculated threshold value less than 80) does not pass the second-tier test. A site is considered toxic if the t-test shows that sample means are significantly lower than control means (p-value $\leq$ 0.05), and if the calculated threshold value (100 – percent effect value) is less than the evaluation threshold value (80%).[3]

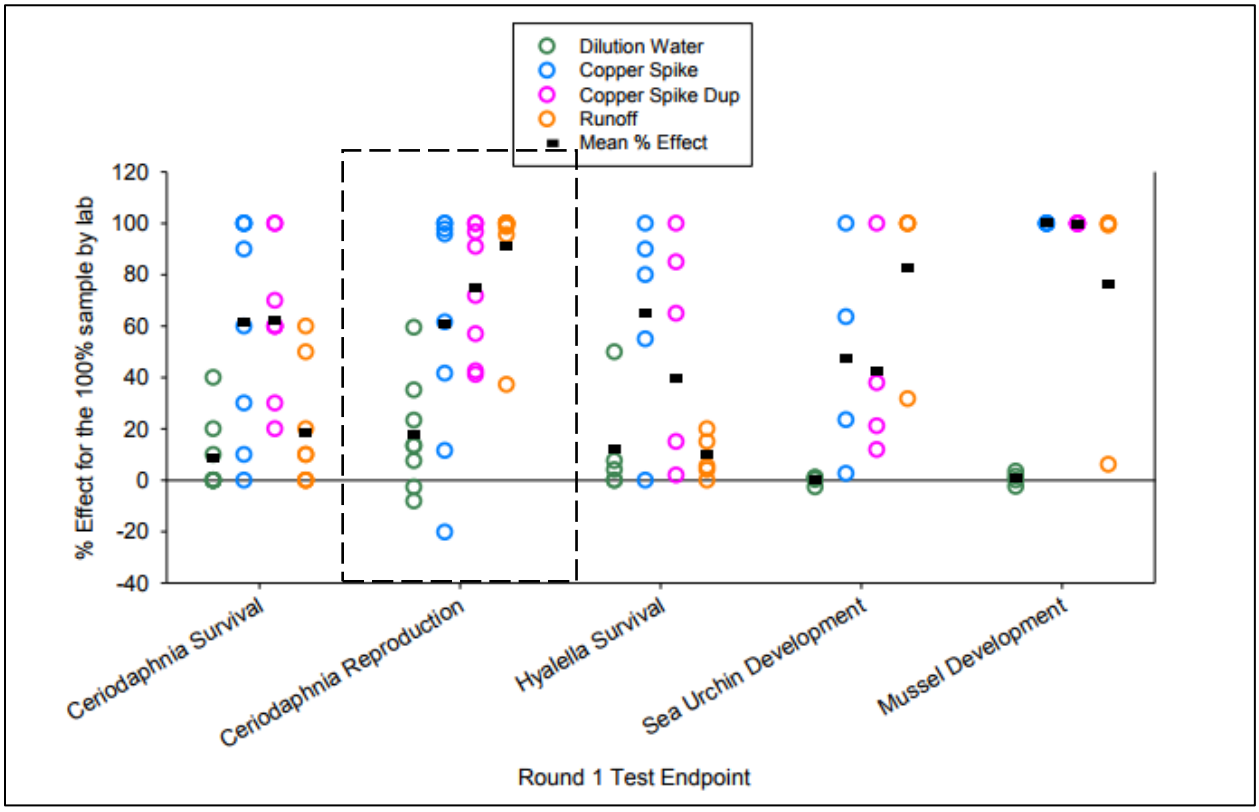
Figure 1 presents the graphical results from Round 1 of the SMC Intercalibration study; the data from the *Ceriodaphnia* reproduction test (surrounded by dashed outline in the figure) were used to quantify variability for application to the sample data for the current analysis. The range of effects that was applied to the data from the eight sample sites were determined using the magnitude of variability from (A) sample results, and (B) laboratory dilution water controls. The percent effect concentrations from each laboratory summarized in Figure 1 of the SMC Toxicity Testing Laboratory Guidance Document were used to calculate the standard deviation from the mean in Laboratory, Copper Spiking, and Runoff treatments. The resulting three levels of variability, two for the sample results (A) and one for the control water (B), are described below:

A. The environmental samples were examined using the standard deviation of percent effect concentrations found in Copper Spiking and Runoff treatment sample results.
    1. Adjust sample values to reach a percent effect of +/– 23% based on Runoff treatment.
    2. Adjust sample values to reach a percent effect of +/- 46% based on Copper Spiking treatment
B. Individual test controls were investigated using the standard deviation in percent effect concentrations observed in laboratory dilution water control results
    3. Adjust control values to reach a percent effect of +/- 22% based on Laboratory control

These levels of variability were applied to the individual toxicity test data sets for the eight sites where *C. dubia* reproduction toxicity experienced failure(s). Results from the application of this variability in the control and samples were evaluated to determine if the treatment impacted the result of the test (pass or fail) and thus the potential status on the 303(d) list.

[3] Significant compared to negative control based on statistical test, alpha of less than 5%, AND less than the evaluation threshold (Both criteria met)

Figure 1. SMC Intercalibration Study Round 1 results. Results from each laboratory are represented as a single point. Using the results from the eight laboratories, the standard deviation for each endpoint (dilution water, copper spike, runoff), was calculated and used as the variability applied to the data sets used in the current analysis. For example, the standard deviation of the copper spike from the eight sample points is 46%. Toxicity test used for data (Ceriodaphnia reproduction) is indicated by the dashed box (Figure from Schiff and Greenstein 2016).



Findings

Table 3 lists the number of pass/fails for each species and endpoint for the eight prioritized water bodies where *C. dubia* was tested in a review of the data obtained from CEDEN. The analysis reported here primarily focuses on the *C. dubia* reproduction endpoint as it showed the greatest variability in the inter-laboratory study and thus likely the largest potential for impacting impairments. The eight sites containing *C. dubia* reproduction toxicity tests reported as passes and failures were evaluated as a demonstration of the potential impact of the observed variability (Table 3). These 8 sites represent 12 failing and 16 passing individual sets of the individual *C. dubia* reproduction toxicity tests.

Table 3. Species and endpoints used as basis for toxicity testing for 303(d) listing of water bodies. All samples were collected between 2001 and 2003.[4]

Species	H. azteca		C. dubia		C. dubia		S. capricornutum	
Endpoint	10-day Survival		Reproduction		Survival		Count	
WATER BODY	Pass	Fail	Pass	Fail	Pass	Fail	Pass	Fail
Poggi Canyon			2	1	3	0	0	3
Santa Ysabel			4	2	6	0	1	5
Moro Canyon Creek	2	0	1	1	2	0	0	4
Poway Creek			3	1	4	0	0	4
Loma Alta Creek			1	3	4	0	0	4
Encinitas Creek			1	2	0	1	0	1
Oso Creek	1	0	2	1	2	1	0	4
Rose Canyon			2	1	3	1	1	3

Ceriodaphnia dubia Reproduction

Data treatments based on the three variabilities described above were applied to each of the individual *C. dubia* reproduction toxicity tests, where one of the variables from Equation 1 (sample or control) is altered while the other is unchanged, to determine if there is a resulting status change. The runoff (+-23%) was considered a lower range of variability to apply to the data samples, while the copper spiked laboratory water sample (+-46%) was considered a high range to apply to the data samples. The laboratory dilution water variability seen in the intercalibration study (+-22%) is the only variability applied to the laboratory control water. Our methodology was to use the variability seen in a study that shouldn't have had variability; it was in controlled conditions with application of the same methods and procedures. This was therefore considered representative variability.

Assuming these variabilities could be positive or negative two potential sets of scenarios result:

1. Fail to Pass (Scenario 1),
   Water sample: variability applied -22%, -46%; control sample variability applied 23%.
2. Pass to Fail (Scenario 2).
   Water sample: variability applied +22%, +46%; control sample variability applied -23%.

The first set of scenarios would result in failures becoming passing results by bringing the two results into greater agreement by either increasing the sample result or decreasing the control. Table 4 shows the resulting number of passing and failing tests for each of the demonstration sites for the data treatments.

Example – Poggi Canyon, May 15, 2003

This study used results presented in the SMC intercalibration study to compare eight data sets against the observed variability, where data was presented as percent effect for the test endpoints for each laboratory. Individual test values were adjusted to elicit a change in percent effect by the previously described variability ((±22 & ±46%); ±23%). Equation 1 was applied to see if results passed or failed the percent effect test. The mean of the reproduction values for the original data set for Poggi Canyon using the May, 2003 data is 7.25 (young females produced per test organism), whereas the mean of

[4] This is the only data obtainable on the CEDEN website.

the control sample was 18.2. Applying Equation 1, this results in a percent effect of 60%, or failure of the toxicity test. (This site passed both January and April sample events, but failed this May event).

(18.2-7.25)/18.2 * 100 = 60%

To apply the quantified variability of -22% effect, the original data values (mean of 7.25) were multiplied by a factor (1.55) to elicit a -22% effect change, which resulted in an increase of the mean to 10.87; subsequently, the calculated percent effect decreased to 38%, which still fails the test.

(18.2-10.87)/18.2 * 100 = 38%

This was similarly done for the 46% copper spiking treatment; the individual values were multiplied by 2.15, resulting in an overall mean of 15.58, for a -46% effect increase. This resulted in the sample passing the toxicity test with a percent effect value of 14%.

(18.2-15.58)/18.2 * 100 = 14%

Table 4. Resulting number of toxicity test results for C. dubia reproduction under different Scenario 1 variability conditions.

Scenario 1 Variability Treatments (C. dubia Reproduction)			Sample				Control	
	Original Sample		-22%		-46%		+23%	
	Pass	Fail	Pass	Fail	Pass	Fail	Pass	Fail
Poggi Canyon	2	1	2	1	2	1	2	1
Santa Ysabel	4	2	4	2	6	0	4	2
Moro Canyon Creek	1	1	2	0	2	0	0	2
Poway Creek	3	1	4	0	4	0	3	1
Loma Alta Creek	1	3	2	2	4	0	1	3
Encinitas Creek	1	2	2	1	3	0	1	2
Oso Creek	2	1	3	0	3	0	1	2
Rose Canyon	2	1	3	0	3	0	2	1

Table 5 summarizes the number of individual test results that would change result and shows that in the first scenario when the lower sample variability treatment (-22%) is applied, 7 of the 12 *C. dubia* reproduction toxicity test failures met the pass requirements and thus would have changed the test result. 12 of the 12 test failures could have passed based on the higher variability treatment (-46%). Similarly, when the variability is applied to the control water (+23%) 7 of the 12 failures could have passed, changing the test result.

The second scenario would increase the relative difference of the control and sample and thus could result in a passing test failing by lowering the sample count results or raising the control results. Table 6 shows the resulting test outcomes for these treatments. When the sample variability is applied, 10 of the 20 results changed from pass to fail with the lower degree of variability applied (+22%) and 15 would change based on the higher degree of variability (+46%). The -23% treatment for the control values resulted in 11 shifts from pass to fail.

Table 5. Resulting number of test result toxicity status changes for C. dubia reproduction under different variability conditions. Based on 8 demonstration sites with 12 failed tests and 20 passed tests.

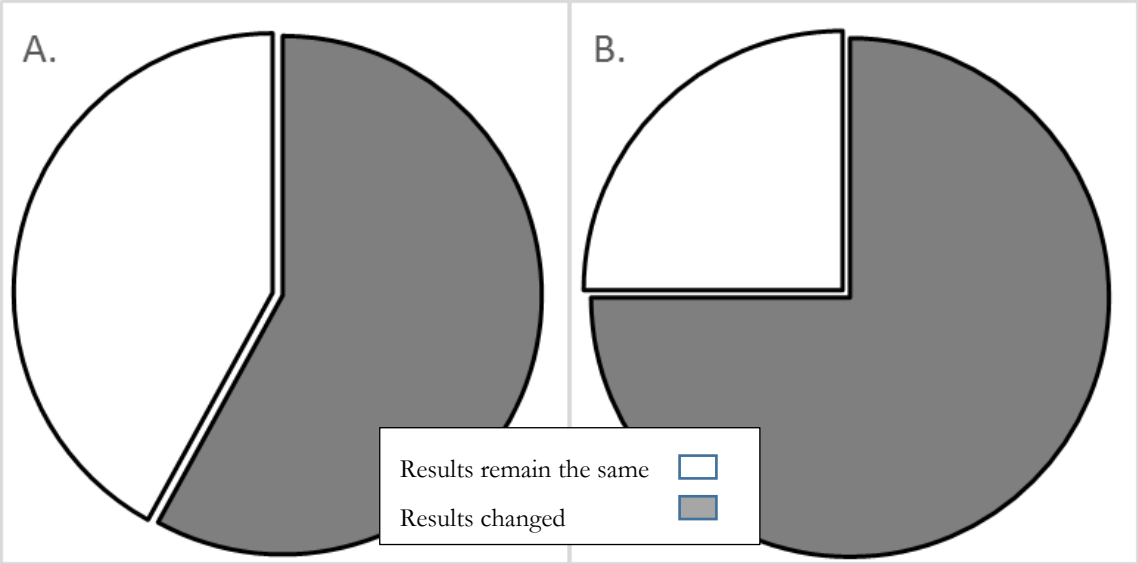
Summary of Test Status Changes Based on Data Variability Treatments	**Samples**		**Control Water**
	22%	**46%**	**23%**
Scenario 1 - Fail to Pass (n=12)	7	12	7
Scenario 2 - Pass to Fail (n=20)	10	15	11

Table 6. Resulting number of toxicity test results for C. dubia reproduction under different Scenario 2 variability conditions.

Scenario 2 Variability Treatments (C. dubia Reproduction)			Sample				Control	
	Original Sample		+22%		+46%		-23%	
	Pass	Fail	Pass	Fail	Pass	Fail	Pass	Fail
Poggi Canyon	2	1	1	2	0	3	1	2
Santa Ysabel	4	2	1	5	0	6	1	5
Moro Canyon Creek	1	1	0	2	0	2	0	2
Poway Creek	3	1	1	3	1	3	1	3
Loma Alta Creek	1	3	1	3	1	3	1	3
Encinitas Creek	1	2	1	2	1	2	1	2
Oso Creek	2	1	1	2	1	2	1	2
Santa Margarita	4	0	3	1	0	4	3	1
Rose Canyon	2	1	1	2	1	2	1	2

These results show the potential impact to the listing status of water bodies. Particularly as even under the lower variability scenarios, more than 50% of the test results could be overturned in either direction, and under the higher scenario 58 – 75% of the samples could be overturned (Figure 2). These demonstration sites can be considered analogous to potential impacts on all water bodies listed and non-listed for impairments as the pass/fail results could potentially be affected in either direction.

Figure 2. Pie charts showing the number of individual toxicity tests that could change from (a) fail to pass and (b) pass to fail based on the intercalibration copper spiking variability applied to representative environmental samples where white remain the same and the shaded portions change test results.



Applying the results of these variations to the 303(d) listing criteria reveal that not only the individual test results can change but also the 303(d) listing status could be affected. A demonstration of this is Encinitas Creek, where under Scenario 1 treatments the number of failing events would be reduced from 3 to 1. The reduction in *C. dubia* reproduction failures leaves one event failure (for *C. dubia* Survival and *S. capricornutum*) and thus the listing criteria of 2 failing events is not met. If *S. capricornutum* toxicity, which was detected at all eight sites is not considered (discussed below), 7 of the 8 sites (all but Loma Alta Creek) would not meet the two failing event criteria to be listed as impaired under the Scenario 1 treatments. Thus, the potential variability observed in *C. dubia* reproduction presents a significant challenge to compromise real world outcomes of individual toxicity tests as well as impairment listings.

Other Potential Variability in Species/Endpoints

Hyalella Azteca

The first round of intercalibration tested found low comparability in *H. azteca* samples. There was only one failure at three sites in this dataset (Jamul Creek), whereas it should be noted that these data were collected and analyzed between 2001 and 2003 when this species was used infrequently. A review of these results only calls this one water body into question. However, the fact that Jamul Creek passed the two *C. dubia* tests, and the small margin by which it failed the *H. azteca* test, do bring potential variability into question. The one failure had a survival percent mean of 70 percent and a p=0.001. The passing sample had a mean of 78 percent and a p=0.051(tests were run with alpha of 5%). The samples were taken approximately 3 weeks apart in spring 2003, with the failing sample taken during a higher base flow. Jamul Creek is noted as potentially impacted based on *H. azteca*

potential variability. The importance of determining if this site is subject to variability is due to the fact that it is only 303(d) listed for toxicity, no other constituents.

Selenastrum capricornutum

The algae species *Selenastrum capricornutum* was not included in the intercalibration study; however, it was found toxic in all but one site in this analysis. Thus, a closer look at the impacts of potential variability similar to the intercalibration species should be considered.

An assessment of the ecological health of the 11 San Diego Region hydrologic units conducted in 2002-2003 included a review of water chemistry, toxicity, and fish. Toxicity was found at nearly all the sites, predominantly due to this algae species, with occasional toxicity to *H. azteca* or *C. dubia*. Based on the variability in the intercalibration study for other species and the frequency of toxicity observed for *S. capricornutum,* intercalibration for algal species should be considered. This is particularly important because multiple species can be required to be tested for toxicity, and toxic results from only one species are required for listing. Therefore, many of these water bodies would have been on the 303(d) list regardless of whether they showed toxicity to *C. dubia* or *H. azteca.*

Known interferences that can result in *C. dubia* variability includes contamination by glassware, testing equipment, sample handling, pathogenic or predatory organisms in the dilution water, food, and especially pH drift. These same interferences can be found in *S. capricornutum* testing, with additional interferences such as the frequency of shaking the algae samples (2x/day or continuous), which can have a significant outcome on test results due to suspension and growth; type of cell count applied (flourometer, microscope); internal competition by native algae; and conductivity. Effluent-dominated systems can be highly affected by interferences due to the polymers and clarifiers used in the coagulation process that can produce false toxicity. The presence of metals can also have differing effects on the results. These additional confounding issues may require additional guidance for laboratories as well.

Discussion

This study shows that a significant number of the individual toxicity test results could potentially change based on the variability observed in the intercalibration study. Most significant was the *C. dubia* reproduction test, where as many as 75% of the passing results could have changed to failures, and 58% of the failing results changed to passing results based on the intercalibration copper spiking variability. Furthermore, this study has revealed that based on these changes, seven of the eight water bodies considered in this study contained individual tests for which the change in results would have affected the site's 303(d) listing status based on intercalibration variability.

These results present an important potential impact to regulatory impairment status. This is particularly impactful when considering that listing a water body only requires two samples with toxicity exceedances, and delisting requires 28 samples with no more than two exceedances. This means that based on the variability of 22 – 46% used in this study, there is a high probability that almost any sample is susceptible to switching results and thus of at least one sample used for delisting could be affected. This highlights the potential expenditure of significant costs to assess whether these sites are

toxic, or if the toxicity was a result of interferences in testing or variability in laboratory methods or laboratories. Therefore, sample collection, laboratory analysis, and variability must be assessed to ensure that sites are correctly listed, or can reasonably be delisted. This variability makes it difficult to determine if toxic results are due to the sensitivity of the different species, an artifact due to variability in testing procedures, or perhaps the sites are truly exhibiting toxicity. If the toxicity was due to method or laboratory variability, re-evaluation of these sites using the updated standardized protocols could be considered as part of the anticipated Total Maximum Daily Load (TMDL) development within the next 5 years. This reevaluation, however, would have the caveat of being reflective of current conditions and not the time of original sampling.

This uncertainty affects the management and regulatory actions on water bodies not just to SMC organizations, but throughout California. This should be a primary concern to dischargers and regulatory agencies that rely on toxicity testing for management responses such as permit compliance, toxicity identification evaluations, or TMDLs. According to the Regional Board (SWAMP 2011), in monitoring conducted between 2001 and 2010, greater than 50 percent of the collection sites have shown some degree of toxicity in fresh water. Correlation analyses and toxicity identification evaluations (TIEs) used to determine the likely causes of surface water toxicity suggested that toxicity to invertebrate test species was most often caused by pesticides. Results of statewide water toxicity tests with the three standard EPA test species show that more samples were toxic to fish larvae and algae than to water fleas (*C. dubia*) (Water Board, 2011). It is of interest that the findings have significant algae toxicity results, indicating the potential presence of herbicides, metals, as well as pyrethroid pesticides. Because most 303(d) listings due to toxicity are based on "unknown source," and no TIE or TMDL has been conducted, it is hard to attribute cause.

The variability observed during the SMC intercalibration study for *C. dubia* reproduction is not uncharacteristic of the variability observed by others. Moore et al. (2000) used split samples of lab dilution water among 16 laboratories for *C. dubia* reproduction tests and observed a mean response of 16% effect and a standard deviation of 28% effect, not dissimilar to the intercalibration study laboratory dilution water split samples where responses ranged from 16 to 27% effect, and standard deviation of 19 to 27% effect.

Outcomes

The continued variability observed in the intercalibration study has been demonstrated here to have the potential to directly affect the listing or non-listing of waterbodies as impaired for toxicity on the 303(d) list. This presents difficult implications and costs to SMC members and the broader storm water community. These revelations present a clear need to address this variability as the status quo represents uncertain results. There are two choices the community has to address these identified uncertainties:

1) Discontinue use of *C. dubia* and use alternate species approved by EPA, or
2) Conduct additional interlaboratory intercalibration studies to improve them.

Cooperation and coordination between the regulators and the agencies will be required to effectively open a channel of communication to jointly explore what policy or procedures need to be changed to rectify 303(d) listings as we move forward. Consideration must also be made as to how to feasibly recover from incorrect inclusions on the list.

The choice to discontinue the use of *C. dubia* reproduction toxicity means that results of other toxicity tests would need to carry more weight in future tests, as well as in the historical record. *C. dubia* does represent the most common species utilized for toxicity by SMC members over the past 20 years and thus its continued use would be valuable. This could be accomplished through the survival test by which using lessons learned in the intercalibration study has shown to significantly increase comparability.

The second choice to improve this test offers opportunities to understand the factors causing the observed variability and thus interpret historical results. This could potentially allow for confidence in historical records or the culling of samples suspected to be affected. Similar efforts to those undertaken following the first round of the intercalibration study to reduce variability in *C. dubia* survival and *H. azteca* can be applied to *C. dubia* reproduction toxicity. An intercalibration study would be supplemented by comparing real-world ambient water samples, in addition to blanks, spiked samples, or artificial samples. Recommendations for such a study include improved study designs, testing frequency, and onboarding new laboratories.

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