Appendix A

FINDINGS REPORT

From A Critical Review of:

Full Life-Cycle Bioassay Approach to Assess Chronic Exposure of *Pseudodiaptomus forbesi* to Ammonia/Ammonium - Final Report

Dated August 31, 2011

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Submittal Date:
December 26, 2011

1. INTRODUCTION

On behalf of the Central Contra Costa Sanitary District (CCCSD), Larry Walker Associates has contracted Pacific EcoRisk, Inc. (PER) to perform a critical review of the "Final Report: *Full Life-Cycle Bioassay Approach to Assess Chronic Exposure of Pseudodiaptomus forbesi to Ammonia/Ammonium*" authored by Teh S, Flores I, Kawaguchi M, Lesmeister S, and Teh C (dated August 31, 2011).). As requested by CCCSD, the primary focus of this review were the experiments described as Subtasks 3-3 and 3-4-1 in the Teh *et al*. report. Additional comments on study methodology and data analysis were developed and can be provided to interested parties on request as evidence that additional study is needed.

2. COMMENTS ON SUB-TASK 3-3 (CHRONIC [31-DAY] LIFE CYCLE TOXICITY TESTING)

Comment #1. Teh *et al*.'s analysis of the number of nauplii and number of juveniles produced during the chronic (31-day) exposure is believed to be flawed at a very fundamental level. It is apparent in Teh et al.'s derivation of 'mean number of nauplii, juveniles, and adult *P. forbesi* produced per female' (in Teh *et al*.'s Table 11) and in the 'sum total number of nauplii, juvenile, and adult *P. forbesi* produced' (in Teh *et al*.'s Appendix III table) that they summed the counts of nauplii and juveniles that were counted on the progressive 2-3 day intervals (the raw data for these counts were provided in Teh *et al*.'s Appendix I) as if each new progressive count was of new individuals that had not been counted on the previous count day. So when 17 nauplii were counted in Control replicate A on Day 5 of the test, and 20 nauplii were counted on Day 7, and 17 were counted on Day 10, and so on, Teh *et al*. summed these up as if they were different nauplii that had been produced during the progressive 'count days'.

This would be correct had the nauplii and juveniles that were counted on each 'count day' been removed from the original replicate container and transferred to a new replicate container such that any nauplii or juveniles observed and counted in the original replicate containers on subsequent days would have been new organisms separate and distinct from the organisms that had been counted during the previous count day(s). Note that this approach would have created a logistical challenge, with a doubling of the number of experimental replicate beakers on Day 3 of the test (going from the original n=20 to n=40), a tripling of the beakers on Day 5 (n=60), a quadrupling of beakers on Day 7 (n=80), and so on and so on. This would then be compounded as nauplii that had transformed into juveniles would again need to be transferred to new replicates so as to allow observation of new juveniles produced by the remaining nauplii. The number of necessary beakers rapidly becomes logistically improbable.

However, it is not believed that this is what happened. Unfortunately, their report's inadequate description of test methodology is not explicit on this. However, it can be deduced from the nature of the study that the neonates were left in place in each replicate, as these were the source of the subsequent juveniles, which were similarly left in place to serve as the source for the

subsequent adults. This was confirmed by inquiry made with one of the other authors of the report (M Kawaguchi, pers. comm.). As a result, when 20 nauplii were counted in Control replicate A on Day 7, some (if not most) of these organism were the very same organisms that had been counted on the earlier Day 5 count, and the nauplii that were counted on Day 10 were some of the same as had been counted on Days 7 and Day 5.

This conclusion is also supported by the following observations made for closely-related congener *Pseudodiaptomus annandalei* (Golez et al. 2004):

1. hatching of the first brood of nauplii occurs within 24-hrs of spawning;
2. females produced new ovisacs at ~ 1 day intervals, again with hatching occurring within that 24-hrs;
3. "females that were isolated from males produced only two clutches of viable eggs". Additional ovisacs were produced (making it appear that the female is reproductive), but the "succeeding clutches of eggs were aborted or shed off within 48 hrs and never hatched out".

Of course, the reproductive biology of *P. forbesi* may differ from that of the congener *P. annandalei*; however, in the absence of contradictory empirical evidence, Occam's razor would dictate otherwise.

We are left to conclude that **Teh *et al*.'s reported results for 'total number' and 'mean number per female' for the nauplii and juveniles are incorrect, and that their analyses of that data are similarly incorrect**.

Interestingly, in Teh *et al*.'s analyses of the 'total number' and 'mean number per female' of adults produced during the study, the number of adults counted on each progressive 'count day' were **NOT** summed in similar fashion, with Teh *et al*. instead evaluating on the count data from a single 'count day' (Day 31).

Comment #2. While it is believed that Teh *et al*.'s count data are incorrect, let us assume for a moment that they are in fact correct. The organism counts using Teh *et al*.'s summation method are summarized in Table 1 below. When their juvenile count data are analyzed using CETIS (a statistical software specifically designed to analyze aquatic toxicity data), the NOEC and LOEC are shown to be 0.79 mg/L TAN and 1.62 mg/L TAN (Table 2 below), NOT the lower concentrations reported by Teh *et al*.

It should noted that CETIS is the statistical software most commonly used by toxicity testing labs to analyze toxicity test data, and is believed to be the statistical software used at the UC Davis Aquatic Toxicology Lab; indeed, Teh *et al*. used CETIS to analyze their Subtask 3-4-1 and Subtask 3-4-2 experimental data as evidenced in Appendices IV and V of their report.

It should also be noted that our assessment of problems with Teh *et al*.'s statistical analyses should not be interpreted as indicating that there was no effect resulting from the ammonia, but

simply that the experimental data do not support any differences that were observed as being statistically significant.

Table 1. Production on *Pseudodiaptomus forbesi* nauplii, juveniles, and adults (from Appendix I in Teh *et al*. report)

Test Treatment (mg/L TAN)	Test Replicate	Total # of *Pseudodiaptomus forbesi* Life Stage Counted			
		Nauplii[A]	Juveniles[A]	Adults[A] (counts made only on Day 31)	Adults[B] (counts made as for nauplii & juveniles)
Control	A	86	38	11	93
	B	100	73	26	178
	C	68	45	7	122
	D	75	52	3	52
0.36	A	60	27	0	1
	B	62	57	3	36
	C	83	79	18	167
	D	71	43	7	77
0.79	A	24	48	10	77
	B	64	31	4	45
	C	41	17	1	17
	D	52	22	8	77
1.62	A	47	1	0	0
	B	32	0	0	0
	C	46	14	5	28
	D	54	23	19	108
3.23	A	15	1	1	4
	B	39	1	1	6
	C	42	18	13	83
	D	30	13	5	34

A - For the nauplii and juveniles, Teh et al. summed the progressive counts on successive days as separate individuals; as explained in our review, this is believed to be erroneous, and is inconsistent with the counts of the "produced" adults which consist of the number of adults that were alive on Day 31 of the test.

B - Counts of "produced" adults using the summation of the progressive counts on successive days as separate individuals (as used by Teh et al. for the nauplii and juveniles); as explained in our review, this is believed to be erroneous.

Table 2. Comparative analyses of juvenile and adult production in the 31-day test (from CETIS analysis of juvenile data using Teh et al. summation method)

Statistical Endpoint	Juveniles		Adults	
	Teh et al. Analyses	CETIS Analyses	Teh et al. Analyses	CETIS Analyses
NOEC =	0.36 mg/L TAN	0.79 mg/L TAN	**<0.36 mg/L TAN**	**3.23 mg/L TAN**
LOEC =	0.79 mg/L TAN	1.62 mg/L TAN	**0.36 mg/L TAN**	**>3.23 mg/L TAN**
Chronic Value =	1.13 mg/L TAN	1.13 mg/L TAN	<0.36 mg/L TAN	>3.23 mg/L TAN

Chronic Value = geometric mean of NOEC and LOEC.

Comment #3. Teh *et al*.'s apparently erroneous statistical analysis of the adult data is even more significant (Table 2). Teh *et al*. reported that the NOEC and LOEC for adults were <0.36 mg/L TAN and 0.36 mg/L TAN, respectively. However, their inter-replicate variability for that endpoint is so high (CVs ranged from 70% to 150%) that even qualitative evaluation suggests otherwise. CETIS analysis indicates that the NOEC and LOEC are 3.23 mg/L TAN and >3.23 mg/L TAN.

Again, it should be noted that our assessment of problems with Teh *et al*.'s statistical analyses should not be interpreted as indicating that there was no effect resulting from the ammonia, but simply that the experimental data do not support any differences that were observed as being statistically significant. Certainly, the NOECs and LOECs resulting from this experiment should not be considered suitable for use in a regulatory framework.

3. COMMENTS ON SUBTASK 3-4-1 (EFFECTS OF AMMONIA ON NAUPLII PRODUCTION OVER 3 DAYS)

Comment #4. In this test, Teh *et al*. exposed individual gravid female copepods to TAN concentrations of 0 (control treatment), 0.38, and 0.79 mg/L for 3 days after which the number of nauplii produced were counted. The results of this test have been summarized in the Table 3 below.

From data reported in Teh et al.'s Table 12 and Appendix V:

Table 3. Effects of ammonia on *P. forbesi* production of nauplii over 3 days (Teh *et al*. Subtask 3-4-1).

TAN Concentration (mg/L)	Mean # of Nauplii per Female
Control	7.6
0.38	5.5
0.79	5.4

The results from this test are somewhat troubling in that, while technically monotonically increasing as the ammonia concentration increases, no apparent concentration-response relationship is observed between the 0.38 mg/L treatment and the 0.79 mg/L treatment. One would expect that as the TAN concentration increases from 0.38 mg/L (a presumably toxic concentration) to 0.79 mg/L (a two-fold greater concentration), there should be an increase in the toxic response – this is a fundamental paradigm of toxicology.
We have already seen in the data evaluations presented above that there is variability in toxic responses made by these organisms. Indeed, in some cases, the variability has been so extreme as to preclude a meaningful statistical analysis (as in the case of the adult data from the 31-day test). The absence of the expected concentration-response in the current test (Table 3) suggests that variability in organism response is occurring (the CV was 48% in the 0.38 mg/L treatment) such that the treatment means may be deviating from the true population mean (in statistical terms, this is referred to as a "false positive" or a "false negative").

In the present case, it is impossible to determine which of the two test responses is deviating most from the true population mean response. However, it is worth noting that:

1. there were two replicates at the 0.38 mg/L treatment that had 10 nauplii (the highest number observed in ANY replicate) whereas there was only one replicate at the control treatment that had 10 nauplii, and
2. the CV at the 0.38 mg/L treatment was 48%, which was markedly higher than at the Control or 0.78 mg/L treatment.

This is suggestive that the variability at the 0.38 mg/L treatment was elevated and may have resulted in a false positive, such that the observed mean response of 5.5 nauplii per female was lower than the true population mean. If correct, then the conclusion(s) drawn from the test data may not reflect true conditions, and the true LOEC could be 0.79 mg/L, and not 0.38 mg/L. At a

minimum, the absence of the expected concentration-response should cast enough uncertainty on the test results as to make them inappropriate for regulatory decision-making.

Comment #5. It is fortunate that multiple sets of test data from the study allow comparison of results between tests; for instance, the results of Subtask 3-4-1 can be compared to those generated in the earlier Subtask 3-3 (31-day) test in which gravid females were exposed to varying concentrations of TAN and counts of nauplii produced after 3 days were counted, but were also counted after 5 days and 7 days (recall that counts made on progressive count days are not believed to be all new organisms). The Subtask 3-3 data are summarized in Table 4 below, along with the data from Task 3-4-1.

If one were to "cherry-pick" the Day 3 data and exclude the additional data, then Teh *et al.*'s conclusion for the Subtask 3-4-1 might stand. However, by extending the observation period beyond 3 days, it becomes evident that not only is there no reduction in nauplii production at 0.36 mg/L TAN, but nauplii production actually appears to be *increased* relative to the control treatment (the maximum mean # of nauplii on Day 5 at the 0.36 mg/L TAN treatment is **31% greater** than the highest mean # of nauplii produced in the Control treatment on any of the count days). Furthermore, CETIS analysis indicates that there were no statistically significant reductions in nauplii production at the 0.36 mg/L (Table 5). Even if we use the count summation used by Teh et al., by extending the counts beyond 3 days, it becomes apparent that there is no statistically significant difference between the response at 0.36 mg/L TAN and the Control treatment. This certainly creates a very significant uncertainty over the results of the Subtask 3-4-1 test of the effects of ammonia on nauplii production over 3 days.

It could be argued that this phenomenon is the result of ammonia having caused a delay in egg hatching, and the 31-day data are certainly suggestive of that. However, the only way to address that would have been to have some information from the scientific literature on the egg gestation period for this species, coupled with testing being performed under the current test conditions using females with egg sacs of the same age.

Table 4. Effects of ammonia on *P. forbesi* nauplii produced over 3 and 5 days.

Teh *et al.* Study Task	TAN Treatment (mg/L)	Mean Number of Nauplii per Female		
		Day 3	Day 5	Sum through Day 5 (Day 3 + Day 5)[A]
Subtask 3-4-1	Control	**7.6**	not counted	not counted
	0.38	**5.5**	not counted	not counted
	0.79	**5.4**	not counted	not counted
Subtask 3-3	Control-A	5.67	6.67	12.33
	Control-B	6.67	6.67	13.33
	Control-C	5	5	10
	Control-D	5	5	10
	treatment mean	**5.6**	**5.8**	**11.4**
	0.36-A	3	5	8
	0.36-B	2.33	8.33	10.67
	0.36-C	3.33	8.33	11.67
	0.36-D	3.33	3.33	6.67
	treatment mean	**3.0**	**6.3**	**9.3**
	0.79-A	0.33	1.67	2
	0.79-B	6.67	3.33	10
	0.79-C	2.67	2.67	5.33
	0.79-D	6.67	4	10.67
	treatment mean	**4.1**	**2.9**	**7.0**

A – These counts are made using method of Teh *et al.*, which assumes that the progressive counts on successive days are separate individuals; as explained in our review, this is believed to be erroneous.

Table 5. Comparison of nauplii production test results (all results expressed as mg/L TAN) (from CETIS analysis of data)

Statistical Endpoint	**Subtask 3-4-1**	**Subtask 3-3**				
	Day 3	Day 3	Day 5	Day 3 + Day 5[A]	Total (31 days)[A]	Total (31 days)[B]
NOEC =	<0.38	3.23	0.36	0.36	0.36	0.79
LOEC =	0.38	>3.23	0.79	0.79	0.79	1.62
Chronic Value =	<0.38	>3.23	0.53	0.53	0.53	1.13

Chronic Value = geometric mean of NOEC and LOEC.

A – These counts are made using method of Teh *et al.*, which assumes that the progressive counts on successive days are separate individuals; as explained in PER's review, this is believed to be erroneous.

B – These counts are made using what is believed to be the best remaining method: identifying the maximum number of nauplii observed on any given day for each replicate (this assumes that the individuals were left in the replicate beakers and were counted again and again on progressive days [i.e. repeated measures]).

4. FINAL COMMENT

The reviewer is troubled by the absence of any discussion by Teh et al. regarding the variability in their test response data, either between tests or within tests (i.e., inter-replicate variability). Without such acknowledgement, it is left for the non-scientist to assume that the data as presented are definitive. Moreover, it raises the question of whether the data from this study are adequate (or 'ready') for use in regulatory decision-making. However, it is important to note that this critical review is not intended to negate Teh *et al*.'s general observations that ammonia is toxic to naupliar, juvenile, and/or adult *P. forbesi* at elevated concentrations and that this toxicity is strongly influenced by pH. Indeed, the primary question of 'what are the effects of ammonia on *P. forbesi*' is relevant and Teh *et al*.'s study results certainly compel a more thorough examination of this. However, the problems associated with Teh et al.'s experimental methodology for Subtasks 3-3 and 3-4-1 and significant questions regarding the analysis of the resulting data do indicate that the quality of the work should preclude the resulting 'critical threshold' data (i.e., NOECs, LOECs, and point estimates [e.g., ECx, LCx, and ICx values]) from being used for regulatory purposes.

References Cited:

Golez MSN, Takahashi T, Ishimaru T, Ohnoa A (2004) Post-embryonic development and reproduction of *Diaptomus annandalei* (Copepoda: Calanoida). Plankton Biology & Ecology 51(1):15-25.

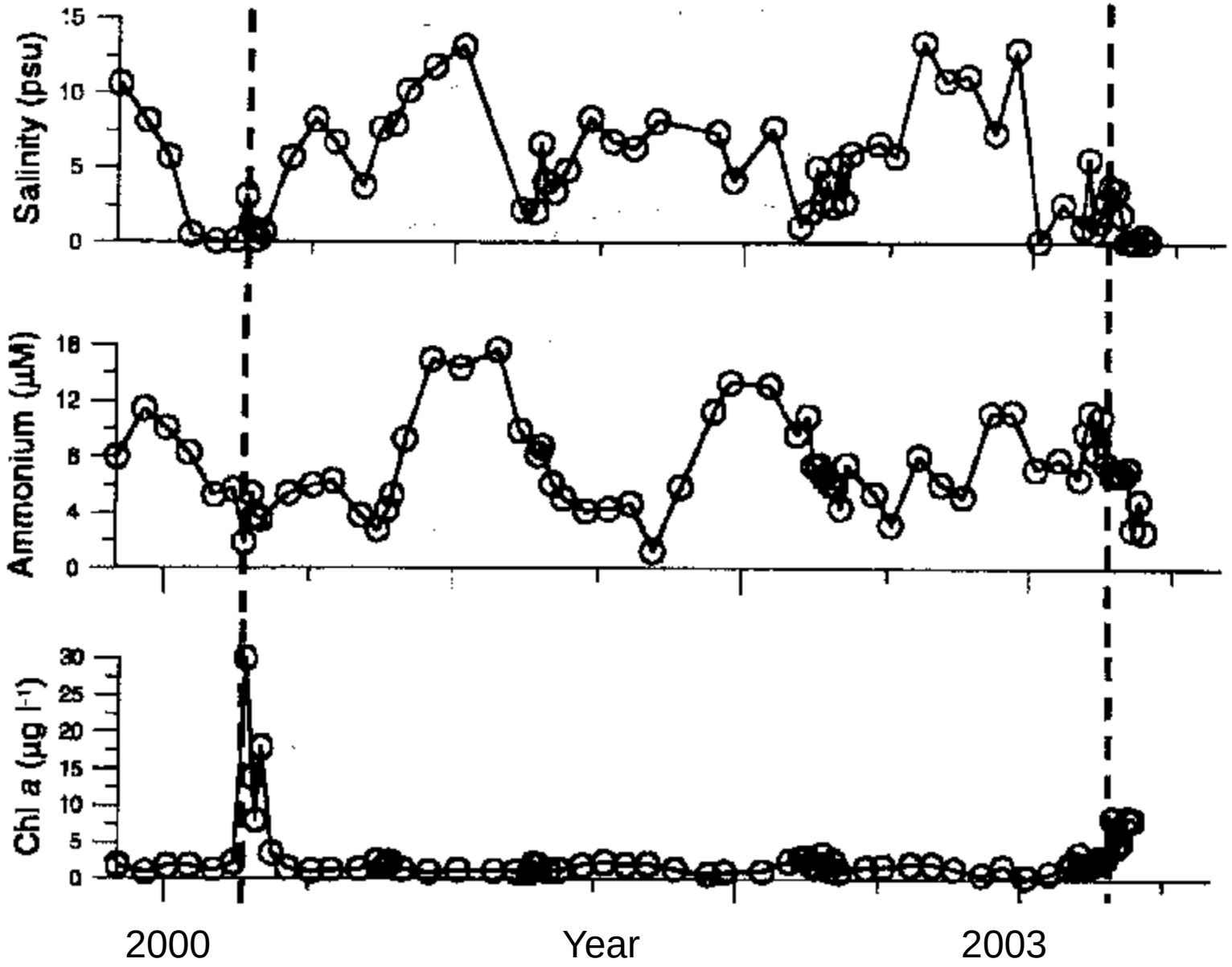
Appendix B

Phytoplankton (Chlorophyll a) versus Ammonium Concentrations

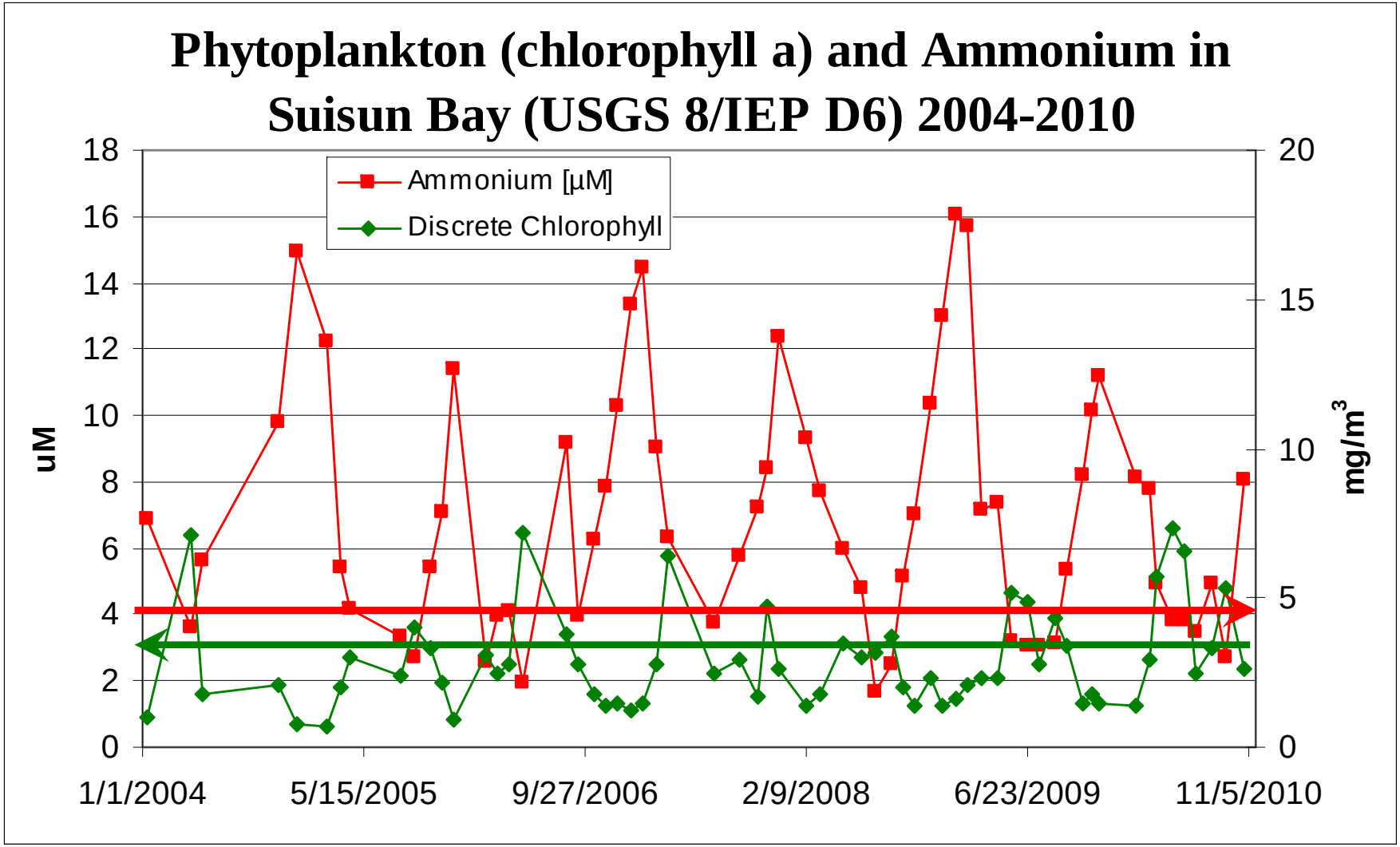
in Suisun Bay [1977-2010]

Dr. Dugdale et al., hypothesis that ammonia-N has to drop below 4 µ moles for phytoplankton bloom to occur is not fully supported by ambient data.

(1) In four years of monthly monitoring in Suisun Bay (November 1999–August 2003) by Dr. Dugdale et al., ammonia-N dropped below 4 µ moles 5 times, yet bloom occurred only one time.

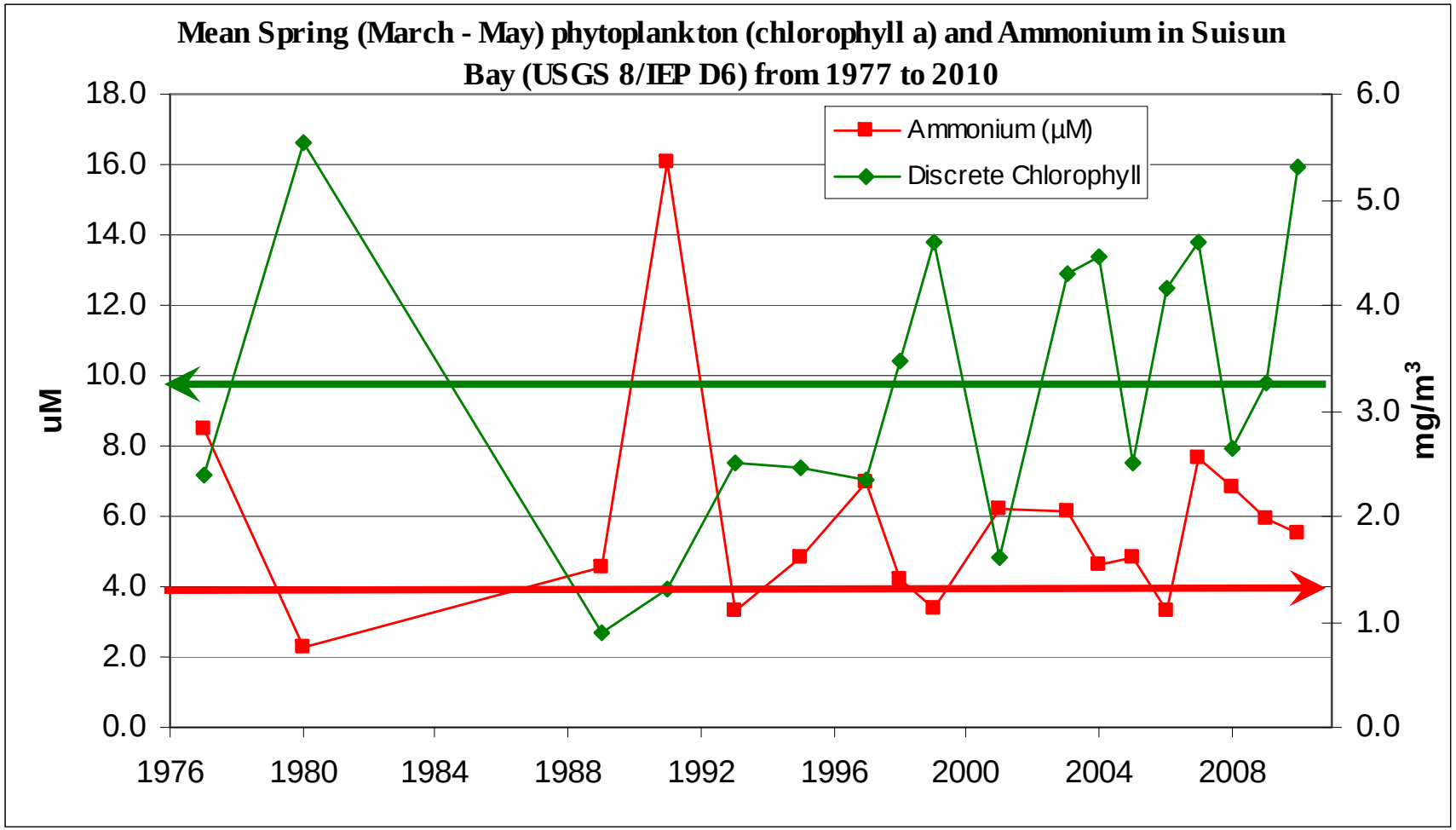

Salinity (psu)
15
10
5
0
Ammonium (µM)
16
12
8
4
0
Chl a (µg l-1)
30
25
20
15
10
5
0
2000
Year
2003

(2) From 2004 to 2010 ammonia dropped below 4 µ moles 20 times, yet there were only 5 blooms.


Phytoplankton (chlorophyll a) and Ammonium in Suisun Bay (USGS 8/IEP D6) 2004-2010
Ammonium [µM]
Discrete Chlorophyll
uM
mg/m3
18
16
14
12
10
8
6
4
2
0
20
15
10
5
0
1/1/2004
5/15/2005
9/27/2006
2/9/2008
6/23/2009
11/5/2010

(3) From 1977 to 2010, the spring mean chlorophyll reached above average only 3 out of 9 times (33%) when ammonia was below 4 µ moles.

This suggests that there are other factors more important than ammonia for increased chlorophyll production.


Mean Spring (March - May) phytoplankton (chlorophyll a) and Ammonium in Suisun Bay (USGS 8/IEP D6) from 1977 to 2010
Ammonium (µM)
Discrete Chlorophyll
uM
mg/m3
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
1976
1980
1984
1988
1992
1996
2000
2004
2008