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August 31, 2011

Ms. Linda Dorn
Environmental Program Manager,
10060 Goethe Road,
Sacramento, CA 95827-3553

Dear Ms. Linda Dorn,

Enclosed are our responses to comments and suggestions from the Sacramento Regional County Sanitation District (SRCSD). We believe that our revisions to the manuscript based upon your suggestions have significantly improved it.

Thank you very much for your comments and suggestions. I apologize for taking so long to get the revisions back to you.

Your Sincerely,

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General Comment 1: *It would be helpful to provide more detailed data in an appendix. At a minimum a more complete description of the methods, test validation criteria and complete data sets are needed. Further peer review should be performed on an updated report with this additional information, in order for the usefulness of these data and conclusions to be evaluated. Repeated testing to reduce uncertainty in these data and to confirm any findings is also imperative before any weight can be given to these findings with non-standard test species. The bioassay testing procedures used throughout this study are not standardized methods and there are no references to where they can be found in the published literature. Furthermore, they are not fully described in this report. Test dates are not presented for any of the reported bioassays. Likewise, no reference toxicant test data are reported.*

See our responses in the specific comments. But to briefly summarize, we have included a more complete summary of test data in the appendix and tables of this report. Methods for acute and chronic toxicity testing and ammonia measurements have also been added in Task 2A. The description and listed citations should be sufficient for another laboratory to repeat our results. Test initiation dates for each task were also added. Please note that the follow up testing was conducted in subtasks 3-4-1 and 3-4-2 to confirm the key findings about the sensitivity of less than 3-day old nauplii obtained from the full life cycle tests conducted several months earlier in subtask 3-3. Test results from subtask 3-4-1 confirmed the earlier results and suggest that ammonia levels in the Sacramento River are at potentially toxic levels to less than 3-day old nauplii. We would welcome a second independent laboratory repeating our study.

As *P. forbesi* is a non-standard resident species, the test methods that we employed, although considered in the developmental phase, are quite similar to the procedure used in Augspurger et al. (2003) for initial toxicity testing of the freshwater mussel. Toxicity testing followed our published laboratory culture techniques and conditions (Ger et al, 2009a and 2009b) as well as USEPA methods. Acute toxicity protocols were based on standard acute toxicity testing procedures as outlined in USEPA (EPA/821/R-02/012) and chronic test protocols were based on standard chronic toxicity testing procedures as outlined in USEPA (EPA-821/R-02/013). Both of our acute and chronic toxicity protocols were modeled after USEPA test conditions, in terms of the number of replicates, number of organisms per replicate, frequency of feeding and water renewals, water quality measurements, temperature and photoperiod used. Moderately hard synthetic freshwater was prepared according to methods published in EPA-821/R-02/013 and was used as the culture and testing medium for all tests: pH 7.8, alkalinity 80 mg/L; beaker was aerated to maintain dissolved oxygen >8 mg/L; salinity 0.5 ppt for acute exposures, 2.0 ppt for chronic exposures; hardness approximately 100 mg/L, however hardness of testing medium was affected by pH manipulation and varied slightly among exposures.

Acute test methods were comprised of four replicate 600 ml test chambers, each containing 500 ml of moderately hard synthetic control water (0.5 ppt) and 20 organisms per replicate. Tests were initiated with juvenile-stage *P. forbesi*. Eighty percent of the test solution was renewed daily, at which time debris and dead organisms were removed. pH were measured daily before and after water renewals. Ammonia measurements were taken on test solutions daily prior to feeding and water renewal. Organisms were fed 500 μg $C.L^{-1}day^{-1}$ of IA diet daily before water renewal. Test chambers were incubated in a temperature controlled environmental

chamber/water bath maintained at 20 ± 0.1°C with a 16h L: 8h D photoperiod under natural and fluorescent light. Mortality was measured daily.

Test methods for chronic toxicity testing of full life-cycle for *P. forbesi* consisted of four replicate 1L test chambers, each containing 900 ml of moderately hard synthetic control water (2.0 ppt) and three organisms per replicate. Three gravid-stage females were employed per replicate chamber at test initiation and allowed to reproduce over the 31-day testing period, during which time the life stages of nauplii were monitored and recorded. Eighty percent of the test solution was renewed daily; organisms were identified and enumerated every 2-3 days for life stages. Organisms were fed 500 µg $C.L^{-1}day^{-1}$ of IA diet before daily water renewal. Water quality including pH, hardness, salinity and ammonia was measured daily prior to feeding. At test termination organisms were preserved for identification and enumeration of life stages.

We understand that reference toxicity tests and interactions of ammonia with other environmental factors such as temperature and salinity are important factors that may affect the outcome of this study; however due to budgetary and timeline limitations, as outlined in this project's study plan, we were unable to perform reference toxicant tests for the duration of the study. We propose including reference toxicant tests in future ammonia-related studies.

General Comment 2: *Multiple ammonia controls do not serve as a kind of reference toxicant. If they did, then the EPA methods would accept control tests and do not require reference toxicant testing. Control survival does not give an indication of relative organism sensitivity to a toxicant, as is determined by reference tox testing. Organism sensitivity can change considerably among tests, which is why there is an acceptable variability for reference toxicant testing of ± 2 standard deviations of the running mean, and this variability is not captured by control performance. In addition, the reported test and controls were performed under variable water quality conditions (i.e., different pH values, conductivities, salinities, alkalinity and hardness) so that comparisons between control survival is not a good indication of relative sensitivities that would be performed under consistent conditions in reference toxicity tests.*

We agree that multiple controls cannot serve as a reference toxicant test. As mentioned under General Comment 1, we were unable to perform reference toxicant testing due to budgetary limitations. We agree that reference toxicant testing should be performed in future ammonia studies. The district is correct that reference toxicity testing is required when assessing compliance with an NPDES permit. However, our study was not for that purpose. It is important to note that the USEPA does not mandate reference toxicity testing when screening and accepting test results for development of ammonia criteria (U.S. EPA 2009). Likewise, the Standard Guide for Conducting Acute Toxicity Tests on Aqueous Ambient Samples and Effluents with Fishes, Macroinvetebrates, and Amphibians by American Society for Testing and Materials (ASTM E1192-97-Reapproved 2008) does not require reference toxicity testing as part of their study procedures. We agree that inclusion of reference toxicant testing would have improved our study. But their absence does not invalidate our results or does outside body require it for the type of research study we performed.

General Comment 3. *Considering the many sampling locations and time periods of sampling throughout the Delta SRCSD has many spatial and temporal questions regarding the study that are detailed in our specific comments*

This was a laboratory study and did not involve any field sampling. We did employ water quality results from Foe et al. (2011) to establish the environmental relevance of the water quality parameters used in our toxicity tests. SRCSD has commented separately on the field study by Foe et al. Their response to SRCSD comments can be viewed in an appendix at the back of that report. Also, see our response to specific comment 2.

General Comment 4: *Caution should be exercised when extrapolating lab test findings to the field given the inherent variability associated with bioassay testing in general, even when standardized, well-established test methods that have clearly documented QA/QC requirements and have undergone rigorous validation are used. Even more caution is called for when nonstandard bioassay procedures are used that lack the quality control checks that are instrumental in ensuring the validity of test results. Thus, statements like "These results demonstrate that toxic events are quite common up to nine miles below the outfall of the SRWTP" (pages 3 and 27) are imprudent and unsubstantiated on the basis of one set of tests, performed by one lab, using in-house methods – regardless of the quality of the work and the lab in general.*
Further, given the general lack of details describing test methods it is not possible for others to replicate this testing, nor is it possible to determine the acceptability of these non-standard tests. The information provided is at times in conflict with the stated conclusions and with alternative presentations of the same data within the report (i.e., figure data do not match data for the same test presented in tables). Therefore, in our opinion the data and conclusions presented in this report are not suitable for use in a regulatory or policy arena in their current form.

See General Comment 1. We have included a more detailed materials and methods section in Task 2A and believe that an independent laboratory could replicate our study from a combination of information in the citations and in our methods and material section. We have removed the sentence "These results demonstrate that toxic events are quite common up to nine miles below the outfall of the SRWTP" from the text. Nonetheless, we measured acute toxicity to less than 3-day old nauplii at ammonia concentrations repeatedly measured by others in the Sacramento River.

P. forbesi was selected for testing because it is one of the primary food organisms for a number of fish species in decline in the estuary including delta smelt, a federally listed species. We acknowledge that limited bioassay testing has occurred with the species but it is a very ecologically important organism and so the results are environmentally relevant. Lack of an established testing protocol should not preclude investigations of the sensitivity of this organism to contaminants present in the estuary. The lack of data on the effects of ammonia on *P. forbesi* is an important data gap that we wanted to address in this investigation. Our goals in this study were to 1) integrate ammonia/pH data with abundance and distribution data of *P. forbesi* in the delta; 2) establish laboratory cultures of *P. forbesi* for the bioassays; and 3) examine acute and chronic effects of ammonia on *P. forbesi*. We believe we have met the objectives of the study.

General Comment 5: *The report presents several sets of "measured" unionized ammonia (UIA) data. It appears that these data were actually analyzed rather than being calculated from measured Total Ammonia Nitrogen (TAN) data, as is usually done. The methods for measuring and determining UIA and TAN should therefore be clarified.*
The measured concentrations of TAN and UIA were consistently less than the nominal (calculated) concentrations and the difference seems higher than one would expect from unavoidable losses. Testing uncertainties, including those associated with the inability to obtain the target TAN and UIA concentrations should be discussed.

We have added the methods for how TAN was measured and UIA calculated from these measurements in Task 2A. The reviewers may be aware that ammonia is a volatile chemical. We have followed the proper methods in the preparation of ammonia concentration for our study and did not expect that measured concentrations would be so much lower than the nominal concentrations. To validate the accuracy and precision of our analytical methods, we split ammonia samples of 5 mg/L TAN nominal concentration collected at 0 and 24 hours into equal volumes for interlaboratory comparison. Two of the split samples were analyzed in our lab and the other two were analyzed by Dr. Randy Dalhgren at UCD. Our measured concentrations at 0 and 24 hours were 3.71 and 3.66 mg TAN /L and Dr. Dalhgren lab's measured concentrations were 3.73 (0 hour) and 3.67 (24 hours) mg TAN/L indicated their results were identical to our. Furthermore, our ammonia analytical methods and measurements are also very similar to those published in Wang et al. (2007) on freshwater mussel toxicity study. In their study nominal concentrations were 0.5, 1, and 2 mg/L while measured concentrations were 0.44, 0.79, and 1.6 mg TAN/L. These differences are also very similar to ours.

General Comment 6: *Increases in measured mean TAN and IUA concentrations between water renewals resulted in higher exposure concentrations than were used for calculating effect levels (e.g., NOEC, LOEC, LCx). Therefore, the reported effect levels are lower than those that are likely to have caused the indicated effects. Time weighted averages of measured exposure concentrations should be used in calculations to more accurately estimate the concentrations that cause adverse effects.*

We used the average measured TAN concentration between water renewals in our report and these measured values are the basis for the calculation of effect levels.

General Comment 7: *Concentrations of important water quality parameters vary substantially among tests were effect levels are compared directly. For example, data from Task 3-1a resulting in a 6-day LC50 of 6.014 mg/L TAN for juvenile copepods (alkalinity of 50 mg/L, conductivity of 975 umhos, 206.7 mg/L CaCO3 hardness) are compared to data from task 3-1b that resulted in a 96-hour LC50 of 2.96 for juvenile copepods (alkalinity of 20 mg/L, conductivity 580 umhos, 140 mg/L CaCO3 hardness). The stated conclusions should be more strongly qualified as "preliminary" while lacking data or any discussion to address uncertainties regarding the affect variable water quality parameters can have on the results.*

Delta toxicity tests with non-standard organisms have found that various water quality parameters can affect test results. For example, Werner et al (2008) report that "Turbidity and EC/Salinity were the two most important factors determine the survival of delta smelt larvae

overall". Given the possible influence of water quality parameters on Delta organisms, what is the potential influence of variable conductivities reported for several of these tests on the results? Results from testing with non-standard test species should be interpreted with caution before their sensitivities are thoroughly understood.

We agree that water quality parameters can affect test results. We feel that future study plans should allow for future exploration into the effects of salinity/conductivity tolerances on this organism.

General Comment 8: *The results from Subtasks 3.1A and 3.1B show that the lowest acute effect levels provided for juvenile P. forbesi were well above ambient concentrations of total ammonia nitrogen (TAN) at Hood. This deserves to be placed in such a context in the Executive Summary, where it should also be noted that no adverse effect concentrations and the lowest acute threshold provided for 3-day old nauplii were also above mean concentrations of TAN at Hood. Given the highly preliminary nature of the chronic test results and methods, the environmental relevance of acute test thresholds, conducted more frequently at the University of California Davis Aquatic Toxicity Laboratory, should be discussed (Werner et al. 2008).*

Changes have been made in the Executive summary to indicate that juvenile and older nauplii were not killed by ammonia concentrations being measured in the Sacramento River. Only less than 3-day old nauplii appear at risk from the ambient ammonia concentrations in the river. You are correct that Werner and others at the University of California Davis Aquatic Toxicology Laboratory have performed similar ammonia toxicity testing with delta smelt. Delta Smelt and copepods are different kinds of organisms and the results of the delta smelt fish tests do not provide information on the toxicity of ammonia to copepods. We have not changed the text as a result of this suggestion.

General Comment 9: *The arguments presented for two of the paper's main conclusions in the Executive Summary and Discussion and Conclusions sections are not supported by the entirety of the data set. The report contains conflicting results and conflicting statements regarding the relative sensitivity of P. forbesi to unionized ammonia (NH3, or "UIA") and ionized ammonia (NH4+, or "IA"). Some of the conflicting results and statements are contradictory to the main conclusions in this report that P. forbesi is more sensitive to ionized ammonia (IA) than UIA and P. forbesi is more sensitive to TAN at low pH.*
Results from Tests 3-1A and 3-1B (compare Tables 5 and 7) are referenced in the Executive Summary and the Discussion and Conclusion Sections as evidence that IA is more toxic than UIA (i.e., ***that copepods died faster at pH 7.4 than pH 7.8, per a given TAN concentration****). However, the results of Task 3-2 (in which TAN was held constant, but pH varied) directly contradict the conclusion that copepods are more sensitive to IA than UIA. In Task 3-2, juvenile copepods were exposed separately to 0 mg/L at pH 7.8 (control) and 5 mg/L nominal TAN concentration at pH 7.0, 7.4, 7.8, 8.2, and 8.6 for 4 days. As Table 9 indicates, copepod survival was inversely related to pH; as pH increased, and as NH3 constituted a higher proportion of TAN, copepod survival decreased. This contradiction in study results is not overtly acknowledged in the report, but is inadvertently illustrated in the Executive Summary. Also, the Discussion and Conclusion unambiguously states:*

"Because P. forbesi is more sensitive to ionized ammonia as we have demonstrated in our studies, exposure to the dominant ionized form in the environment may render relatively more adverse effects to copepods compared to fish," (p. 26, emphasis added)
Experiment 3-2 demonstrated the opposite effect.

Our results do not contradict the conclusions of the study. The misunderstanding of our results stem from the lack of understanding on the mode of toxic action and physiological differences between fish and copepods which can be clearly illustrated below based on the underlying facts that total ammonia when in solution consists of IA + UIA and the ratio concentration of this mixture is a function of pH and temperature. We have added a new section to the manuscript to attempt to clarify the physiological differences between fish and copepods.

Results of Task 3-1A and 3-1B indicated ammonia is more toxic at pH 7.4 than 7.8.

This is based on the equilibrium equation of total ammonia (TAN)
LOW PH← → HIGH PH
NH_4^+ (Ionized Ammonia) + $H_20 \leftrightarrow NH_3$ (Unionized Ammonia) + H_30^+

TAN (Total ammonia) = Ionized ammonia (IA) + Unionized ammonia (UIA)

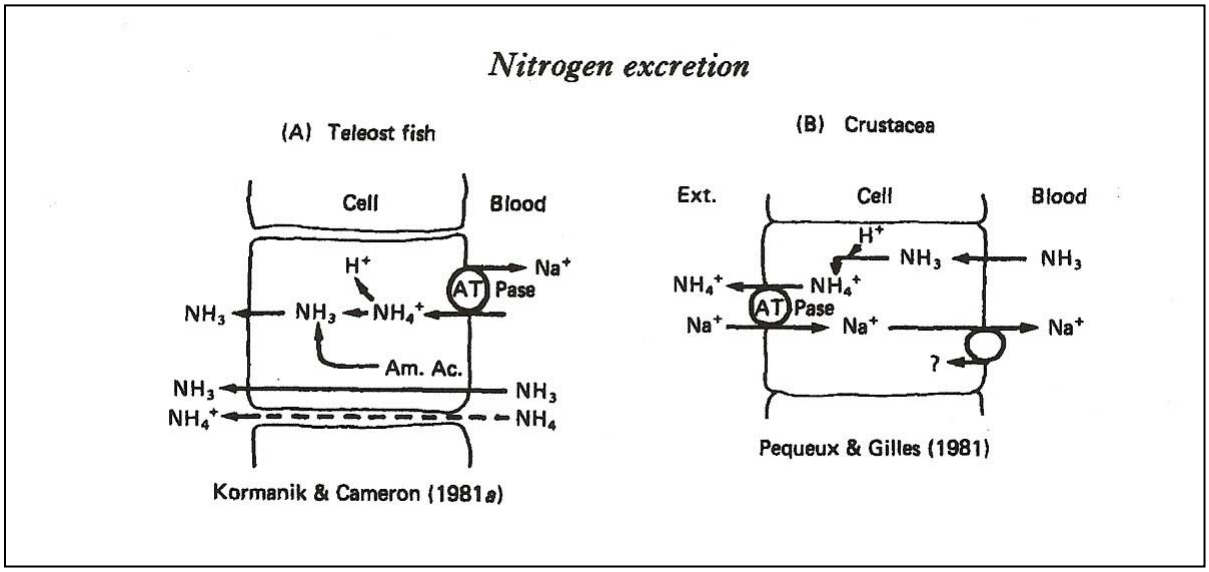
In Task 3-1A, we do not see LC50 for *P. forbesi* until 6-day post-exposure to nominal 0, 1, 2, 4, 6, and 8 mg TAN/L at pH 7.8. However, when exposed to similar concentrations of ammonia at pH 7.4, the 4d-LC50 is 2.96 mg TAN/L, 0.029 mg UIA/L, and IA = TAN (2.96)-UIA (0.029) = 2.931 mg IA/L. Results of this study support our previous study presented in the Ammonia Summit (Teh et al 2009) and confirmed the studies of Armstrong et al 1978; Borgmann, U. 1994; and Ankley et al 1995 that invertebrates are sensitive to ammonia at low pH.

The objective of Task 3-2 is to determine the 96hrLC50 of UIA and one way to do this test is to keep TAN constant and change the pH so we will have various concentrations of UIA for acute toxicity testing (see Table 8). Assuming *P. forbesi* in not sensitive to pH changes (see response to specific comments 30 and 33), UIA concentrations from pH 7.0 to 8.6 are 0.014, 0.037, 0.094, 0.227, 0.514 mg/L and the 96LC50 for UIA is 0.303 mg/L.

Task 3-2 shows the threshold toxicity of UIA (0.303 mg UIA/L) to *P. forbesi* but this toxic threshold is approximately 10 times higher than UIA (0.029 mg/L) at pH 7.4 in Task 3-1B. Therefore, we concluded that *P. forbesi* is more sensitive to IA than UIA (see explanation below). Furthermore, compared to IA, the UIA form is present in a very small fraction in the total ammonia concentration that depends on pH and temperature. We do not want to rule out the sublethal effect of UIA in ammonia toxicity, for simplicity, we suggest reporting ammonia copepod study using TAN. Again, we have made significant changes to the text to better explain our conclusions.

Physiological and Mode of Toxic Action differences between fish and Copepods
Unlike fish that excrete unionized ammonia through gills, copepods have no gills and ionized ammonia is excreted primarily through their maxillary glands. The following schematic

physiological processes involved in ammonia excretion through epithelial cell of fish gill and copepods maxillary glands are from Regnault 1986:



In Task 3-2, *P. forbesi* exposed to ammonia at higher pH will be mostly affected by unionized ammonia (UIA) which is nonpolar and highly soluble in lipids therefore it can readily diffuse through the cell membrane into the maxillary gland or passively absorb directly into other parts of their bodies. The TAN found toxic at high pH (>8.2) is ~3.71 mg/L with 92% (3.497mg/L) exist as IA and 8% (0.303 mg/L) exist as UIA. The passive diffusion of UIA into their body may be the major cause for ammonia toxicity at high pH.

In Task 3-1A, *P. forbesi* exposed to ammonia at low pH (7.4) will be mostly affected by Ionized Ammonia (IA). Ionized ammonia is a polar compound and has inhibitory effect on sodium influx. The TAN found toxic at pH 7.4 is 2.96 mg/L with 99.02% (2.931 mg/L) exist as IA and 0.98% (0.029 mg/L) exist as UIA. By competing with sodium ions, the IA would reduce the influx of Na+, thereby diminishing body concentrations of this important salt. In addition, disruption of Na+/NH4+ transport system also cause body levels of ammonia to rise by itself, riding the transport mechanism in or preventing metabolic NH4+ from riding it out, therefore resulting in autointoxication.

Comparison of Task 3-1 and 3-2 indicate that the 96LC50 of TAN (3.71 mg/L) and UIA (0.303 mg/L) at high pH is higher than 96LC50 of TAN (2.96 mg/L) and UIA (0.029 mg/L) at low pH and lead us to conclude that *P. forbesi* is more sensitive to IA than UIA.

It has been demonstrated in these studies that sufficiently high concentrations of IA in water of low pH is lethal to copepods, even though the UIA concentration may be sublethal. It is probably an over-simplification to attribute the toxicity of ammonia only to unionized ammonia at high pH and to ionized ammonia at low pH. There may be a contribution from each ionized and unionized fraction at a total ammonia concentration found to be toxic. It should be noted that our result does not suggest that copepods are not sensitive to UIA, but that more UIA is needed

to decreased copepod survival compared to less IA at lower pH. Accordingly, we suggest caution in the interpretation of results on ammonia-induced responses of different test organisms to consider the contribution from both UIA and IA fractions. Our overall intention is to suggest using TAN instead of IA or UIA alone despite the sensitivity differences in ammonia between copepods and fish. We strongly agree that more study is needed to support this hypothesis. Therefore, we remove the comparison between Task 3.1 and Task 3.2.

General Comment 10: *In ammonia toxicity testing pH may be an independent stressor. Nowhere in this report, or related reports, is there any discussion of the potential for pH to affect zooplankton fitness in ammonia toxicity testing, independent of TAN concentrations or other water quality constituents. Zooplankton survival and reproduction can be affected by decreases in pH alone – a phenomenon which has been addressed in acid deposition research. Before conclusions are drawn regarding whether ammonia/ammonium speciation explains differences in copepod survival at different test pH, additional experiments should be conducted with Delta copepods in which pH is varied but TAN is not added.*

We agree that future study plan should include pH as an independent stressor and we have added a sentence in the test to indicate that we assume that our responses are not due to pH shifts (See response to specific comments 30 and 33). However, changes in pH used in this study are well within the range of values observed in the field where these animals live. Please see specific comments 30-33 for additional explanations.

Specific Comment 1. The interpretation of the report is hindered by grammatical errors and imprecise language throughout. For example, because the report describes results for several life stages of copepod, the age/stage of P. forbesi should be specified each time a test result is specified – including in the executive summary, experiment descriptions, and figure and table captions.
Examples of ambiguous language from the Executive Summary:
Example 1. What is meant by "this study" in the following sentence from Result 2?
"Time to 50% mortality of P forbesi was faster at pH 8.6 (66 hr) when compared to pH 8.2 (87 hr) at 3.71 mg/L TAN. The 4d-LC50 was 0.303 mg/L UIA. However, The 4d-LC50 of 0.303 mg/L UIA concentration in this study was approximately 10 times higher than the 4d-LC50 of 0.033 mg/L UIA in the pH 7.4 studies (see above) when copepods were exposed to different concentrations of TAN." (bolding added) Do the authors refer to the pH 8.2 test, the pH 8.6 test, or both?
Example 2. The bolded conclusion for Result 1, "These results suggest that P. forbesi is sensitive to TAN at low pH" This conclusion would be more precisely stated "These results suggest that P. forbesi is more sensitive to TAN at low pH (7.4) than high pH (7.8)."
Example 3. It should be made clear which combination of pH and temperature produced the percentage referred to in the following sentence (Result 2): "Furthermore, since 98.9% of the total ammonia is in ionized form which is the most toxic fraction to P. forbesi…"
Example 4. In the sentence below do the authors imply that pH is the main cause of copepod mortality or UIA?: "P. forbesi is sensitive to higher pH when TAN is maintained at constant concentration indicated that higher pH fostered higher UIA concentration, which is the main caused of the copepod mortality." Are the authors implying that pH is the main cause of copepod

mortality or UIA? This is important, because pH (alone) can affect zooplankton reproduction and mortality, all other factors being equal.
Example 5. Should we interpret the sentence "A 31-d life-cycle study indicated significantly lower numbers of nauplii and juveniles in gravid females exposed to 0.79, 1.62, and 3.36 mg/L TAN when compared to control and 0.36 mg/L TAN." to mean that "A 31-d life cycle study indicated that gravid females produced significantly lower numbers of nauplii and juveniles when exposed to 0.79……"?
Example6. "There is significantly lower number of new born nauplii surviving to 3-day old when exposed to 0.38 mg/L TAN" – compared to what?

The language for all comments has been revised in the final report. I apologize for the fact that English is my second language.

Specific Comment 2:
Why should pH and temperature in the Sacramento River at Hood in June define the most appropriate conditions for an evaluation of the population-level effects of any water quality constituent for P. forbesi?

Temperature and pH are important water quality factors that modify ammonia toxicity. We used water quality measurements characteristic of the northern Delta including the Sacramento River and the Cache Slough complex to help make the results of our bioassay study more environmentally relevant. As described in the background of our report, copepods is one of the primary food sources to higher trophic level organisms (Kimmerer 2004) and calanoid copepods comprise the main zooplankton component (60-80% of all metazoan zooplankton) in the SFE (Lopez et al., 2006). Gut contents of larval fish further reveal the calanoid copepods *Eurytemora affinis* and *Pseudodiaptomus forbesi* to be the dominant prey organisms highlighting their importance in the pelagic foodweb of the SFE (Steve Slater, California Department of Fish and Game (CDFG), unpublished data). As shown in the 2007-2009 CDFG 20 mm survey, *P. forbesi* population increase in April and peak in the month of June, therefore, it is important for us to evaluate ammonia, pH and temperature between April and July in the Sacramento River at Hood (Table 1) and Cache slough complex (Table 2).

Why is one season of zooplankton townet data (7 tow samples at two stations in a single water year 2008) sufficient to determine seasonal abundance patterns for P. forbesi?

The reviewers most likely are very well aware of the constant shifting and changing in the environmental factors and water qualities from year to year in the delta. Due to budget constraint, instead of compiling multiple years of zooplankton data, we chose the most recent year data when we submitted our proposal to Dr. Chris Foe as an example of the changes in *P. forbesi* and *E. affinis* population dynamics. The purpose of the 20 mm townet data is to indicate *P. forbesi* population increases at stations 716 and 719 when *E. affinis* population decreases. These data have yet to be published but were generously provided by staff at California Department of Fish and Game (Erin Gleason, Julio Adib-Samii, and Bob Fujimura). We are now including 2007 and 2009 and adding station 711 for *P. forbesi* and *E. affinis* in the North Delta. The 3-year surveys clearly indicated that *P. forbesi* population increases as the *E. affinis*

population decrease and are at a maximum in the summer. We used summer water quality conditions of temperature and pH in our bioassay testing regime.

Why does the report state in the Executive Summary that P. forbesi were most abundant at Hood in the North Delta, when data are only described for CDFG stations 719 (Sac. Deepwater Ship Channel) and 716 (Cache Slough near Liberty Island)?

Thank you, our error. We have removed the statement from the Executive Summary.

Given that hundreds of recent measurements of TAN and pH are available from monitoring stations in the North Delta from the IEP, USGS, DWR, UCD-ATL and other monitoring programs, why was the range of TAN concentrations from 7 water samples collected by Regional Board staff between Apr-July 2009 at Hood considered sufficient to characterize the frequency of "toxic" events in the Sacramento River (see last paragraph of Executive Summary), or to claim that they are "quite common"? Why are pH, temperature, and TAN in 9 samples each from 4 Cache Slough sites during one 4-month period in 2009 considered a characterization of conditions in the Cache Slough complex (Table 2)? More data from sites in the "Cache Slough complex," spanning 2006-2009, is available from the UCD-ATL POD study (e.g., at Cache Slough at Ulatis Creek, Cache Slough at Lindsey Slough, Sacramento DWSC). Was the conductivity of test water in the acute tests (variable from test to test; 580, 975, 983, 3238 µMho) environmentally relevant?

We have removed "quite common" from the text. Please see answer to specific comment 2 for our reason to focus on the month of April – July. We agree that there are hundreds of recent measurements available from different studies in the delta. It is not to imply that other water measurements by IEP, DWR, USGS, UCD-ATL are not relevant. However, a data set that includes a simultaneous measurement of temperature, pH and ammonia throughout the northern delta is quite rare. All this information was available twice a month for over 20 sites in the Delta both above and below the SRWTP in the Foe et al study. The scope of work for this contract required that we consider the most recent Regional Board water quality data when designing our study. Since the focus of our study is *P. forbesi* where these copepods are most abundant between April and July in the North delta and with limited funding and time constrain, we believe that it is most cost effective to compare available *P. forbesi* data to the 4-month period in 2009 where extensive water monitoring have been performed by Dr. Chris Foe.

Because the San Francisco Delta is tidal, environmental relevant conductivity can fluctuate between 300 to 980 µMhos in Cache Slough region. All these data are available at California Data Exchange Center at Department of Water Resources. (http://cdec.water.ca.gov/jspplot/jspPlotServlet.jsp?sensor_no=8869&end=03%2F01%2F2010+11%3A00&geom=medium&interval=30&cookies=cdec01). Higher environmental relevant conductivity can be seen at Point Sacramento, Antioch, and Chipp Island (Foe et al 2010). In addition, the physiological and toxicological responses of copepods and fish to ammonia are different (see response to general comment 9) and we have not yet investigated their effects on the survival of copepods, which remains to be determined in future studies. We do not have the budget and time to include hundreds of additional data that will not significantly change the outcome of our study.

Why was the 31-day life-cycle test done in significantly more saline water (3244 µMho) than most of the acute tests? Answering these questions in the final report would be helpful.

See specific comment 5

Specific Comment 3: *The scientific article referred to in the sentence quoted below should be properly cited in the normal fashion (author, year in the body of text, complete citation as a footnote or in references cited).*
"Controversial arguments are being debated regarding the effects of pH on ammonia toxicity in aquatic organisms (http://www.jstor.org/stable/25041284?origin=JSTOR-pdf)." (page 8, Draft Report)
The article is from 1982 and thus (alone) does not provide support for a current controversy regarding pH and ammonia sensitivity, nor does it likely serve as a reflection of the current state of science regarding pH and ammonia toxicity for zooplankton.

Article has been properly cited.

We respectfully disagree with the statement that "*The article is from 1982 and thus (alone) does not provide support for a current controversy regarding pH and ammonia sensitivity, nor does it likely serve as a reflection of the current state of science regarding pH and ammonia toxicity for zooplankton"*. Most recently, the USEPA published a draft of the 2009 ammonia report (USEPA 2009). The current state of science on ammonia has been focused mainly on UIA toxicity based upon the general understanding that UIA is 100% more toxic than IA. Because most studies on ammonia toxicity to aquatic organisms suggest that toxicity is principally due to UIA (USEPA 1985 and 1999), most of recent published data mainly focus on UIA toxicity. There is little or no study focusing on copepods more so, on the putative role of IA and UIA on overall ammonia toxicity. The controversial argument is not to reflect on the current state of ammonia science but to provide compelling evidence that there are significant differences in species response to ammonia toxicity. These differences of organism response to ammonia toxicity are very well affected by various environmental parameters (in addition to pH and temperature) that alone or by virtue of their interaction with biotic and abiotic components of the recipient ecosystem are to say the least, downright complex.

Specific Comment 4. *Null hypotheses H1-H3 should be stated more precisely, along the lines suggested by bold type:*
H1*: P. forbesi is more sensitive to ammonia at lower pH.* ***The survival rate*** *(or state other endpoint(s))* ***of P. forbesi will be significantly lower when exposed to ammonia at pH 7.4, compared to 7.8.***
H2*: The fecundity and fertility of adult copepods is likely affected* ***will be lowered*** *by exposure to TAN at environmentally relevant concentration****s, compared to control concentrations****.*
H3*: The survival of nauplii to juvenile or adult is likely affected* ***will be lowered*** *by exposure to TAN at environmentally relevant concentration****s, compared to control concentrations****.*

We have made changes in the hypotheses.

Specific Comment 5: *Why are two salinities listed for culture conditions (0.5 and 2.0 ppt)? Which salinity was used to rear the copepods used in this research? Please comment on the validity of chronic testing at 2 ppt salinity when this is not an environmentally relevant condition for assessing the potential for adverse effects to P forbesi from ammonia in the upper Delta. Specifically, why were tests 3-3, 3-4-1, 3-4-2 performed using significantly more saline water (3244, 3000, 3238 µMho) than the acute tests using juvenile copepods (pages 18-24)?*

See response to general comment 1 for the descriptions of our culturing methods. We have two cultures of copepod at two salinity levels: one at 0.5 and the other at 2.0 ppt for this study.

In Teh et al. 2008 report, we found that *Eurytemora affinis* survivals were significantly lower 3 of 3 times in station 711 and 2 of 3 times in Sacramento River at Hood at a salinity of 1.5 ppt and TANs were between 0.5 and 0.53 in station 711 and. 0.34 to 0.59 mg/L in hood. Subsequently, all of our acute toxicity studies of ammonia were performed at 2ppt salinity and approximately 3000 µMhos conductivity (Werner et al 2010). Based on this information, when we propose the *P. forbesi* ammonia study to SWRCB, we decided to use 2 ppt for the *P. forbesi* full life-cycle study. Since early life stage was found to be sensitive to ammonia and we would like to determine if ammonia is also toxic to nauplii at 2 ppt, therefore we used 2 ppt for task 3-4-1 and 3-4-2.

Increases in salinity have been shown to decrease ammonia toxicity (Losordo et al. 1992; Masser et al. 1992; Sampaio et al. 2002) thus, we performed several studies at 2ppt salinity. We decided to test the ammonia toxicity at lower salinity hence we used 0.5 ppt for our acute toxicity testing. We propose that additional ammonia-related tests will include different salinity levels in future study plans.

Specific Comment 6: *How was it determined that 20 individuals per beaker was appropriate (and other densities used in beaker tests)? What volumes were the 20 individuals placed in? If the acute tests followed a static renewal method, how often were the copepods transferred to new media? Was this done by pipetting or screening? Where are detailed methodologies published? Where are the "culture techniques and conditions of copepod cultures that we developed in our laboratory" publicly described?*

Ideally, numbers of test replicates, volume of test replicates, numbers of animals per replicate chamber and other testing parameters would have been optimized during a process of test validation including power analysis; however a long-term data set including many experiments is required to run that analysis. Generally speaking, US EPA recommends that toxicity tests in both the acute and chronic manuals have a minimum of four replicate test chambers with a minimum of 10 organisms each. We felt that 20 organisms per replicate would give this test adequate sensitivity. The 20 organisms were placed in 600 ml beakers, with 500 ml of test solution. Eighty percent of the test waters were renewed daily, with animals remaining in the test chambers. See response to General Comment 1 for our culture methods.

Specific Comment 7: *Test dates are not presented for any of the reported bioassays and no reference toxicant test data are reported. References toxicity testing describes the relative sensitivity of test organisms among test dates to a consistent toxicant under identical test*

conditions and is required by the referenced EPA methods as part of the quality assurance/quality control data. These data are even more important for tests with non-standard species that we know little about than they are for standard toxicity test organisms. Reference toxicant results and test dates should be reported for use in data interpretations and comparisons among the reported tests and any future repeated testing.

We have included bioassay initiation dates in the final report. As mentioned under General Comment 2, we were unable to perform reference toxicant testing due to budgetary limitations. We agree that inclusion of reference toxicant testing would have strengthened our conclusions and we recommend that this testing be performed should these tests be repeated. Nonetheless, we do not believe that their absence invalidates any of our results.

Specific Comment 8. *Bioassay testing procedures used throughout this study are not standardized methods, have not been published, and are not described in this report. The report says only that "toxicity testing conditions in this study will closely follow the US-EPA standard procedures… and culture techniques and conditions of copepod cultures that we developed in our laboratory" (starting at bottom of page 8). Thus, apparently some combination of standardized EPA methods and in-house culturing methods was used, but none of the procedures are described. It is not clear if any method validation work has been performed as none are referenced, or if the methods included proper quality assurance/quality control checks, such as acceptance criteria for controls, variability in pH, total ammonia nitrogen (TAN) concentrations, or other test conditions. Consequently, there is no way to judge the validity of the methods used and not possible for other researchers to reproduce the testing.*

Based upon your request we have expanded the description of testing procedures in our report; see response to General Comment 1. We believe that another independent laboratory could repeat our experiment from a combination of the descriptions in our method and materials section and other citations on toxicity testing provided. We agree that further refinement in the copepod bioassay procedure would be helpful. Nonetheless, we believe that any changes in experimental protocol will not compromise any of the results reported here.

Specific Comment 9. *No acceptance criteria are reported to have been established for control survival in these tests. The 82.25% survival observed for the controls seems low (other tests showed control survival of 90, 91.25, and 83%). Please describe the test acceptability criteria for these tests, how they were determined for this non-standard test species, and if consistent among different test durations and life stages tested? The referenced methods for short-term acute tests (EPA-821-R-02-012; EPA 2002) indicate a minimum required survival of 90% or greater in controls for C. dubia, D. pulex, and D. magna, which some of the reported tests do not meet.*

Augspurger et al. 2003 in their initial ammonia study with freshwater mussel have evaluated data from all sources for acceptability using guidance modified from the U.S. EPA (Stephan et al. 1985). They stated that "*Because no U.S. EPA or American Society for Testing and Materials standard methods exist that have specifically been developed for freshwater mussel toxicity tests, studies that demonstrated acceptable survival in control treatments (≥80%) used measured rather than nominal values for ammonia test concentrations, and documented test water pH and*

temperature to allow calculation of total and un-ionized ammonia concentrations were deemed acceptable and were used in our analysis".

In our copepod study, the referenced EPA manual was used to help guide us in determining appropriate test protocols, which were optimized for use with *P. forbesi*. As *P. forbesi* is a sensitive species, we would expect that overall survival would be lower than what is listed in the manual for acute toxicity testing of *C. dubia*, *D. pulex* and *D. magna* species. Ideally, acceptance criteria would be determined through evaluation of a long-term data set. As this is a developmental protocol with a non-standard species, we are lacking such a data set at this time. In our best professional opinion, until a long-term dataset is available for evaluation, we set the acceptance criteria at 80% control survival for the acute 96-hour test. We believe that although these criteria may not be sufficient for regulatory testing of effluents, they are strict enough for evaluating alternative hypotheses in a research context.

Specific Comment 10. *The following statement in the Discussion is unwarranted: "Missed opportunities for larval fishes to predate on nauplii and juvenile copepods, and for juvenile and adult fishes to graze on adult copepods, can render serious consequences in the growth and reproduction of native fishes of California." Adult native pelagic fish and native non-pelagic fishes in the Delta prey on a wide variety of invertebrates – inclusing Mysids and amphipods. Pelagic species such as Longfin smelt have shown habitat and foraging shifts toward the littoral zone and littoral prey as the availability of pelagic habitat with the appropriate salinity has diminished. In addition, no literature is cited or reviewed which warrants any statements regarding the native fishes of California.*

It is possible that adult fish can prey on a wide variety of prey. However, studies by Moyle et al 1992, Nobriga 2002, Kimmerer 2004, and recently by Slater (CDFG, unpublished data) indicated that *E. affinis* and *P. forbesi* are the dominant prey organisms for larval native fish of California. Reductions in prey size and availability may lead to starvation and reduced growth which ultimately affect larval fish survival (Houde 1987). References have been added to support our statements.

Specific Comment 11a and b*: The arguments presented for two of the paper's main conclusions in the Executive Summary and Discussion and Conclusions sections seem unconvincing.*
1: P. forbesi is more sensitive to TAN at low pH. The first part of the argument cites a faster time-to-50% mortality at pH 7.4 than at a similar TAN dose at pH 7.8 (Tasks 3-1B versus 3-1A), but conventional views of toxicity are not normally concerned with rates except whether the toxicity occurs within the specified test period or not. We suggest presenting all these data as 96-hour LC50s as follows (2.96 at pH 7.4 and >6.25 at pH 7.6. Additional data from previous studies could also be inserted into the discussion where 96-hour LC50s were 5.87 mg/L at pH 7.6).
The second part of the argument is that the 4-d LC50 at pH 7.4 (2.96 mg/L TAN and 0.033 mg/L UIA) was lower than the 6-d LC50 at pH 7.8 (6.014 mg/l TAN and 0.150 mg/L UIA)1. While this comparison supports the conclusion, it is contradicted by the test results for Task 3-2. In that test, TAN concentration was kept constant in the test series and pH was varied (7.0-8.6), which resulted in a range of UIA concentrations that increased with increasing pH. In this study, toxicity also increased with increasing pH (Table 9 in report). The reported final conclusions

should be revised after consideration of all test data and the differences between test results should be explained in light of a working hypothesis.
Conclusion 2: P. forbesi is more sensitive to ionized ammonia (IA) than UIA. The first two parts of the argument given for this conclusion seem to refute rather than support it. These refer to results from Task 3-2 in which toxicity was higher or faster in tests using higher pH values and therefore having higher UIA concentrations (TAN concentrations were held constant). The third part of this argument points out the LC50 expressed as UIA determined in Task 3-2 (pH=7.0-8.6, measured TAN~3.5 mg/L, LC50=0.303 mg/L UIA) was higher than that determined in Task 3-1B (pH=7.4, measured TAN=2.96, LC50=0.033 mg/L UIA1). The meaning of this is difficult to decipher. Among other things, differences in the relative sensitivity of test organisms from reference toxicity test data would be helpful in comparing data between these tests if performed at different times. Differences in control data at 96 hrs in both tests, although performed at different pHs, seem to indicate variable responses due to the test pH and should not be ignored. See specific comments 12-16 for additional concerns related to the determination of toxicity endpoints with these data.
It seems more likely to the reviewers that the ammonia species responsible for toxicity can be illustrated by the idealized graph presented in the 1999 Update of Ambient Water Quality Criteria for Ammonia document (EPA, 1999) and reproduced here on the right. This graph assumes that IA and UIA jointly determine toxicity and UIA is 100 times more toxic than IA. At sufficiently low pH (e.g., roughly pH 8 and less in this example), IA (NH4+) becomes the predominant species and begins to dominate toxicity. At higher pH levels, UIA becomes increasingly responsible for toxicity.
Thus, the report includes data contradictory to the summary and conclusion statement (pages 25-27) that "Because P. forbesi is more sensitive to ionized ammonia as we have demonstrated in our studies, exposure to the dominant ionized form in the environment may render relatively more adverse effects to copepods compared to fish." The reported final conclusions should be revised after consideration of all test data.

Page Number Specific
Specific Comment 12. *Page 2, Executive Summary, Result #2 contains a contradiction. The first sentence contradicts the final two sentences,*
"P. forbesi is sensitive to higher pH when TAN is maintained at constant concentration indicated that higher pH fostered higher UIA concentration, which is the main caused of the copepod mortality,"
"Therefore, these tests concluded that P. forbesi is more sensitive to ionized ammonia (IA) than UIA. Furthermore, since 98.9% of the total ammonia is in ionized form which is the most toxic fraction to P. forbesi, it is highly recommended that all future ammonia studies will be expressed in terms of TAN."

Specific Comment 13*.The first sentence states that UIA is the main cause of copepod mortality (presumably as compared to IA). The final two sentences state that IA is more toxic than UIA.*

Specific Comment 14*. Page 2, 1st paragraph.. The statement saying "…IA is more toxic to fish…" is incorrect. It is widely understood that UIA is more toxic than IA.*

Specific Comment 15. *Page 2, 2nd paragraph. "- (UIA or UIA)" is redundant. Bold statement is missing "more" between "is" and "sensitive" (surmised from repeat of this conclusion in Summary section).*

Specific Comment 16. *Page 2, 3rd paragraph. The first sentence is incomprehensible. "P. forbesi is sensitive to higher pH when TAN is maintained at constant concentration indicated that higher pH fostered higher UIA concentration, which is the main caused of the copepod mortality."*

Specific comments 11-16 have been addressed under response to general comment 9 and the Final report.

Specific Comment 17. *Page 3, Executive Summary, Result #4 refers in a confusing manner to results from at least two different tests using nauplii. The 1stsentence of the paragraph quoted below appears to refer to the acute test using newly hatched nauplii. It is not possible to decipher which test or which endpoint is referred to as a NOEL and LOEL for "P. forbesi reproduction" in the 2nd sentence. The 3rd sentence appears to refer to the 31-day life cycle test, which was distinct from the acute test using newly hatched nauplii.*
"There is significantly lower number of new born nauplii surviving to 3-day old when exposed to 0.38 mg/L TAN. Using Dunnett's Multiple Comparison Test, the No Observed Effect Level (NOEL) and LOEL were <0.38±0.011 mg/L and 0.38±0.011 mg/L TAN respectively for P. forbesi reproduction. These results further demonstrate that ammonia concentration of ≥ 0.36 mg/L affect the survival and reproduction of P. forbesi in the 31-d life cycle study"(emphasis added)
Specific Comment 18. *Results #4 and #5 appear to provide different NOELs and LOELs for nauplii. Are the NOEL and LOEL listed in Result #4 for nauplii survival, or for some other endpoint?*

We have revised the executive summary. See results #4 and #5 on page 3 of Final report

Specific Comment 19. *Page 8, It is not known what reference is being indiated by "http://news.discovery.com/animals/best-animal-jumper-crustaceans.html" or "http://www.jstor.org/stable/25041284?origin=JSTOR-pdf". While it is nice to provide web links to reference documents, it would be more helpful to provide references in the standard format (author, Year) in the main text and add web links in the references section after the complete citation.*

Two citations have been added, see final report.

Specific Comment 20. *Page 8, last line continuing onto p. 9. Not clear what elements of EPA methods and in-house methods are used. The report gives no description of bioassay testing methods used.*

See response to General Comment 1

Specific Comment 21. *Page 9, 1st paragraph under Subtask 3-1A heading. Says water samples were analyzed with an ISE and meter and that "The meter was calibrated using the EPA method 350.3…" This is the only description of analytical testing methods given in the report (other than repeated references to using an ISE), and it is not even clear whether EPA 350.3 was used in its entirety or just for calibration. EPA 350.3 and other ammonia-selective electrode methods (e.g., APHA et al., 2005) are intended for analysis of TAN; not for UIA. The report fails to make clear how UIA was measured.*
Page 9, last line continuing onto p. 10. Refers to "measured UIA concentrations". It appears that these were actually analyzed instead of being calculated from measured TAN concentrations (and pH and temperature), but this is not clear. Also, on p. 8 is was implied that samples were analyzed daily, but only one set of measured TAN and UIA data are reported per test. The report fails to say if these are average values, maximums, minimums, or initial or final values. These comments are applicable for the data presented for all tests done in this study.
It is unclear what the measured values presented for TAN and UIA represent. For the experiments conducted under Task 3-1A, it appears that TAN and UIA were analyzed at time 0 and at 24-h intervals for the 6 days of the tests. However, only one measured value per test is presented for TAN and UIA. Are these values averages, maximums, or single measurements from either the beginning or end of the tests? The full variability in results (if averages) should be shown. This same comment applies to all of the subsequent tests. Measured mean TAN and IUA concentrations representing each treatment (e.g., Table 8 for Subtask 3-2; Table 11 for Subtask 3-3) increased over each 24-hour interval between water changes by >30 percent in some treatments. However, exposure concentrations that effect levels were based on seem to have been calculated using the initial concentration. These calculated effect levels (e.g., NOEC, LOEC, LCx) underestimate the actual exposure and are overly conservative (i.e., lower than the concentrations organisms were exposed to).

See response to general comment 1 and methods sections in Task 2A. Mean ± Standard Errors have been added to the tables of all tasks. Also, see response to specific comment 36.

Specific Comment 22. *Page 10, Table 5, Subtask 3-1A. The reported survival rate at the 6 ppm concentration for 4-days is listed as 87.75%. The correct calculation is 87.5%.*

Revised.

Specific Comment 23. *Page 11, Subtask 3-1A. The data plotted on Figures 1A and 1B do not seem to agree with the data presented in Tables 4 and 5. For example, the number of data points, the axis values corresponding to the points, and the apparent LC values implied by the data all seem to be at odds with the data presented in the table, text, and figure title. It's almost as if transformed data are plotted with incorrect axis titles and units. This comment is also applicable to the plots in Figures 2A and 2B.*

There are no errors in Figures 1A, 1B, 2A, and 2B. The LC data above 80% are tabulated from USEPA PROBIT program analysis.

Specific Comment 24. *Please define "juvenile." Use the term copepodites (preferably molt stage also; CI, CII, etc.) or nauplii.*

Juvenile was already defined on page 10. Juvenile and copepodites is interchangeable term used commonly by most researchers studying zooplankton. We choose to use juvenile to help the general audience.

Specific Comment 25*. The report states that water samples were collected at 0 and 24 hr for 6 days. Does ths imply that the copepods were transferred to fresh beakers at the end of each 24-hr period? Otherwise, this implies that just one sample was taken per day (at midnight) for days 2-5.*

See response to General comment 1 and Task 2A method section in the final report.

Specific Comment 26*. What statistic is meant by "Daily ammonia variation in control treatment…" (range? standard deviation?)*

See Table 8 showing the variation of pH between 0 and 24 hrs. Means and Standard errors were added to the table.

Specific Comment 27. *Page 12, Subtask 3-1B, last line of text. Omitted "more" between "are" and "sensitive".*

Revised.

Specific Comment 28. *Page 12, Table 6, Subtask 3-1B. The measured TAN concentrations are between 31 and 41% lower than the nominal (calculated) values. This discrepancy seems greater than normally expected due to uncontrollable losses, and suggests the possibility of experimental error. There is considerable variance between measured and nominal values throughout this study, with the measured values being consistently lower.*

See response to General comment 5.

Specific Comment 29*. Page 15, Subtask 3-2, Data supporting the following conclusion is not provided "Despite minor fluctuation of TAN and pH over 24 hr period, concentration gradients of UIA exist and are maintained by 80% water changes every 24 hours during the 96-hour exposure." There are no measured pH values or ranges provided in Table 8, but variability is implied. While TAN did not change markedly over the 0-hour and 24-hour means presented, mean UIA concentrations varied substantially. Please present the raw data or variability data for all parameters. It would also be very informative to present error bars for mean concentrations presented in figures.*

See revised text and revised Table 8. Actually, error bars have already been included in the mean concentrations presented in the figures. Because error bars are so small, it is hard to see the error bar on each data point when we plotted the figures.

Specific Comment 30, Specific Comment 31, and Specific Comment 33

30. Please explain why a control ammonia treatmentwas only provided for pH 7.8 (as opposed to pH used for the other with-ammonia treatments: 7.0, 7.4, 8.2, 8.6)?

31. Regardless of the original purposes of the various tests, Subtask 3-2 is a definitive check on the conclusion that P. forbesi are more sensitive to TAN at lower pH than higher pH. These data clearly demonstrate that at a constant TAN, effects are greater at a higher pH; thus contradicting one of the main study conclusions.

33. Page 16, Table 9, Subtask 3-2. Only one set of controls was run, at the middle test pH of 7.8; therefore, there's no way to judge whether the different pH levels used had any effects independent of ammonia (for instance, pH 8.6). Consequently, it doesn't seem entirely valid to report LC values for UIA (considering there were two variables, UIA concentration and pH). Nevertheless, the data indicate that toxicity increased with increasing pH and UIA concentration, contradicting one of the report's main conclusions.

pH 7.8 was chosen as control because it is the pH where copepods were culture in this study. A better experimental design in the future would be to evaluate copepod survival over the entire test range. However, in previous testing we have determined the 96 hours survival of *P. forbesi* at pH 7.2, 7.4, and 7.8 and found them to be the same (88.33%, 90.00%, and 91.25%, respectively).

In regard to your comment 33, we do not believe there is a contradiction in our conclusion; see response to general comment 9 for additional explanations.

Specific Comment 32 and Specific Comment 34

32. It is unclear what concentrations of UIA were used to calculate the LC values reported under this task. Table 8 lists mean UIA concentrations observed at 0-hours and 24-hours, but how these ranges of concentrations, or the raw data, were used to calculate the reported effect concentrations. It should be noted that measured mean TAN and UIA concentrations representing each treatment in (Table 8) increased over the 24-hour period. Therefore, effect levels (e.g., NOEC, LOEC, ECx) calculated with 0-hour measurements would underestimate the actual exposure and are overly conservative. Time weighted averages of measured exposure concentrations should be used to more accurately estimate the appropriate effect levels. Please present all data (in an appendix would be appropriate) to allow a complete review of the data and conclusions.

34. Page 17, Figure 4, Subtask 3-2. Similar to an earlier comment, the data plotted do not appear agree with the tabulated test data (for instance, the number and data points and their corresponding values).

There are multiple Figures 3 and 4 related to separate Subtasks. Figure 3 and 4 are presented on

See response to general comment 9 and specific comment 23. Thank you, we have corrected the Figures numbers.

Specific Comment 35*. Page 17, There are duplicate figure numbers. Figure 3 and 4 are presented on Page 17 as part of Subtask 3-2, and again on pages 20 and 21 related to subtask 3-3.*

Changes have been made in the final report.

Specific Comment 36. *Page 18, Subtask 3-3. It is not clear how the final TAN concentration presented in Table 11 were determined. Mean TAN concentrations presented in Table 10 were 5 to >30% higher than at 0-hours than when measured at 48-72 hours. However, Measured TAN concentrations representing each treatment in Table 11 and in Figure 4a-c were based on the lower 0-hour measurements. Effect levels (e.g., NOEC, LOEC, ECx) calculated with these 0-hour measurements underestimate the actual exposure and are overly conservative. Time weighted averages of measured exposure concentrations should be used to more accurately estimate the appropriate effect levels.*

Most studies including those reported in U.S. EPA 1985, 1999 and 2009 draft ammonia report that we have reviewed use either average of initial or final measured concentration as exposure concentration.. We have provided the average concentration over the 48 or 72 hour time period for the readers review but have used the initial measured concentration in this report.

Specific Comment 37. *Page 19, Table 11, Subtask 3-3. Please present the raw data, or at a minimum present the variability associated with means presented in this Table, where single numbers are provided to represent 4 replicates of 3 gravid females/replicate.*

We have includes raw data in the appendix.

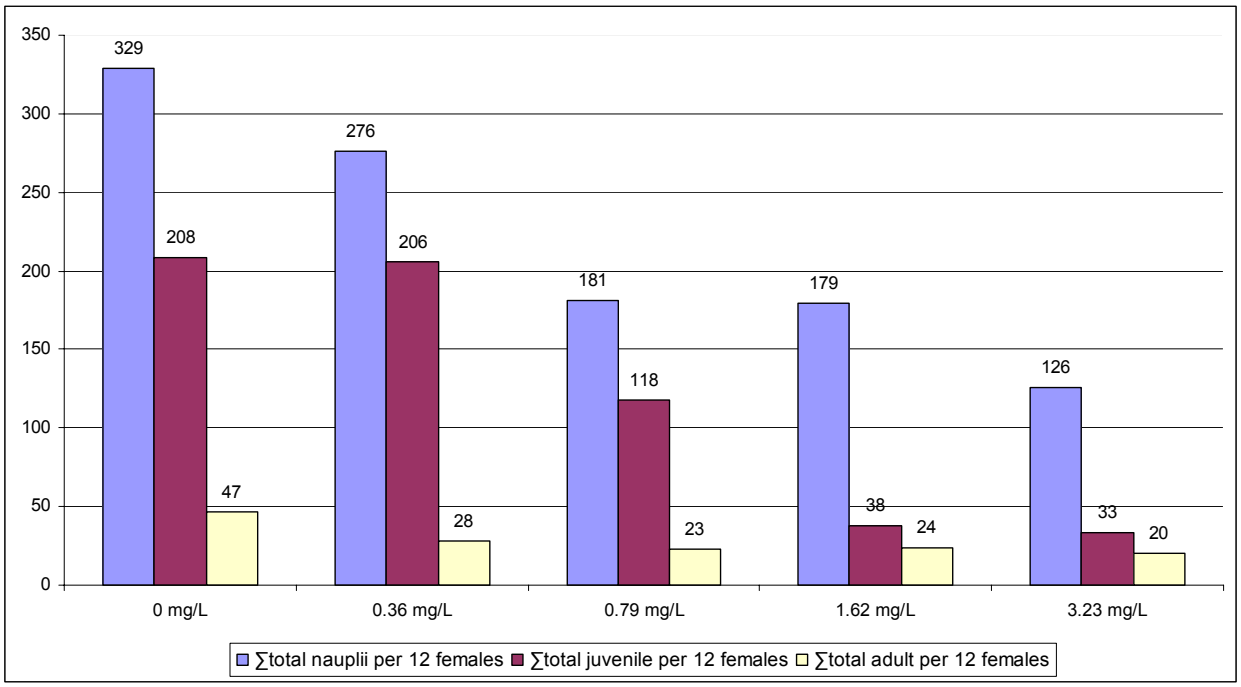
Specific Comment 38. *Page 19, Table 11v. What amount of reproductive success and recruitment, from gravid female to adult, would be considered "good" for copepods, under favorable environmental conditions and no predation? Is 3.9 adults per gravid female a "good" value for controls?*

This is a good population exponential growth question for copepods. Currently, there are no laboratory *P. forbesi* reproductive strategy data to compare to our laboratory culture data. We are currently collaborating with Drs. John Duran and Wim Kimmerer on investigating the population dynamic of *E. affinis* and *P. forbesi* in the Delta.

Considering everything is equal such as favorable environmental conditions and no predation as described by the reviewers, 3.9 adult per gravid female in control is relatively better than 2.3 adult per gravid female in 0.36 mg/L. As copepods are low on the trophic level, their reproductive strategy is to produce lots of offsprings to ensure their survival to adult stages.

Specific Comment 39. *Page 18 and Figure 4, Subtask 3-3. It is not clear how the data presented in Figure 4 matches the data in Table 11 from the same test. For example, Figure 4a presents values for the number of nauplii/beaker ranging from approximately 5 to 14 that are lower than the number per female presented in Table 11. This is inconsistent with the text description that there were "4 replicates of 3 gravid females" per replicate. Please clarify this inconsistency.*

There is no inconsistency. We were presenting analyze data in two different ways. We have included the raw data in the Appendices I, II, and III now for your review. The following histogram shows the total number of nauplii, juvenile, and adult produced by 12 females. Table 11 is based on the average number of nauplii, juvenile, and adult produced in a single female, i.e divided by 12. Regardless of how we presented the data, Table 11 and figure 6 in our final report both indicate dose-response relationship between TAN exposures and *P. forbesi* survival and reproduction.



Specific Comment 40. *Page 22-23, Subtask 3-4-1. The significance level used in the statistical analysis represents critical information that should be presented. Considering the high intra-concentration variability exhibited by the results presented in Table 12, it is surprising that there would be statistically significant differences in the data sets, leading to the reported NOEL and LOEL values. Please present the level of significance and statistical results. This novel test should also be repeated to confirm and reduce uncertainty with these findings, especially given the high variability in these data.*

We have included the level of significance and statistical results in Appendix IV of the final report.

Specific Comment 41. Page 24, Subttask 3-4-2, Table 14. *No acceptance criteria are reported to have been established for control survival in these tests. The 82.25% survival observed for the controls seems low (other tests showed control survival of 90, 91.25, and 83%). Please describe the test acceptability criteria for these tests, how they were determined for this non-standard test species, and if consistent among different test durations and life stages tested? The referenced methods for short-term acute tests (EPA-821-R-02-012; EPA 2002) indicate a minimum required survival of 90% or greater in controls for C. dubia, D. pulex, and D. magna, which some of the reported tests do not meet. The concluding statement in this section, recommending testing with*

"as young a copepod life form as possible" may be misleading if adequate control survival cannot be achieved.

We have addressed the question of acceptance criteria under Specific Comment 9. Our concluding statement regarding sensitivity of younger-age organisms was meant as a recommendation for future testing, based on our results and also on the general knowledge that early life stage organisms are typically more sensitive than juvenile or adult organisms. We agree that acceptability criteria should be determined for this species once a long-term dataset is available.

Specific Comment 42*. Page 24, Subtask 3-4-2. Please present the level of significance and statistical results and comment on the statistical significance of the results. The data presented do not produce 95% confidence limits on effect levels (i.e., the LC5, LC10 and LC50) determined with the Probit model. This indicates a relatively poor fit. Also note that the LC50 value was calculated to be greater than the highest concentration tested. This extrapolation beyond the observed data leads to low accuracy. Neither of these statistical comments invalidate the data, but the accuracy of the effect concentrations* have low certainty. Repeated testing is needed to validate and reduce uncertainty related to these findings.

The 95 percent confidence limits for the LC5 and LC10 results are now included. No confidence belt is possible for the LC50 value as it is extrapolated from outside our data set. We agree with your comment about additional testing.

Reviews in Fisheries Science

Long-term Changes in Nutrient Loading and Stoichiometry and their Relationships with Changes in the Food Web and Dominant Pelagic Fish Species in the San Francisco Estuary, California

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Running head: *Relating nutrient loading and stoichiometry to fish in the CA Bay Delta*

Key words: nutrient ratios, nutrient stoichiometry, eutrophication, plankton trophodynamics, ammonium, CUSUM charts, delta smelt, pelagic organism decline

ABSTRACT

Nutrient enrichment is an important stressor in coastal ecosystems. This analysis tests the hypothesis that changes in nutrient loads, imbalances in nitrogen:phosphorus, and changes in nitrogen form, especially shifts to increasing loads of chemically reduced, rather than oxidized nitrogen, can have major impacts on food webs, from primary producers through secondary producers to fish. The application of cumulative sums of variability, the running total of deviations from normalized values over time, is a sensitive method for comparing rates of change between and among all parameters, including organisms of all trophic levels. This approach was applied to the San Francisco Estuary, California, demonstrating that abrupt changes in nutrient loads and nutrient form over the past several decades were correlated with food web changes, including pelagic fish collapse. Remediation of pelagic fish populations should be centered on reduction of nitrogen loads and reestablishment of balanced nutrient ratios delivered from point source discharges.

INTRODUCTION

The San Francisco Estuary, California, which encompasses the Sacramento-San Joaquin Bay Delta, is one of the largest estuarine systems on the Pacific Coast as well as one of the largest managed and engineered water systems in the United States. It is the largest source of municipal and agricultural fresh water in California and is home to economically important fisheries. Major modifications to this system have occurred over the past century, including drainage of marshes to support agriculture, installation of dikes to prevent farmland flooding, expansion and deepening of shipping lanes, and significant diversion of water to various users throughout the state (Atwater et al., 1979). The Bay Delta system, an inverse delta, receives the majority of flows from the Sacramento and San Joaquin Rivers, of which the Sacramento is the largest (Atwater et al., 1979; Nichols et al., 1986). The Bay Delta ecosystem has also been significantly modified by invasive species, including clams, bay grasses, various species of copepods, and fish over the past several decades (Cohen and Carlton, 1998; Kimmerer, 2002).

The Bay Delta is the subject of considerable national public awareness due to the sociopolitical and socioeconomic tension surrounding the plight of the endemic delta smelt (*Hypomesus transpacificus*), a small (length ca. 6 cm) fish whose decline has been taken as a sign of adverse environmental conditions in the region. The delta smelt was put on the Threatened Species list in 1993 (Wanger, 2007a,b) and has since undergone further significant population decline along with longfin smelt (*Spirinchus thaleichthys*), threadfin shad (*Dorosoma petenense*) and young-of-the-year striped bass (*Morone saxatilis*; Manly and Chotkowski, 2006). Accelerated losses during the last decade have been termed the “Pelagic Organism Decline” (POD) period (Sommer et al., 2007). In recent years, the Federal court, under the Endangered Species Act, has ordered modification of water diversion projects to protect the smelt (Wanger, 2007a,b). Presently, a National Academy of Sciences panel has been convened in order to prepare a report for the U.S. Congress on sustainability and the planned water management options for the Bay Delta (NRC, 2010).

To date, no single ultimate cause of the POD has been identified, and the interpretation of data has favored a conclusion that multiple stressors combined to cause a population collapse (Sommer et al., 2007; MacNally et al., 2010, Thompson et al., 2010). Among the major factors that are thought to stress the delta smelt and other pelagic organisms are modification of the natural hydrology of the system, including export pumping for domestic and agricultural water use, habitat changes that affect recruitment (reproduction), invasion of exotic species including toxic algae, toxin loading, climate change, and food web modification through changes in species and predation (Linville et al., 2002; Lehman et al., 2005; Bennett, 2005; Sommer et al., 2007; Davis et al., 2008; Jassby, 2008). Because each of these physical, chemical and biological factors potentially influences and modifies other factors, the system as a whole is highly complex and prior efforts that used standard multifactor correlative analyses of 30 years of ecosystem data have not been successful at identifying causality with any degree of certainty (Bennett and Moyle, 1996; Sommer et al., 2007; MacNally et al., 2010; Thompson et al., 2010).

Of considerable interest has been the effect of export pumps on pelagic fish. The extent of withdrawals of water for human and agricultural consumption is on the order of 20-25% of the inflowing water (Jassby, 2008), and there is no question that these operations have had large effects on the ecosystem. Flow is rigorously managed through engineering of the isohaline where salinity is equal to 2; this isohaline is measured as the distance from the Golden Gate Bridge and is locally referred to as X2 (Jassby et al., 1995; Kimmerer, 2004). Given the current state of decline of the pelagic fish, restrictions on water pumping have been imposed in recent years, resulting in public, economic, and political tensions (NRC, 2010). It has been thought that regulation of flow will lead to improved conditions for endangered fish.

It is also well recognized that the flows of energy and materials through the food web of the San Francisco Estuary are complex and not well understood. The frequent changes, invasions, and effects of engineering and other management actions also make these relationships complicated to interpret. Some investigators have suggested that the food web of the Bay Delta is sensitive to alterations in nutrients. However, no effort to date has focused on regulation of nutrients as a means to improve the declining fish.

Yet, it has been reported, based on experimental data, that high ammonium (NH_4^+) levels inhibit diatom growth, thus potentially restricting the availability of a preferred food source in the food chain that supports fish (Wilkerson et al., 2006; Dugdale et al., 2007). Moreover, changes in nitrogen:phosphorus (N:P) ratios of nutrients in the water have been correlated with overall declines in water column chlorophyll a (chl *a*) of the Bay Delta in the mid-1990s (Van Nieuwenhuyse, 2007).

The possibility of 'bottom up' control of fish populations in this system has been largely dismissed for several reasons: most nutrients are at levels that saturate (maximize) phytoplankton growth; phytoplankton growth is considered to be regulated primarily by light limitation (Cole and Cloern, 1984); NH_4^+ is generally a preferred form of nitrogen for phytoplankton uptake; the pH of the receiving waters prevents formation of the toxic compound, ammonia gas (NH_3); and the NH_4^+ levels are typically below the criteria considered by the U.S. Environmental Protection Agency (EPA) for fish habitat (McCarthy et al., 1977; Millero, 2006; Jassby, 2008; U.S. EPA, 2010). In addition, some analyses of nutrient effects have considered only total N or P and chl *a*, rather than nutrient form and phytoplankton composition (e.g., Jassby, 2008). As a consequence, relationships between nutrients, production or food web effects have been ambiguous, leading to indecisive conclusions, as noted by Jassby (2008), "The physiological effect of ammonium …may well play a role in the dynamics of specific phytoplankton events…But it is one factor among many, and its ecological effect relative to other sources of variability underlying long-term phytoplankton patterns is not yet clear."

In contrast to conditions in the 1960s and early 1970s in this system, when hypoxia was more frequently noted (Nichols et al., 1986), there are presently no widespread classic symptoms of eutrophication (e.g., Cole and Cloern, 1984; Kimmerer, 2004) although localized hypoxia has been reported and increased frequency of cyanobacterial blooms in the past decade has been noted (Lehman et al., 2005; 2008). Improvements in sewage treatment in the 1980s, in response to the U.S. Clean Water Act, as well as other nutrient removal efforts, are generally credited with minimizing symptoms of eutrophication. Ironically, as will be shown below, these changes in sewage treatment and other nutrient removal efforts may have had unintended consequences on the food web, that, while reducing the classic

symptoms of eutrophication, may have resulted in significant biotic responses that propagated through the food web.

Shifts in algal composition and food availability have been suggested as an important factor in fish decline, especially in the past decade, not only because of the increasing frequency and range of blooms of the cyanobacterium, *Microcystis aeruginosa* (Lehman et al., 2005, 2008), but because of declines in diatoms and increases in flagellates (Lehman, 1996; Müller Solger et al., 2002, Brown, 2010). Yet, how and why these phytoplankton groups have changed has not been understood. As noted by Kimmerer (2004), "we do not really understand the controlling factors of some of the important fishes and invertebrates of the estuary. We have almost no information on the dynamics of energy flow in higher trophic levels, or how these levels are limited by productivity at the base of the food web." The current analysis attempts to begin to understand these relationships from a broad, long-term perspective. Key pathways of nutrient effects were discerned from the analysis herein. Nevertheless, Kimmerer's (2004) analysis continues to stand correct in that much remains to be understood with regard to understanding the dynamics and mechanisms of nutrient flow and its effect on trophodynamics.

The quality (form) of N has long been recognized to influence the relationship between primary producers and fish. Within the field of oceanography, NO_3^--based food webs are thought to lead to fish (export) production while those based on NH_4^+ more generally support retentive or microbial food webs in nutrient- depleted marine systems, based on the classic concept of "new" and "regenerated" production (Dugdale and Goering, 1967; Eppley and Peterson, 1979; Glibert, 1998). However, the extent to which this dichotomous control of food webs applies in nutrient-enriched coastal systems is unclear. These systems receive significant inputs of "new" N in reduced form and therefore the question remains as to whether total nutrient load or form controls food webs when loadings are high (e.g., Nixon and Buckley, 2002). The fundamental ecological question is: How does the nutrient signal propagate through the food web? It has previously been suggested that variance in state variables changes with regime shifts or disturbance (e.g., Carpenter et al., 2007), but examples of trophic changes due to nutrient changes in highly impacted coastal systems are rare.

This analysis focuses on those pelagic species whose populations have changed significantly in the San Francisco Estuary over the past decade or more: delta smelt, longfin smelt, striped bass, largemouth bass (*Micropterus salmoides*), inland silversides (*Menidia beryllina*), threadfin shad (*Dorosoma petenense*) and sunfish (*Lepomis* spp.). The delta smelt has undergone significant population declines in the past few years, along with longfin smelt, threadfin shad, and striped bass, while largemouth bass, inland silversides, and sunfish, among other species have recently increased in abundance (Kimmerer et al., 2000; Bennett, 2005; Rosenfield and Baxter, 2007). The latter trends have led to suggestions that increased predation is another factor contributing to declines in smelt and other POD species.

The goal of this analysis is to identify key relationships between nutrient concentrations, forms, ratios, and sources and the major components of the food web, from phytoplankton to zooplankton, clams and fish, based on the 30-year term time series from the San Francisco Estuary. In particular, the hypotheses that increased NH_4^+ loading relative to NO_3^-, as well as changes in nitrogen (N) and phosphorus (P) stoichiometry, are related to the changes in dominant fish over time because of their effects on the dominant primary producers. As the dominant functional groups of primary producers in the system changed, so too did the food web leading to fish. Accordingly, this analysis also explores the relationships between nutrient availability and form on the invasive clam, *Corbula amurensis*, as well as its relationship to the food web.

This analysis applies the cumulative sums of variability (CUSUM) approach (Page, 1954) to identify trends in nutrients, plankton communities, and fish over time. Comparisons of CUSUM charts of time series data can detect changes not readily apparent in mean values (e.g., Manly and Mackenzie, 2003; Mesnil and Petitgas, 2009), and they are sensitive to the timing and directional change in trends. This approach, displaying a running total of deviations from normalized values, was used to compare changes between and among all parameters, i.e., from nutrients to the trophic links to fish. The CUSUM approach, commonly used in some other disciplines, has not been previously applied in an end-to-end (*sensu* Steele et al. 2007) ecological assessment. It provides a powerful tool to test whether a particular

variable drives ecosystem change. It is an alternative to complex, multi-parameter, nonlinear models of food web interactions that are often limited by available data or knowledge of key interactions.

These nutrient and trophodynamic relationships developed here are also contrasted with those of system water flow. In total, nutrient availability and stoichiochiometry were found to be more strongly correlated with long-term changes in dominants in each tropic level than was water flow over a multi decadal period. Lastly, given the scientific, management and legal issues related to water management in the Bay Delta, specific management recommendations are suggested for nutrient control that have high probability for success in restoring endangered pelagic fish.

MATERIALS AND METHODS

Site Description

The estuary consists of South San Francisco Bay, Central Bay, San Pablo Bay, Suisun Bay and the Sacramento-San Joaquin Bay Delta, a complex of rivers, channels, wetlands, and floodplains (Fig. 1; Atwater et al., 1979; Nichols et al., 1986; Müller Solger et al., 2002). With exception of the deeper Central Bay, the mean depths of the various sub-embayments in the estuary range from 3.3 to 5.7 m (Kimmerer, 2004). In the context of estuarine typology (e.g., Madden et al., 2010), Suisun Bay is river-dominated, while the South Bay is lagoonal (Kimmerer, 2004). The focus of this analysis is on Suisun Bay, and its main river source, the Sacramento River. Nutrient data are provided from the lower San Joaquin River for comparison.

The upper reaches of the Sacramento River drain 61,721 km^2, while the upper San Joaquin River drains 19,030 km^2 (Sobota et al., 2009). On a long-term basis, the Sacramento River contributes >80% of river inflow to the Bay Delta, while the San Joaquin delivers ~12%, the remainder coming from minor sources flowing into the Delta from the east (IEP, 2006; Jassby, 2008). River flow has varied by about

ten-fold over the past several decades due to effects of El Niño, prolonged droughts, and ENSO wet years (Jassby, 2008).

Overall Approach

A retrospective analysis was conducted of 26 to 30 years (depending on variable), spanning 1975 (or 1979) to 2005, of monitoring data from the San Francisco Estuary and Bay Delta. Data were obtained from publically available portals that provide long-term monitoring programs of numerous agencies, or, for some recent years, by direct request from state or federal agencies.

Although the sources and brief methodological descriptions are given here of the source data, the reader is referred to the actual sources for more thorough metadata descriptions. The analysis here highlights those species which are either dominant, or which have received considerable attention because they represent invasive species that have had effects on the food web. Note that there are no long-term data available on bacteria, ciliates or most other microzooplankton. Kimmerer (2004) provides a more thorough review of the complexities of the food web for San Francisco Estuary.

Data Sources

Flow data were obtained from the California Department of Water Resources Dayflow record, http://www.water.ca.gov/dayflow/. Dayflow is a computational program that accounts for natural, tidally averaged flows, as well as inflows, exports, and transfers of managed, tidally averaged flow into, within, and out of the Sacramento-San Joaqiun Delta (IEP 2006). The Sacramento outflow data were used here.

All nutrient, chlorophyll *a* (chl *a*) and phytoplankton data were obtained from the Interagency Ecology Program Bay Delta and Tributary project data portal, http://www.bdat.ca.gov/. Nutrient samples were collected from the subsurface on a bimonthly to monthly basis, filtered through Whatman GF/F filters, and frozen until analysis by autoanalyzer techniques. Concentrations of chl *a* were also determined

on subsurface samples on a bimonthly to monthly basis. Samples for phytoplankton composition were collected by submersible pump, preserved in Lugol's solution, and subsequently enumerated microscopically to species level. Nutrient data, as NH_4^+ concentration, ratio of dissolved inorganic nitrogen:phosphorus (DIN:DIP), and the ratio of oxidized to reduced inorganic forms of N (NO_3^-+ NO_2^-: NH_4^+) were examined herein. Values for NO_3^-+NO_2^- are referred to as NO_3^-. Phytoplankton data, while available as individual species counts, were grouped into dominant functional groups: diatoms, green algae, cryptophytes, other flagellates, and cyanobacteria. For each function group, values were calculated as average species cell number mL^{-1}. The cyanobacterium *Microcystis aeruginosa* has increased in this system since ~1999 (Lehman et al., 2005), but these data are not included in this analysis because they are not in the long-term data base. Picocyanobacteria are also not included herein because they are not routinely enumerated. Where frequency of data was greater than monthly for nutrients or phytoplankton, monthly averages were calculated.

Zooplankton data were retrieved from the monthly zooplankton surveys conducted by the California Department of Fish and Game (http://www.dfg.ca.gov/delta/). These samples were collected from spring to fall using a Clarke-Bumpus net (154 m mesh) for meso-zooplankton and, for the micro-zooplankton, a pumped sample was passed through a 43 m mesh net. All samples were preserved with 5% formalin and subsequently enumerated microscopically. This analysis focuses on 3 dominant copepods species, *Eurytemora affinis*, *Pseudodiaptomis forbesi*, and *Limnoithona tetraspina*. The analysis does not include ciliates, other microzooplankton or mysids, nor does it include bacteria.

Data on the abundance of the exotic clam *Corbula amurensis* were also obtained from the Interagency Ecological program database (http://bdat.ca.gov/). Those samples were collected using a hydraulic winch and Ponar dredge, which samples a bottom area of ~ 0.05 m^2 and which penetrates to variable depths depending on local conditions. Repeated samples are collected and slurried before enumeration according to Standard Methods for the Examination of Water and Wastewater (1998). In the

laboratory, identifications were made using a stereoscopic dissecting microscope (70-120x) or a compound light microscope if needed.

Fish data were obtained from the California Department of Fish and Game (http://www.dfg.ca.gov/delta/). The summer townet surveys (delta smelt only) were conducted by undertaking up to 3, 10-min, stepped, oblique tows using gear with 1.5 m mouth opening attached to a hoop frame and mounted on skis. Surveys were conducted from late June to early August. Fall midwater trawl (FMWT) data were obtained from samples that were collected from 10- min diagonal tows using variable meshes starting with 20 cm at the mouth of the net and tapering to 1.25 cm. One survey was conducted each month from September-December from San Pablo Bay into Sacramento-San Joaquin Delta. Volume- weighted catch-per-unit-effort data for each survey were summed to produce the annual FMWT indices of abundance.

Effluent discharges to the upper Sacramento River were compiled by the State Water Contractors (http://www.swc.org/) based on monthly discharger self-monitoring reports to the Regional Water Quality Control Board. Although the Sacramento wastewater treatment plant came on line in 1982, NH_4^+ discharge data are only available beginning in 1984. Annual averages of discharge of N and P prior to 1992 have previously been published (Van Nieuwenhuyse, 2007).

This analysis emphasizes results from the upper Sacramento River station C3, and Suisun Bay stations D8 and D7, although additional NH_4^+ data from the delta region (station D28) are presented for comparison (Fig. 1). The intervening region between the upper Sacramento River and Suisun Bay encompasses much of the natural habitat for the delta smelt and the other pelagic fish discussed above.

Statistical Analysis

CUSUM trends were calculated for the 3-decade data record for flow, nutrient concentrations, nutrient ratios, effluent loadings, phytoplankton abundance as chl *a* and as dominant functional groups, and abundance of the major zooplankton, as well as clams and pelagic fish species. There are numerous

approaches for CUSUM calculations; the approach herein applies the z-score CUSUM method (Page, 1954). All data for which CUSUM scores were calculated were first transformed to z-scores. This involves calculation of a 'population' mean and standard deviation, where population refers to all data of that parameter in the time series. Each data point (either monthly or annual, depending on the parameter) was normalized by first subtracting the population mean and then dividing the result by the population standard deviation. The second step in the CUSUM approach is to sum all of the z-scores over time to obtain a long-term trend.The effect of such manipulation is to filter the short term or seasonal variance, thereby revealing the long-term patterns in the data.

Although not equivalent, the trends in CUSUM over time for time series data are similar to long-term running averages (Glibert et al., in review). It is the change in CUSUM over time, or the comparison of CUSUM changes in one parameter relative to another, that is of interest. Absolute CUSUM values are not important to the understanding of relationships. Absolute CUSUM values will change depending on the length of the time series, as inclusion of additional data will change the 'population' mean and standard deviation. CUSUM curves are particularly useful in identifying change points, or periods when the long-term mean changes from being, for example, above the long-term mean to below the mean. These points in time are identified from inflection points on the curves. Downward trends in CUSUM charts indicate values below the long-term mean and upward trends indicate values above the long-term mean.

If CUSUM charts of two different variables exhibit similar ascendency, descendency and inflection points, the changes in these variables are correlated. In relating CUSUM charts of one variable to another, it is recognized that such correlations do not equate to correlations of the raw data. The former is a comparison of how the long-term trends in the variables compare, whereas the latter is a comparison of how the concentration of individual parameters compare at any one point in time. Relationships between CUSUM trends for different nutrients or between different components of the food web, as shown herein, allow investigators to infer mechanistic relationships supported by known physiological or trophic relationships, or can lead to further testable hypotheses of the relationships between trophic

components. It is in this context that they are used here. As with all correlations, the variables may have a cause-and-effect relationship or both may be related to another variable.

All correlations between CUSUM plots were fit to linear models. No attempt was made to examine the fit of the relationships with a temporal offset of one variable relative to another. Refining the fit to these relationships is an ongoing effort; here the goal was to identify broad patterns. All reported CUSUM relationships herein are significant at $p<0.0001$ unless otherwise indicated; they were not corrected for autocorrelation; all short term variance in these data were removed through the standardization calculation (transformation and summing of z-scores).

RESULTS

Overview

The data presentation in the following sections follows a 'bottom-up' approach. Beginning with flow, then nutrients, phytoplankton, zooplankton, clams and fish, each major section starts with the changes over time, followed by their relationships with lower trophic levels and/or nutrients and flow.

Flow

The time series encompassed varying Sacramento River flows (Fig. 2A). The early to mid 1980s was a period of relatively high flow and the late 1980s a period of lower flow. The early 1990s was a period of very low flow, but flow increased in the late 1990s and decreased in the early 2000s, but this latter period of low flow was not as low as in the early 1990s. The X2 metric, the isohaline where salinity is 2, is related to flow, as X2 moves inland when flow is low and seaward when flow is high (Fig. 2B). Thus, the CUSUM calculations of flow and X2 are inversely related (Fig. 2A,B).

320 ***Nutrients***

321

322 Concentrations of NH_4^+, the dominant inorganic N form in the upper Sacramento River (station
323 C3) and in Suisun Bay (stations D8, D7), approximately 75-80 km downstream, were lower before the
324 mid-1980s than in later years, as evidenced in both the raw data and in the declining CUSUM trends
325 (Figs. 3A-C). NH_4^+ concentrations in the Sacramento and Suisun Bay were high in the late 1980s to early
326 1990s, coincident with the dry period, declined in the late 1990s, and then increased significantly after the
327 year 2000; the upward trend in the CUSUM charts of these sites after 2000 indicates that the NH_4^+
328 concentrations were well above the long-term mean. In contrast to these sites, the concentrations of NH_4^+
329 in the lower San Joaquin River have not fluctuated over the time series to the same degree, and thus the
330 CUSUM trends for this site are different than those of stations C3, D7 or D8 (Fig. 3D). The CUSUM
331 trends for this site show no indication of increasing NH_4^+ since 2000; instead, there has been a decline in
332 these values.

333 The fluctuating concentrations of NH_4^+ are also are reflected in the changing NO_3^-:NH_4^+ ratio in
334 the upper Sacramento and Suisun Bay (Fig. 4). For the upper Sacramento River, the CUSUM trend
335 increased until the mid-1980s, declined, and then increased again in the late 1990s (Fig. 4A). In contrast,
336 the CUSUM chart for this ratio for the Suisun Bay stations declined until about 1990, increased for the
337 next several years, and then declined from 1993 onward (Fig. 4B,C). The CUSUM charts of flow and the
338 ratio of NO_3^-:NH_4^+ in the receiving waters of the upper Sacramento River revealed similar patterns
339 (compare Figs. 2A and 4A). This is interpreted to mean that under periods of low flow, the point source
340 discharges of NH_4^+ (see below) represented a greater fraction of the total N load in the upper Sacramento
341 River, while under high flow there was greater dilution of the effluent NH_4^+ by other riverine nutrients
342 (Fig. 2). Under very low flow conditions (1987-1993), the ratio of NO_3^-:NH_4^+ changed to a greater degree
343 from upstream (station C3) to Suisun Bay (stations D7 and D8; Fig. 4), suggesting a greater degree of
344 nitrification was occurring when flow was low. Although quite variable within years, the DIN:DIP ratio
345 was below the long-term mean until the mid-1990s, when there was an increase (Fig. 5).

346 Long-term trends in nutrient concentrations and ratios were related to changes in nutrient loading,
347 with a major source being the Sacramento Regional wastewater treatment plant (Van Nieuwenhuyse,
348 2007; Jassby, 2008). The concentration of NH_4^+ discharged increased from ~10 g L^{-1} when the plant
349 came on line in the early 1980s to >20 g L^{-1} in the 2000s (Fig. 6A). Concentration of NO_3^- discharged
350 has remained <1.5 g L^{-1}, except for a few periods in the late 1990s (Fig. 6B). Concentration of PO_4^{3-} in
351 the wastewater discharge declined precipitously in the early to mid-1990s (Fig. 6C), coincident with
352 removal of P from domestic detergents by most U.S. manufacturers (Litke 1999). Total nutrient load, a
353 function of changing concentration and volumetric rate of discharge, also increased over time, now
354 averaging >500 ML day^{-1} (Fig. 6D). The molar ratio of DIN:DIP of the discharge increased from <10
355 prior to 1994 to >20 in recent years, with few exceptions (Figs. 6E). Thus, the change in DIN:DIP in the
356 upper Sacramento River and in Suisun Bay (Fig. 5) in the 1990s occurred around the same time as the
357 DIN:DIP changed in the effluent discharge.
358 CUSUM trends in NH_4^+ concentration in wastewater effluent over time are highly correlated with
359 CUSUM trends in NH_4^+ concentration in the upper Sacramento River (at C3). For the time period over
360 which NH_4^+ discharge data are available (1992-2005), the R^2 correlation between these trends was 0.70
361 (n=167; Fig. 7A), and for the POD period (2000-2005), the R^2 was 0.92 (n=71; data not shaown).
362 Moreover, CUSUMs for NH_4^+ concentration in the upper Sacramento River (at C3) were highly
363 correlated with those in Suisun Bay (at D8; R^2=0.92, n= 246; Fig. 7B) and at D7 (R^2= 0.95, n=246, data
364 not shown) for the period of 1984-2005.
365 The correlations between flow (dayflow values) and nutrients, as total NH_4^+, NO_3^-:NH_4^+ or
366 DIN:DIP were variable for both the upper Sacramento River and Suisun Bay (stations C3 and D8) for the
367 years since the wastewater facility began operation (Fig. 8). In all cases the slope of these correlations
368 were lower in the pre-POD years (1984-1999) than during the POD years (2000-2005). The increase in
369 slope in the POD was related to the increase in NH_4^+ concentrations (Fig. 6).
370

371 ***Phytoplankton***

372

373 Overall phytoplankton biomass as chl *a* was high before 1987, often reaching values >30 g L^{-1}
374 (Fig. 9A). In 1986, these values declined abruptly, and the associated CUSUM chart has an inflection
375 point at this time (Fig. 9A). The CUSUM trend in diatoms indicates that their abundances were above the
376 long-term population mean prior to 1982, but subsequent abundances declined to well below the long-
377 term mean (Fig. 9B). Trends in cryptophytes and green algae were opposite those of chl *a*: these algal
378 groups were not abundant prior to 1986, increased and remained abundant until the late 1990s, and then
379 declined (Fig. 9C). Abundance of other flagellate abundance was high in the mid to late 1980s, and again
380 around 1996 (Fig. 9D). The trend in cyanobacteria was similar to that of cryptophytes through most of the
381 time course, first increasing in the mid 1980s when chl *a* levels were declining, but unlike cryptophytes,
382 cyanobacteria continued to increase since 2000, a trend apparent even when the most recent increase in
383 *M. aeruginosa* was not included (Fig. 9E; Lehman et al., 2005, 2008).

384 When CUSUM charts of NH_4^+ concentration in Suisun Bay and those of major phytoplankton
385 groups were compared, the correlations were all strong, but the relationship was negative for diatoms and
386 positive for the other algal groups (Fig. 10). When these correlations were calculated only for the years
387 since the wastewater treatment plant has been in operation (1984-2005), they were much higher than
388 when the entire period of record was considered. For example, for diatoms, the R^2 with CUSUM NH_4^+
389 increased to 0.83 (n=147), that for cryptophytes increased to 0.76 (n=133) and that for cyanobacteria
390 increased to 0.97 (n=8; not shown).

391

392 ***Copepods***

393

394 The dominant copepod species also changed over time: *E. affinis* declined in 1986, and *P. forbesi*
395 began increasing soon thereafter, and by the late 1990s, both *P. forbesi* and *L. tetraspina* were well
396 established (Fig. 11). For the entire record (1975-2005), the relationship (R^2) between the CUSUM of chl

a and *E. affinis* is 0.93 (n=360; Fig. 12A). Relationships between different algal groups and copepods varied. The most pronounced were those of other flagellates and *P. forbesi* when it was dominant (1986-2000), a period for which the R^2 of their CUSUMs is 0.53 (n=36; Fig. 12B) and cyanobacteria and *L. tetraspina* (1988-2005), a period for which the R^2 of their CUSUMs is 0.96 (n=5, p=0.003; not shown).

Clams

The clam *Corbula amurensis* first appeared in significant numbers in Suisun Bay in 1987 (Fig. 13). It thus appeared around the same time that the copepod *P. forbesi* began to appear (Fig. 11B), and around the time that the phytoplankton assemblage had increasingly become dominated by cryptophytes and green algae (Fig. 9C). Moreover, the CUSUM of NH_4^+ for Suisun Bay was highly and positively related to that of clam abundance (Fig 14A), as was the CUSUM trend in DIN:DIP (Fig. 14B).

Pelagic Fish

Pelagic fish populations changed over time, coincident with changes in lower trophic levels. Delta smelt (estimated from both summer townet or fall midwater trawl indices), as well as longfin smelt, began to decline in ~1982 (Figs. 15A,B). Within roughly a year of the start of the decline in the smelt populations, young-of-the-year striped bass also began to decline (Fig. 15C).

The size of delta smelt changed over time as well, becoming smaller around 1990 (Fig. 16). The timing of the change in smelt length corresponded to the time period when *P. forbesi* became established, replacing *E. affinis* as the dominant copepod (Fig. 11).

In contrast, other fish species increased in numbers over the time series (Fig. 17), including largemouth bass (Fig. 17A), inland silversides (Fig. 17B), threadfin shad (Fig. 17C) and sunfish (Fig. 17D). Largemouth bass and sunfish, in particular, began to increase in the POD years since 2000. Inland silversides and threadfin shad increased in the late 1990s, but subsequently decreased in the POD years.

423 The overall trends in these groups of fish were related to changes in their food. The CUSUM
424 trends in delta smelt (summer townet index), longfin smelt and young-of-the-year striped bass were
425 positively and highly correlated with CUSUM trends in *E. affinis* (Fig. 18), but were negatively correlated
426 with *P. forbesi* and *L. tetraspina* (Fig. 18). The CUSUM trends in delta smelt FMWT index and
427 zooplankton were more complex than those of the summer townet, and these relationships are being
428 developed further elsewhere and thus are not presented here. In brief, they showed a positive correlation
429 with *P. forbesi* for the years after it became dominant, but before the POD collapse. In contrast to smelt
430 and young-of-the-year striped bass, the CUSUM trends in largemouth bass, silversides, threadfin shad
431 and sunfish were all negatively correlated with CUSUM trends in *E. affinis*, but were all (with the
432 exception of largemouth bass and *P. forbesi*) positively correlated with CUSUM trends in the other
433 copepods. Silversides, threadfin shad, and sunfish especially had very strong correlations with *L.*
434 *tetraspina* (Fig. 19F,I,L).
435 Considering that the various planktonic members of the food web were related to nutrient
436 availability and composition, and given that the fish were related to trends in zooplankton, fish
437 abundances were also strongly related to nutrients. CUSUM trends in delta smelt, longfin smelt and
438 young-of-the-year striped bass were negatively correlated with CUSUM trends in NH_4^+ and in DIN:DIP
439 (Fig. 20), while CUSUM trends in largemouth bass, silversides, threadfin shad and sunfish were
440 positively correlated with CUSUM trends in NH_4^+ and in DIN:DIP (Fig. 21)
441 The delta smelt ultimately were related to changes in NH_4^+ of the wastewater discharge in the
442 upper Sacramento River: The relationship between the CUSUM delta smelt summer townet index and
443 CUSUM NH_4^+ discharge was highly significant for the period over which discharge data are available
444 (R^2=0.97; n=13; Fig. 22).
445 There were no significant relationships between CUSUM trends in fish or clam abundance and
446 the CUSUM of X2 (Table 1).
447
448

DISCUSSION

Value of the CUSUM Approach

The CUSUM approach, originally developed in 1954 (Page, 1954) is only beginning to be used in ecological time series analysis (e.g., MacNally and Hart, 1997; Breton et al., 2006; Mesnil and Petigas, 2009). It is more widely used in the manufacturing industry, as well as in public health monitoring of clinical outcomes (e.g., Sibanda and Sibanda, 2007), among other applications. It is similar to other statistical time series approaches involving examination of standard deviations of key variables (Carpenter et al., 2007). The advantages of the CUSUM approach are that it provides visually accentuated patterns making it easy to discriminate timing of shifts in variables, it is insensitive to irregularly spaced data that often occur in long-term time series where collection frequency changes over time, and *a priori* knowledge of relationships is not required, as is the case where parameterization of relationships affects complex multivariate food web models. As there is a great need for ecological models that reliably predict the composition of algal species and assemblages occurring under conditions of changing nutrient loads, the CUSUM approach may allow scientists and managers to investigate relationships and trends that previously were considered too complex to tease apart.

The CUSUM approach has recently been applied in several relevant ecological studies of long-term changes in nutrient loading and/or phytoplankton blooms in coastal lagoons or estuaries. For example, it has been applied to the long-term nutrient and plankton relationships in Florida Bay and ecosystem recovery from the effects of hurricanes (Briceño and Boyer, 2010). It has also been applied to an analysis of a 14 year (1988–2001) data set on phytoplankton in the central Belgian Coastal Zone in order to understand the relationships between nutrient loading and the North Atlantic Oscillation (NAO) and a shift in species dominance of the phytoplankton between diatoms and *Phaeocystis* (Breton et al., 2006). In the Coastal Bays of Maryland, CUSUM has been applied to understand the relationships between freshwater flow and increased nutrient loading over a period of 15 years (Glibert et al., in

review). Where abiotic and biotic factors are changing, often on different scales, CUSUM is a powerful approach to understand their relationships.

Nutrients as a Strong Driver of Trophic Changes Leading to Fish

Enrichment of coastal estuaries by nutrients is a function of population growth and intensified production of food and energy (Howarth et al., 2000, 2002; Smil, 2001; Cloern, 2001; Seitzinger et al., 2002; Glibert et al., 2006). Total quantity and composition of nutrients in coastal waters have changed over time (Seitzinger et al., 2002; Burkholder et al., 2006; Glibert et al., 2006), and this can lead to system changes associated with eutrophication, including hypoxia, harmful algal bloom development and loss of submerged aquatic vegetation (Nixon, 1995; Anderson et al., 2002; Glibert and Burkholder, 2006). While N and P loading have increased globally over time, N loading has increased at a rate faster than P loading in many regions (Seitzinger et al., 2002; Glibert et al., 2006), in some cases leading to expressions of eutrophication that differ from those classically considered, including inhibition of primary production by high N (Yoshiyama and Sharp, 2006). There are several reasons for the disparity in N and P loading: first, use of N fertilizers has increased faster than P fertilizers over the past several decades (Glibert et al., 2006, 2010), and, use of P in detergents has declined in the U.S. and many parts of the world (Litke, 1999). The shift in the form of N loading noted herein has also occurred in many regions throughout the world because of changes in fertilizer composition (Glibert et al., 2006). Both these changes in total N and P loading and in N form can affect food webs by altering phytoplankton species composition.

This analysis has provided an evaluation of the end-to-end, inorganic nutrient-to-fish, relationships in a highly impacted, and historically nutrient-rich estuary. Numerous studies, ranging from whole lake manipulations (e.g., Mills and Chalanchuk, 1987) to oceanic food web analyses (e.g., Steele et al., 2007), have shown that alterations in nutrient loading affect trophic linkages to fish. Here, evidence has been provided that such regime shifts in the San Francisco estuary correspond to periods of abrupt changes in nutrient loading. Regime shifts, fish declines and alterations in zooplankton and

phytoplankton in the San Francisco Estuary have been previously described, but have heretofore been attributed to climate change (e.g., Lehman, 2004; Cloern et al., 2007), introductions of invasive species (Cohen and Carlton, 1998; Kimmerer, 2002, 2004) or other abiotic variables, such as water clarity and temperature (Feyrer et al., 2007; Nobriga and Feyrer, 2008). Understanding the factors changing this ecosystem is crucial to water management, but understanding how aquatic trophic cascades are modified by nutrients and other factors is a key scientific question and a major challenge more broadly (e.g., Carpenter and Kitchell, 1993; Polis and Strong, 1996).

The relationships shown here between nutrient composition, concentration, and dominant plankton and fish for the San Francisco Estuary can be conceptualized as 3 different major food webs over time (Fig. 23): a diatom-*Eurytemora*- delta smelt period prior to 1982, a mixed phytoplankton (cryptophytes-green algae-flagellates)-*Pseudodiaptomus*- bass-shad period from 1982-~2000, and a cyanobacteria-*Limnoithona*-silverside-largemouth bass-sunfish period post 2000. The availability and accessibility of long-term monitoring data at both the species-level and nutrient form-level was fundamental in this analysis.

Before 1982, chl *a* concentrations in Suisun Bay were relatively high, averaging ~9 g L^{-1}, with numerous values exceeding 30 g L^{-1}, and diatoms, *E. affinis*, and delta smelt were all abundant. The decline in diatoms, which began in 1982, was highly correlated with the increase in NH_4^+ loading. This relationship illustrates two well known physiological processes. First, although NH_4^+ may be a preferred N form under N limitation, it can be inhibitory at high concentrations (e.g., Syrett, 1981). Second, diatoms prefer and, under some conditions, physiologically require, NO_3^- over NH_4^+, unlike many other algae which preferentially use NH_4^+ over other N forms (McCarthy et al., 1977; Syrett 1981; Berg et al., 2001; Glibert et al., 2004, 2006). NO_3^- is used in the energy balance of these cells as a photoprotective mechanism (Lomas and Glibert, 1999a,b). As NO_3^- became less available relative to NH_4^+ in Suisun Bay (Fig. 4B,C), the competitive advantage shifted to phytoplankton taxa that can more efficiently use reduced forms of N. Among the phytoplankton groups that replaced diatoms in this system, cyanobacteria

526 and many flagellates have a preference for chemically reduced forms of N (Berg et al., 2001; Glibert et
527 al., 2004, 2006; Brown, 2010). As diatoms declined, so did *E. affinis*. Prey selectivity in zooplankton is
528 well known; diatoms have been shown to support *E. affinis* growth and the proportion of diatoms in their
529 diet, as well as their physiological state, affect copepod egg production and metabolism (Jones and Flynn,
530 2005; Ask et al,. 2006).

531 From 1982-1986 chl *a* continued to decline, as did *E. affinis*. The virtual disappearance of chl *a*
532 from Suisun Bay in 1987 has been attributed to the proliferation of the invasive clam, *C. amurensis*,
533 thought to have filtration rates sufficient to remove most of the chl *a* (Kimmerer, 2002; Jassby et al.,
534 2002). As mentioned, this exotic clam became established in Suisun Bay in ~1986-1987, coincident with
535 the collapse in chl *a* (Fig. 13). Its increase was positively correlated with both NH_4^+ and DIN:DIP changes
536 over time (Fig. 14), suggesting that it thrived when the food web changed due to nutrient loading. While
537 clams may have continued to keep phytoplankton chl *a* low due to their filtering, the earlier decline in
538 diatoms (in 1982) is better explained by the inhibitory effect of the elevated NH_4^+ loading than due to the
539 clam invasion. Clams also have been shown to consume *E. affinis* nauplii (Kimmerer et al., 1994), but *E.*
540 *affinis* was already in decline (Fig. 11A) before clams became well established (Fig. 13).

541 One of the largest changes in nutrient loading occurred in the mid 1990s when the N:P ratio
542 roughly doubled (Fig. 5). The change in N:P ratios is evident in wastewater discharge (Fig. 6E), in the
543 upper Sacramento River (Fig. 5A), and in Suisun Bay (Figs. 5B,C). One of the reasons for the alteration
544 in the nutrient ratios is the reduction in P, most likely a result of the removal of P in domestic detergents
545 (Litke, 1999). However, this was not the only change that occurred in ~ 1990. Concentrations of NH_4^+ in
546 the upper Sacramento River and throughout the estuary declined slightly (Fig. 3), likely due to higher
547 flows, leading to greater dilution of the incoming effluent. Thus, the ratio of NO_3^-:NH_4^+ increased in the
548 upper Sacramento (Fig. 4A) in the mid to late 1990s. This increase led to a very modest increase in
549 diatoms in the upper Sacramento River (not shown), but diatoms were apparently not able to recover in
550 Suisun Bay (Fig. 9B). Their recovery likely was hampered because the DIN:DIP was higher than in the
551 early 1980s. Instead, other flagellates proliferated (Fig. 9D; Brown, 2010), and different copepod species

552 became dominant (Fig. 11). Cryptophytes and green algae were still abundant, but declined in ensuing
553 years (Fig. 9C). The copepods *P. forbesi* and *L. tetraspina* responded to an altered phytoplankton
554 assemblage. *Pseudodiaptomus forbesi* has experimentally been shown to feed on diatoms and
555 dinoflagellates in the laboratory (Bouley and Kimmerer, 2006). In contrast, *L. tetraspina* does poorly
556 when feeding on diatoms (e.g., Kimmerer, 2004; Bouley and Kimmerer, 2006), and it developed after the
557 decline in diatoms. This copepod also consumes ciliates among other food sources, but the available time
558 series data did not allow exploration of this relationship.

559 Cyanobacteria began to increase in the late 1980s (Fig. 9E) although, as noted above, the
560 cyanobacterial abundances reported here are underestimated for the most recent decade (Lehman et al.,
561 2005, 2008, 2010a). Cyanobacteria thus proliferated as the DIN:DIP ratio increased (Fig. 5). It has been
562 suggested that some cyanobacteria can proliferate in low P environments when other algal classes are P-
563 limited, due to their lower P cell quota or their ability to substitute P-containing lipids in membranes with
564 non-P containing lipids under P limitation (Bertilsson et al., 2003; Van Mooey et al., 2009). The
565 proliferation of cyanobacteria during the most recent decade illustrates that nutrient stoichiometry may
566 indirectly, as well as directly, affect phytoplankton assemblages: while cyanobacteria can tolerate
567 elevated N:P levels, its dominance may also reflect the decline in other species without such tolerances.
568 Cyanobacteria do not have to grow faster at elevated N:P than at lower N:P values to become abundant,
569 they merely have to grow faster than competing species groups.

570 Beginning in the early to mid 1980s, the ecosystem was characterized by sharp declines in delta
571 smelt, longfin smelt, and young-of-the-year striped bass (Fig. 15). And, when *P. forbesi* became
572 established and the dominant food for delta smelt, the fish declined in size (Fig. 16). As zooplankton
573 changed, the community of fish did also, with species such as largemouth bass and silversides becoming
574 more prevalent when *E. affinis* began its decline. While smelt is a planktivore, not all the fish studied here
575 are, but all require zooplankton as food at least in their larval or juvenile stages or rely on prey that rely
576 on zooplankton (Kimmerer, 2004). As predators increased, those fish that were in decline due to changes
577 in food supply were subjected to additional stresses of predation. In the most recent decade, there were

further declines in smelt, along with silversides and threadfin shad (Fig. 17; e.g., Bennett, 2005; Sommer et al., 2007; Thompson et al., 2010). During this time, NH_4^+ loading from wastewater discharge increased 25%, from ~ 9 metric tons day^{-1} to 12 metric tons day^{-1} (the product of Figs. 6A and 6D; Van Nieuwenhuyse, 2007), leading to a strong correlation over the time series of CUSUM trends in wastewater effluent NH_4^+ and the delta smelt (Fig. 22).

The elemental composition of fish has been the subject of a considerable number of studies, from fish bioenergetics to whole system nutrient models (e.g., Kraft, 1992; Vanni, 1996; Sterner and George, 2000). Fish composition and fish size previously have been related to nutrient availability. Sterner and George (2000) speculated that the P content of fish "relates to their 'boniness'". Clearly there is much to be examined with regard to the ecological stoichiometry of all the components of the food web and how changes in the nutrient availability may be related not only to the food web of the San Francisco Estuary, but to the metabolism of dominant fishes as well.

Relationships of Fish Abundance with Food vs. Flow

This analysis was not intended to be a review of X2, its relationships, or the management thereof; there have been numerous other such efforts and others are ongoing. Instead, this analysis reviewed nutrients and their food web effects. The overwhelming conclusion here is the fact that relationships between nutrients and fish are stronger than those of flow and fish (comparison of Figs. 20, 21 and Table 1). Furthermore, changes in flow are not correlated with all nutrients and nutrient ratios over the entire time series (Fig. 8), although there were significant, but different, relationships for the pre-POD and the POD years. The slope of the relationship between CUSUM flow and nutrients changed in the POD years, coincident with the increase in effluent NH_4^+ discharge, a major driver of NH_4^+ concentrations and the nutrient ratios shown.

Nutrient Management Implications

Water management in California is challenging and contentious, and a significant fraction of the water supply for state needs is extracted from the Delta. This is done through extensive waterways and engineering projects exporting water from the Delta, via pumps and aqueducts, to the southern, drier, part of the state (Brown et al., 2009). In recent years, restrictions in water use have been mandated through federal court decisions because of declines in delta smelt abundance and its listing as a threatened and/or endangered species (Wanger, 2007a,b). Water restrictions are thought to be required to reduce further loss of these fish by entrainment in export pumps. However, management strategies to date have not reversed fish declines because they have not addressed the ultimate cause of the change at the base of the food web and the complex role of nutrient form and quantity. When food web analyses are not linked to ultimate causes of change, management guidance is inconclusive, as in the MacNally et al. (2010) multivariate analysis of fish decline in the Delta. MacNally et al. (2010) state, "The relatively large proportions of variance explained by interactions among the declining fishes and their prey suggest that trophic interactions also are important, but it is less clear how management actions could modify such relationships."

The present study supports the premise that reduction of the NH_4^+ effluent into the Bay Delta is essential to restoring historic pelagic fish populations and that until such reductions occur, other measures, including regulation of water pumping or manipulations of salinity, as has been the current strategy, will likely show little beneficial effect. By altering nutrient composition and nutrient load, it is likely that a healthy phytoplankton assemblage including diatoms could be restored. A clear management path is the application of nitrification and denitrification processing of the dominant nutrient source, the wastewater effluent, prior to discharge into the estuary to 1) decrease NH_4^+ concentration in the river; 2) reduce N:P ratio of the effluent; and 3) increase NO_3^-:NH_4^+ ratio to a level required to increase diatom abundance to support a more favorable food web for fish production (Fig. 22). Pre-1982 nutrient concentrations and ratios could serve as a management target. Historic data can serve as the "reference

condition" to establish numeric nutrient criteria when, as is the case here, there is knowledge of how the system functioned prior to the nutrient loading impacts (U.S. EPA 2001, 2010).

The findings herein point to an important consideration in the development of numeric criteria for nutrients in estuaries, a challenge that many states are now facing (U.S. EPA 2010; Glibert, 2010). Many such criteria, or integrated indices of water quality status and trends, are based on total N or P, rather than specific forms of N or P (U.S. EPA 2010). These findings show that nutrient form is related to the "quality" of phytoplankton. Thus, nutrient forms or ratios should be considered in criteria development if effects on food webs are to be related to such criteria.

Prior studies in the Bay Delta suggested that phytoplankton assemblage composition and total phytoplankton biomass were related to NH_4^+ availability or dissolved inorganic N:P ratios (Wilkerson et al., 1996; Dugdale et al., 1997; Van Nieuwenhuyse 2007). However, there have been no prior efforts linking these changes through the food web. In fact, the suggestion that nutrient loading (particularly NH_4^+) affects the food web was discounted because it was assumed that NH_4^+ is a preferred form of N for phytoplankton uptake (of all species), and in order to have effects on higher trophic levels, the levels must be in the range causing direct toxicity. The pH of the receiving waters prevents formation of the toxic NH_3, and NH_4^+ levels are generally below levels considered by the U.S. Environmental Protection Agency criteria for fish habitat (McCarthy et al., 1977; Millero, 2006; U.S. EPA 2009). The latter treats NH_4^+ as a toxicant. The more subtle ecological impacts of NH_4^+ loading and the importance of changes in NO_3^-:NH_4^+ in phytoplankton succession have not been appreciated. Moreover, the potential for P limitation (Van Niewenhuyse, 2007) has not been given full consideration because the concentrations are not at levels normally taken to be indicative of limitation, i.e., less than the half saturation constant for uptake (e.g. Reynolds, 2006; Jassby, 2008). The analysis herein reconciles the seeming inconsistencies of the nutrient regulation hypotheses advanced by Wilkerson et al. (2006) and Dugdale et al. (2007) and by Van Niewenhuyse (2007). While Wilkerson et al. (2006) and Dugdale et al. (2007) have suggested that the controlling nutrient is N, especially NH_4^+ inhibition, Van Niewenhuyse (2007) suggested that P limitation was limiting to phytoplankton. From the analysis here, it appears that both have had significant

656 effects on phytoplankton communities, but their major effects have occurred at different points along the
657 time course.

658 The changes in food web structure with changes in nutrient form and/or nutrient ratio suggest that
659 the Eppley and Peterson (1979) paradigm applies in this nutrient rich estuary. That paradigm, which
660 suggests that NO_3^- -based food webs are fundamentally different from those of NH_4^+ -based food webs,
661 was originally developed for oligotrophic, oceanic waters. Here, as in the oceanic condition, NO_3^- -based
662 food webs were supported by higher proportions of diatoms and the NH_4^+ -based food webs were
663 supported by higher proportions of flagellates, cryptophytes and cyanobacteria. Both food webs supported
664 fish, although different species.

665 The analysis described here should be highly relevant to other systems that have been subject to
666 alterations in N and P loading and N form. The fact that chl *a* declined over time as N loading increased
667 has deflected management attention away from nutrients. It is counterintuitive to the normal progression
668 of eutrophication, typically resulting in higher algal biomass and a shift from benthic to pelagic
669 production (Cloern, 2001). The inhibitory effect of NH_4^+ on diatoms seen here has, however, been
670 observed in other estuaries, such as the Delaware Estuary and the inner bay of Hong Kong Harbor
671 (Yoshiyama and Sharp, 2006; Xu et al., 2010). In the Delaware Estuary, inhibition by NH_4^+ was greatest
672 in the colder months, when diatoms dominated (Yoshiyama and Sharp, 2006). From a management
673 perspective, not only is near-field alteration of phytoplankton growth important, but so too is the potential
674 for large down-stream impacts - impacts not often associated with discharges far removed in space. In this
675 study, CUSUM trends in discharge of NH_4^+ from the treatment plant were highly related to those of NH_4^+
676 concentrations far downstream, ~80 km from the treatment plant (Fig. 7). The Sacramento River acted as
677 a conduit for transport of N downstream.

678 Supporting the idea that correct balance of nutrients is important for restoration of delta smelt and
679 other pelagic fish, there is a small but apparently successful subpopulation of delta smelt in a restored
680 habitat, Liberty Island. Liberty Island is outside the immediate influence of Sacramento River nutrients. It
681 has abundant diatoms among a mixed phytoplankton assemblage, as well as lower NH_4^+ levels and higher

682 ratios of NO_3^-:NH_4^+ than the main Sacramento River (Lehman et al., 2010b). Thus, if efforts are made to
683 restore additional habitat, consideration should be given to location of the habitat to be restored relative to
684 the main sources of nutrients. This system demonstrates that alterations in nutrient forms do indeed alter
685 food webs, even when all major nutrients are abundant, as was the case prior to 1994, or when one
686 nutrient (in this case P) is controlled, as is the current condition in the upper Sacramento River.
687 Additionally, nutrients that are abundant when one nutrient is controlled can be displaced in space, having
688 significant effects on the ecology and food chain downstream. The CUSUM approach was an effective,
689 sensitive, simple means to detect these relationships. These relationships also lead to directly testable
690 hypotheses and experiments that can further understanding about the role of changing nutrient loads and
691 composition on the dynamics of the food web in this system
692
693 ***SUMMARY***
694
695 Nutrient changes in concentration and form in the San Francisco Estuary and Bay Delta are
696 significantly correlated with changes in components of the food web over time. These changes are highly
697 related to loadings from a single major point source. The long-term changes in NH_4^+, the dominant N
698 form that is discharged from the Sacramento River effluent plant are similar in the upper Sacramento
699 River (C3) and 80 km in Suisun Bay (D8 and D7). However, they are not similar in lower San Joaquin
700 River (D28), consistent with previous findings that inflow from the Sacramento River and its chemical
701 constituents dominate over those of the San Joaquin. Changes in nutrient loadings and forms were related
702 to changes in the phytoplankton assemblage, which in turn were related to changes in zooplankton, and in
703 turn, related to clam abundance, and to the abundance of various fish species. The invasive copepods *P.*
704 *forbesi* and *L. tetraspina* became dominant when the phytoplankton community shifted from diatom to
705 flagellate and cyanobacterial dominance. Fish species fell into two groups: those whose long-term,
706 CUSUM trends were positively correlated with trends in abundance of *E. affinis* and negatively correlated
707 with *P. forbesi* and *L. tetraspina*, and those whose long-term, CUSUM trends were negatively correlated

with *E. affinis* and positively correlated with *P. forbesi* and *L. tetraspina*. Trends in the former group of fish also were related negatively to trends in NH_4^+ and DIN:DIP, while the opposite pattern emerged for the latter group of fish species. Long-term trends in abundance of the clam, *C. amurensis*, were also highly related to trends in NH_4^+ and DIN:DIP, suggesting that this invasive species was opportunistically responding to a change in ambient conditions when it proliferated. All of these relationships were significantly more robust than relationships with flow or X2; there were no significant relationships between the CUSUMs of X2 and nutrients, phytoplankton species, zooplankton or fish over the entire time series. Thus, a clear management strategy is the regulation of effluent N discharge through nitrification and denitrification. Until such reductions occur, other measures, including regulation of water pumping or manipulations of salinity, as has been the current strategy, will likely show little beneficial effect. Without such action, the recovery of the endangered pelagic fish species is unlikely at best.

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970

971 ***Figure Legends***

972 Figure 1. Map of the Sacramento-San Joaquin Estuary, with sites of the major wastewater discharge site
973 in the upper Sacramento River, and the sites of data analyzed herein indicated.

974

975 Figure 2. CUSUM values (primary axis, filled diamonds) as a function of time for A) flow, estimated
976 from Dayflow (see text) and B) X2, the distance in km from the Golden Gate Bridge where the isohaline
977 is 2. The secondary axis (open squares) for both panels gives the actual data in the units indicated. The
978 vertical dashed lines are guides to delineate the major periods discussed in text.

979

980 Figure 3. CUSUM values (primary axis, filled diamonds) as a function of time for ammonium (NH_4^+)
981 concentrations for A) the upper Sacramento at station C3, B and C) Suisun Bay at stations D8 and D7,
982 and D) the lower San Joaquin at station D28. The secondary axis (open squares) for all panels gives the
983 actual data in g L^{-1}. The vertical dashed lines are guides to delineate the major periods discussed in text.

984

985 Figure 4. CUSUM values (primary axis, filled diamonds) as a function of time for the ratio of nitrate:
986 ammonium (NO_3^-:NH_4^+) concentrations for A) the upper Sacramento at station C3, B and C) Suisun Bay
987 at stations D8 and D7. The secondary axis (open squares) for all panels gives the actual data in g L^{-1}: g
988 L^{-1}. The vertical dashed lines are guides to delineate the major periods discussed in text.

989

990 Figure 5. CUSUM values (primary axis, filled diamonds) as a function of time for the ratio of dissolved
991 inorganic nitrogen:phosphorus (DIN:DIP) concentrations for A) the upper Sacramento at station C3, B
992 and C) Suisun Bay at stations D8 and D7. The secondary axis (open squares) for all panels gives the

993 actual data in g L^{-1}: g L^{-1}. The vertical dashed lines are guides to delineate the major periods discussed
994 in text.

995

996 Figure 6. Nutrient concentrations (mg L^{-1}) of the wastewater effluent as a function of time for A) NH_4^+,
997 B) NO_3^- + NO_2^-, C) PO_4^{3-}, D) the volumetric daily rate of effluent discharge (ML day^{-1}) from the
998 wastewater facility, and E) the molar ratio of dissolved inorganic nitrogen:phosphorus (DIN:DIP) for the
999 major wastewater treatment facility in the upper Sacramento River. Although the plant came on line in
1000 1982, monitoring data are only available from 1992.

1001

1002 Figure 7. (A) Correlation between the CUSUM trend in NH_4^+ concentration in wastewater discharge and
1003 that in the upper Sacramento River for the period of discharge data availability (1992-2005); and (B)
1004 Correlation between the CUSUM trend in NH_4^+ concentration in the upper Sacramento River at station
1005 C3 and that in Suisun Bay at station D8 (1984-2005).

1006

1007 Figure 8. Correlation between the CUSUM trend in dayflow and the CUSUMS of (A) NH_4^+ at C3; (B)
1008 ratio of NO_3^-:NH_4^+ at C3; (C) ratio of DIN:DIP at C3; (D) NH_4^+ at D8; (E) ratio of NO_3^-:NH_4^+ at D8; (F)
1009 ratio of DIN:DIP at D8. All trends are for the period since the establishment of the wastewater treatment
1010 facility in the upper Sacramento (1984-2005). For each panel, the open triangles are for 1984-1999 and
1011 the closed circles are for the POD years, 2000-2005.

1012

1013 Figure 9. CUSUM values (primary axis) as a function of time for A) total chlorophyll *a* (filled diamonds)
1014 in Suisun Bay at station D8, B) diatom abundance (filled diamonds) at station D8, C) cryptophytes (filled

diamonds) and green algae (gray circles) at station D8, D) total flagellates (filled diamonds) and dinoflagellates (gray circles) at station D8, and E) cyanobacteria at station D8 (filled diamonds) and at station C3 (gray circles). The secondary axis (open squares) for all panels gives the actual data in g L^{-1} (panel A) or in average per species cells mL^{-1} (panels B-E). Actual data for green algae (panel C), dinoflagellates (panel D) and cyanobacteria at station C3 (panel E) are not shown. The vertical dashed lines are guides to delineate the major periods discussed in text.

Figure 10. Correlations between the CUSUM trends in NH_4^+ and A) diatoms; B) Cryptophytes; C) Green algae; D) Cyanobacteria; and E) Flagellates

Figure 11. CUSUM values (primary axis, filled diamonds) as a function of time for the major copepods A) *Eurytemora affinis*, B) *Pseudodiaptomus forbesi*, and C) *Limnoithona tetraspina* in Suisun Bay. The secondary axis (open squares) gives the actual abundance data in number of individuals m^{-3}. The vertical dashed lines are guides to delineate the major periods discussed in text.

Figure 12. Correlations between the CUSUM trends in A) chlorophyll *a* at station D8 and the copepod *Eurytemora affinis* in Suisun Bay, and B) flagellate abundance at station D8 and the copepod *Pseudodiaptomus forbesi.*

Figure 13. CUSUM values (primary axis, filled diamonds) as a function of time for the clam *Corbula amurensis.* The secondary axis (open squares) gives the actual abundance data in individuals m^{-2}. The vertical dashed lines are guides to delineate the major periods discussed in text.

Figure 14. Correlations between the CUSUM trends in A) NH_4^+ and B) DIN:DIP and the abundance of the clam *Corbula amurensis*.

Figure 15. CUSUM values (primary axis, diamonds, triangles) as a function of time for the fish A) delta smelt, *Hypomesus transpacificus* (fall midwater trawl- filled diamonds, summer townet-open diamonds), B) longfin smelt, *Spirinchus thaleichthys*, and C) young-of-the-year striped bass, *Morone saxatilis.* The secondary axis (open squares) gives the actual data based on fall midwater trawl index. The vertical dashed lines are guides to delineate the major periods discussed in text.

Figure 16. CUSUM values (primary axis, diamonds, triangles) as a function of time for the delta smelt, *Hypomesus transpacificus* length. The secondary axis (open squares) gives the actual data. The vertical dashed lines are guides to delineate the major periods discussed in text.

Figure 17. As for Figure 15, except for largemouth bass (*Micropterus salmoides*), inland silversides (*Menidia beryllina*), threadfin shad (*Dorosoma petenense*) and sunfish (*Lepomis* spp.). Actual data for threadfin shad are based on the fall midwater trawl survey, and for largemouth bass, inland silversides, and sunfish are based on estimates of the annual average catch per tow across stations regularly occupied by delta smelt.

Figure 18. Correlations between the CUSUM trends in the copepods *Eurytemora affinis*, *Pseudodiaptomus forbesi* and *Limnoithona tetraspina* in Suisun Bay and CUSUM trends in delta smelt

(panels A-C; summer townet index), longfin smelt (panels D-F) and young-of-the-year striped bass (panels G-I). All correlations cover the period 1975-2005 for *E. affinis*, and 1987-2005 for the other copepods.

Figure 19. As for Figure 18, except for largemouth bass (panels A-C), inland silversides (panels D-F), threadfin shad (panels G-I) and sunfish (panels J-L).

Figure 20. Correlations between the CUSUM trends in NH_4^+ and DIN:DIP at station D8 in Suisun Bay and CUSUM trends in delta smelt (panels A-B; summer townet index), longfin smelt (panels C-D) and young-of-the-year striped bass (panels E-F). All correlations cover the period 1975-2005.

Figure 21. As for Figure 20, except for largemouth bass (panels A-B), inland silversides (panels C-D), threadfin shad (panels E-F) and sunfish (panels G-H).

Figure 22. Correlation between the trend in CUSUM in NH_4^+ concentration in wastewater discharge in the upper Sacramento River and the trend in CUSUM delta smelt, estimated from the summer townet index.

Figure 23. Conceptual diagram of some of the hypothesized changes in the food chain from phytoplankton to fish that have occurred in the Sacramento-San Joaquin Estuary over the past 30 years. Each of these hypothesized food chains has different dominant nitrogen forms or amounts relative to phosphorus. This conceptual model is intended simply to highlight some of the major flows of energy and

materials and does not include all organisms, pathways or flows. The size of the symbols is meant to infer relative importance.

Fig. 1

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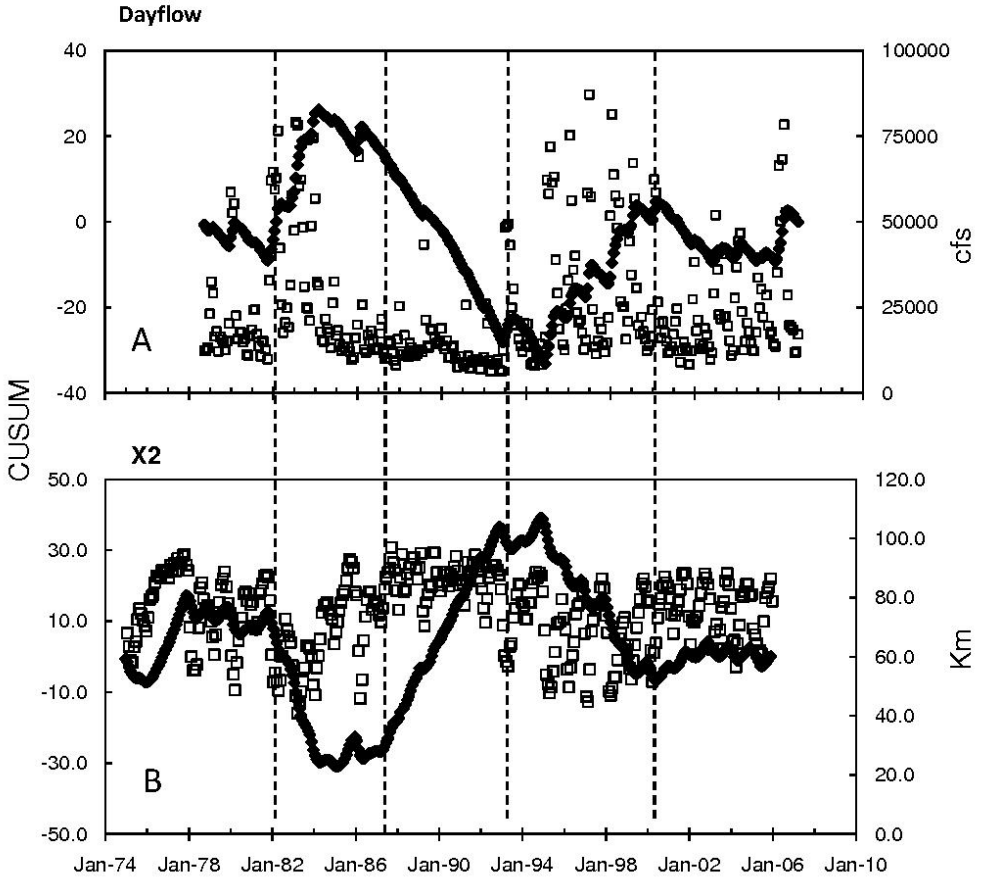

Fig. 2

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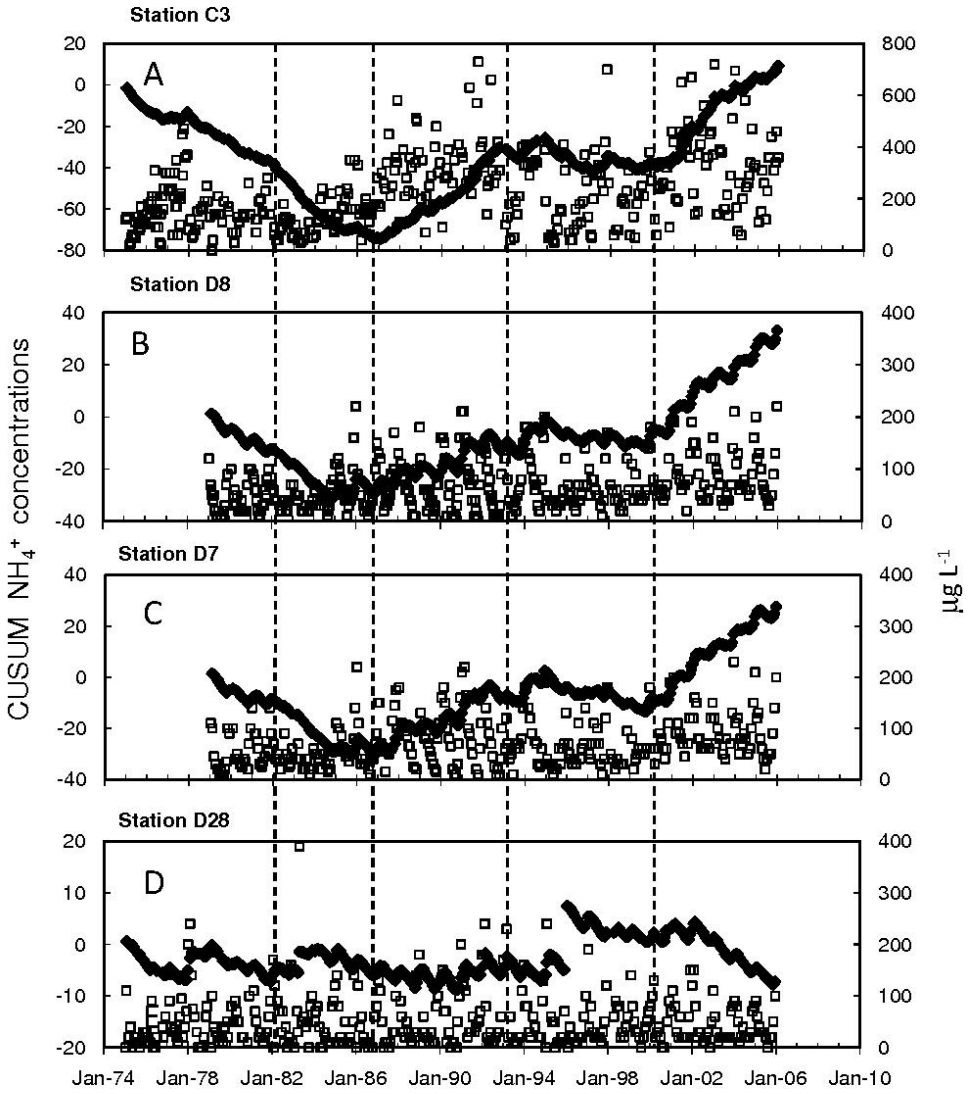

Fig. 3

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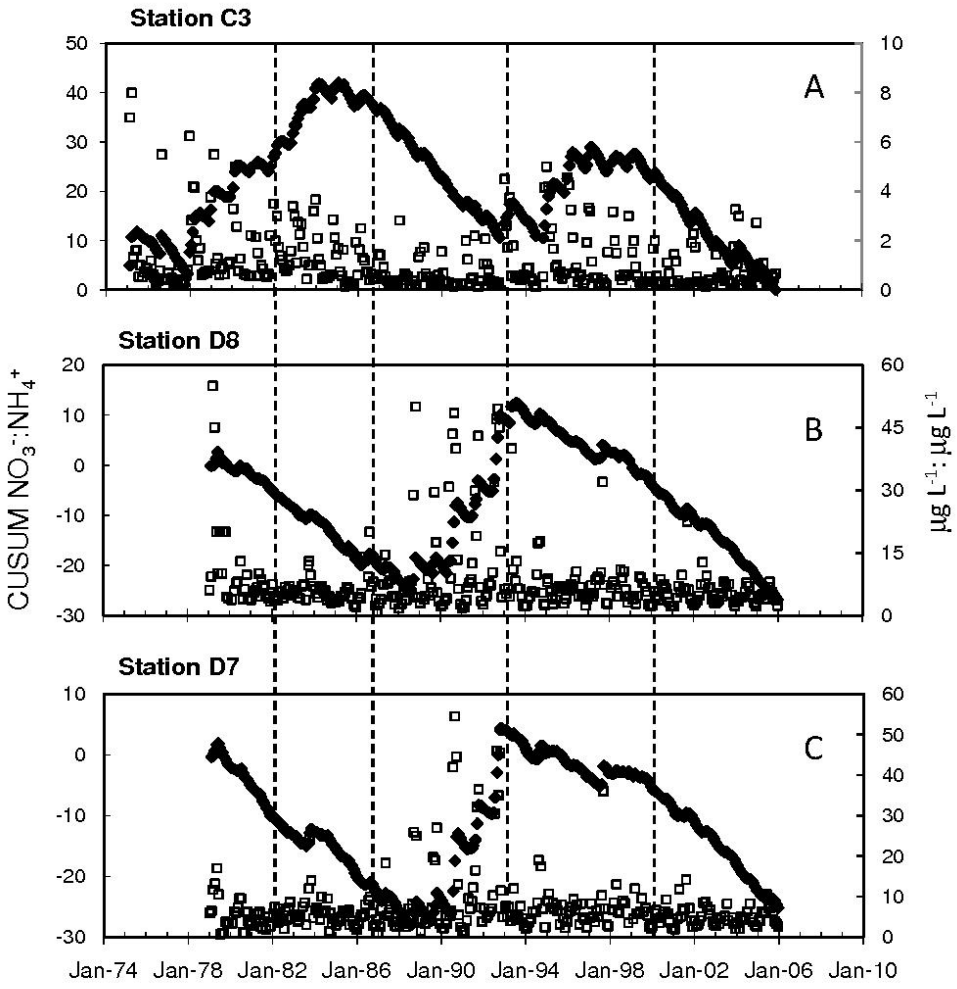

Fig. 4

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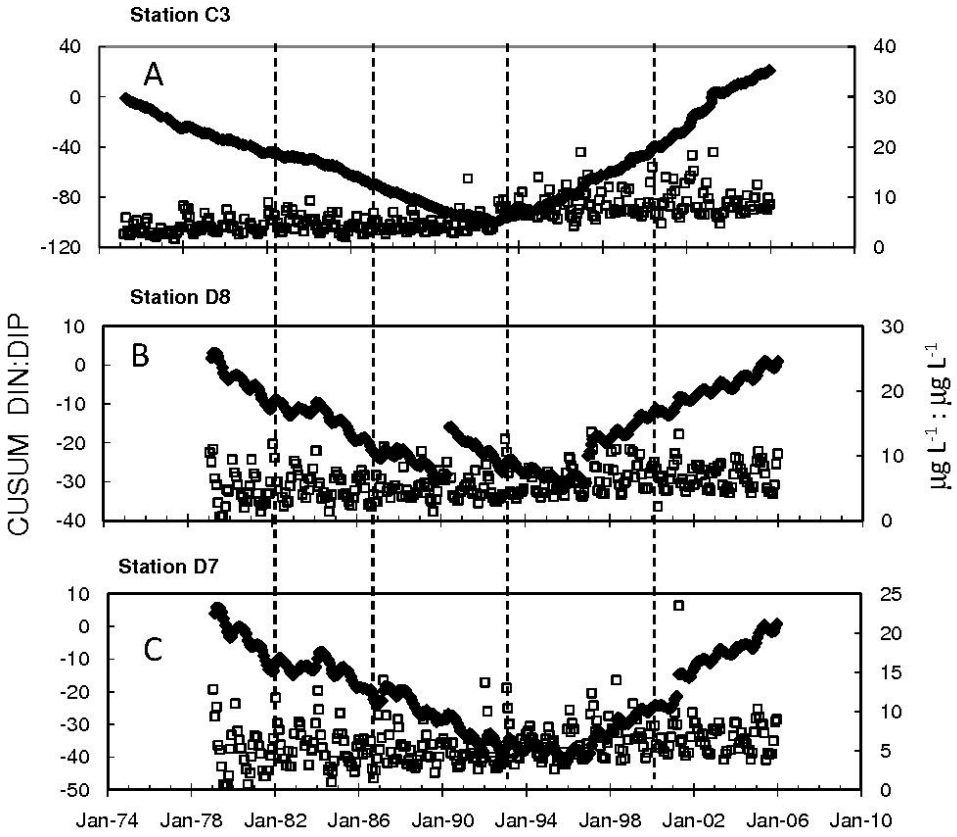

Fig. 5

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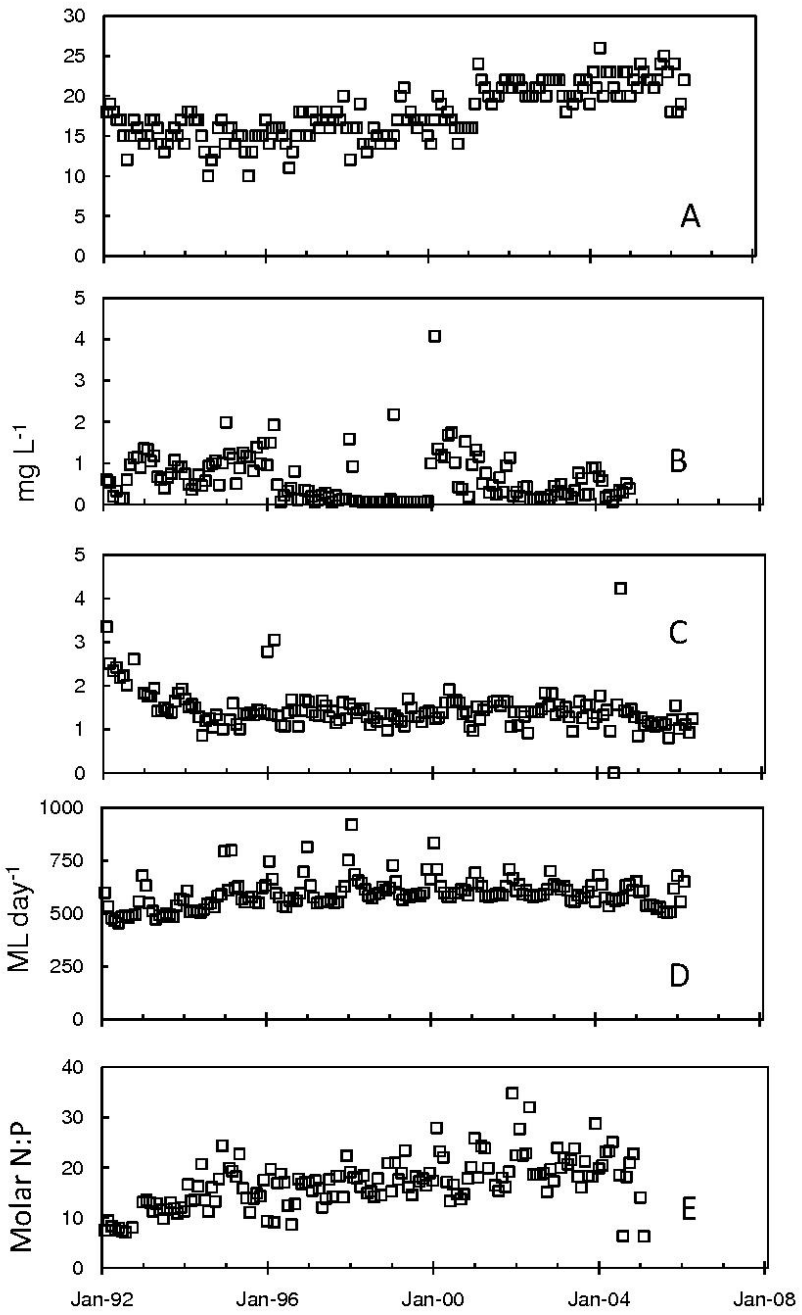

Fig. 6

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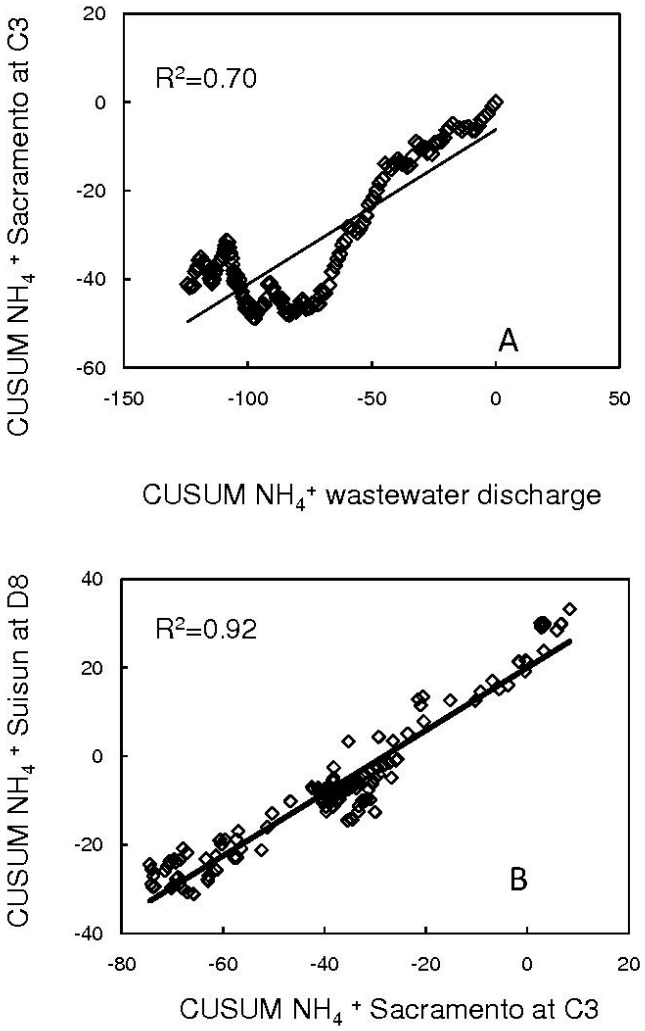

Fig. 7

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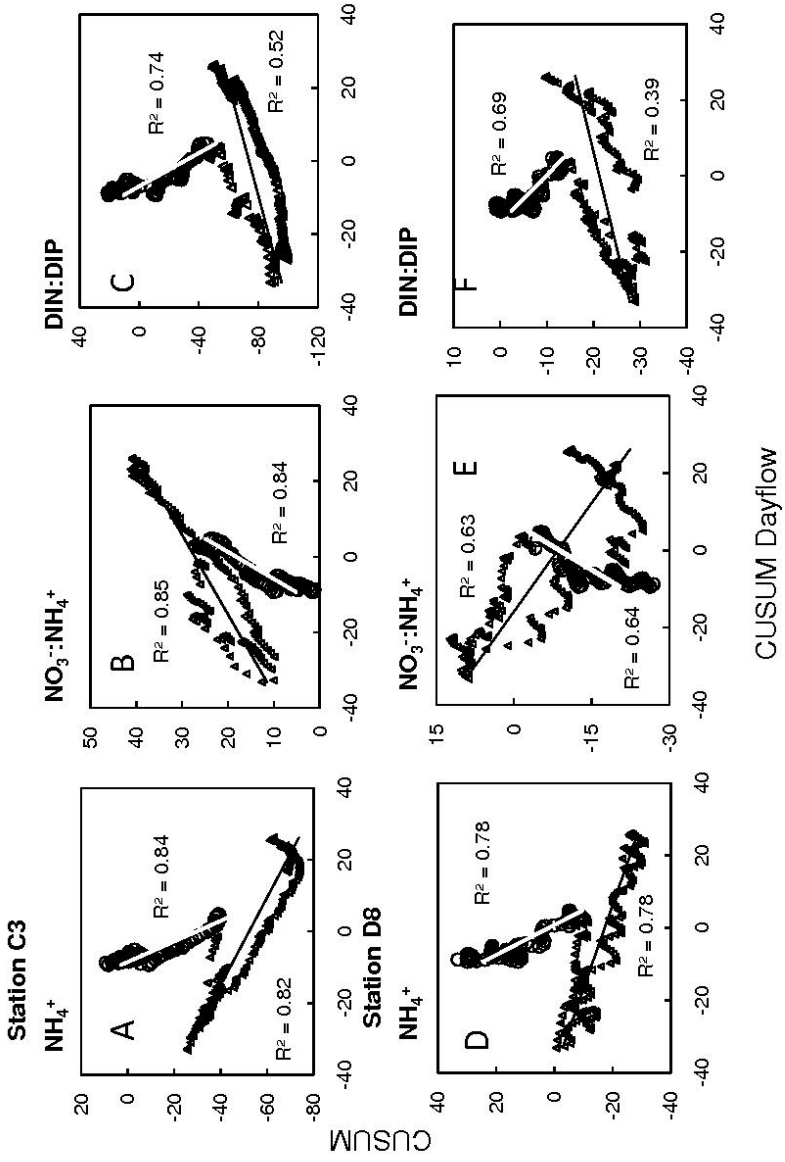

Fig. 8

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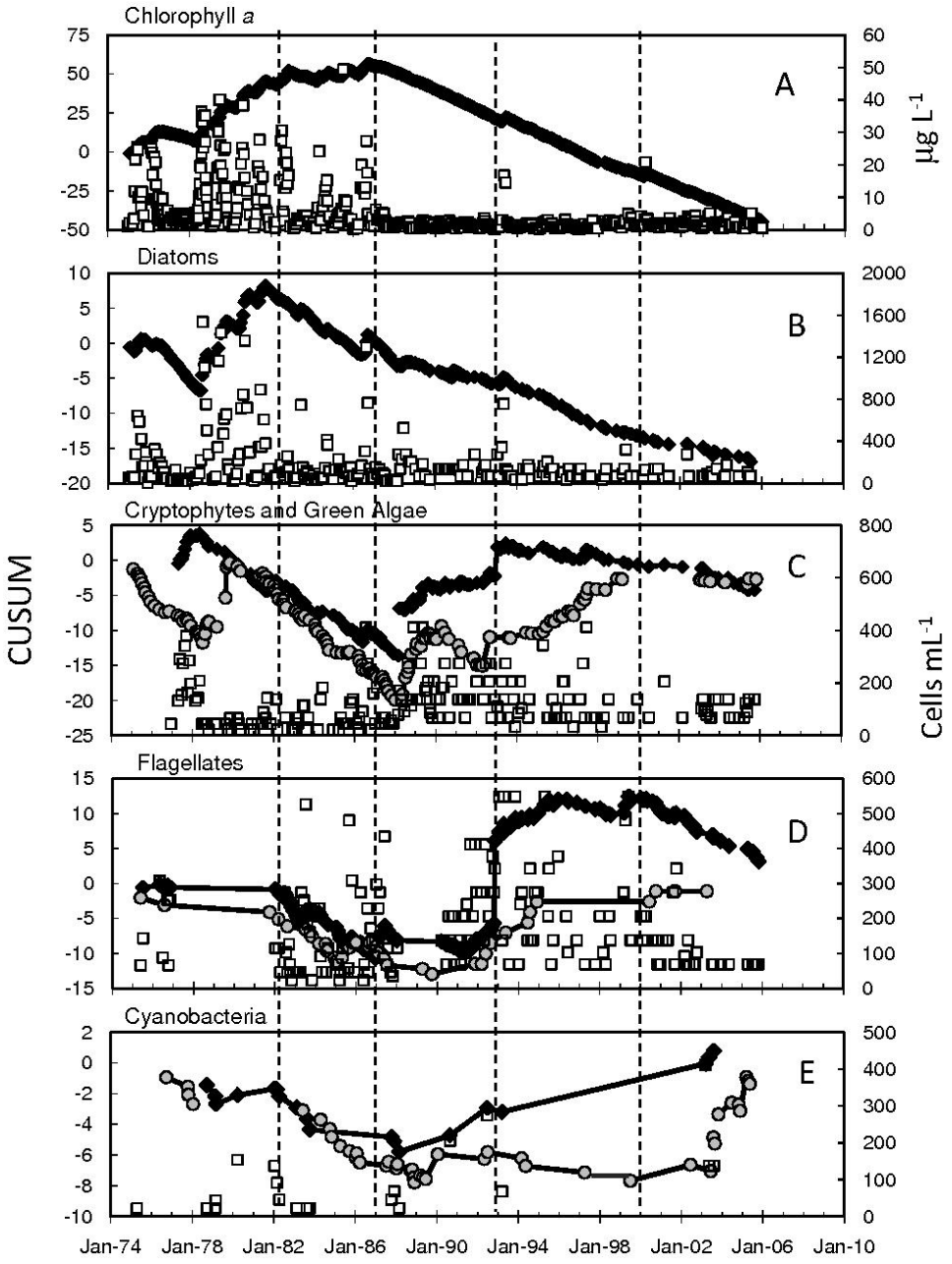

Fig. 9

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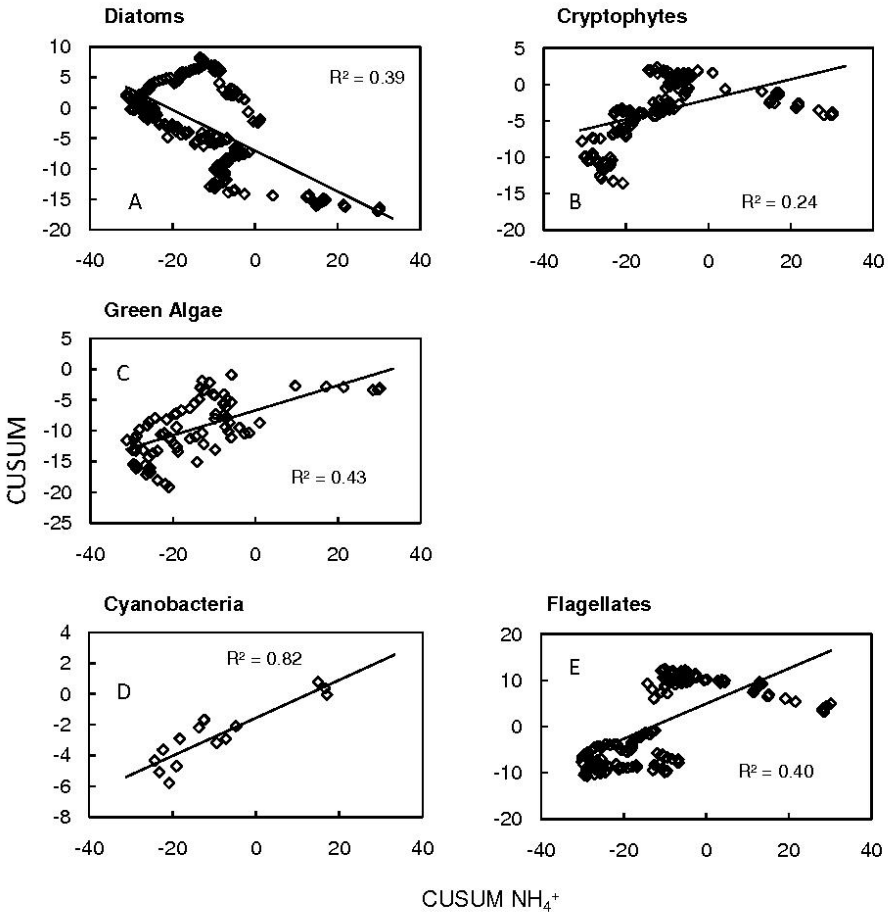

Fig. 10

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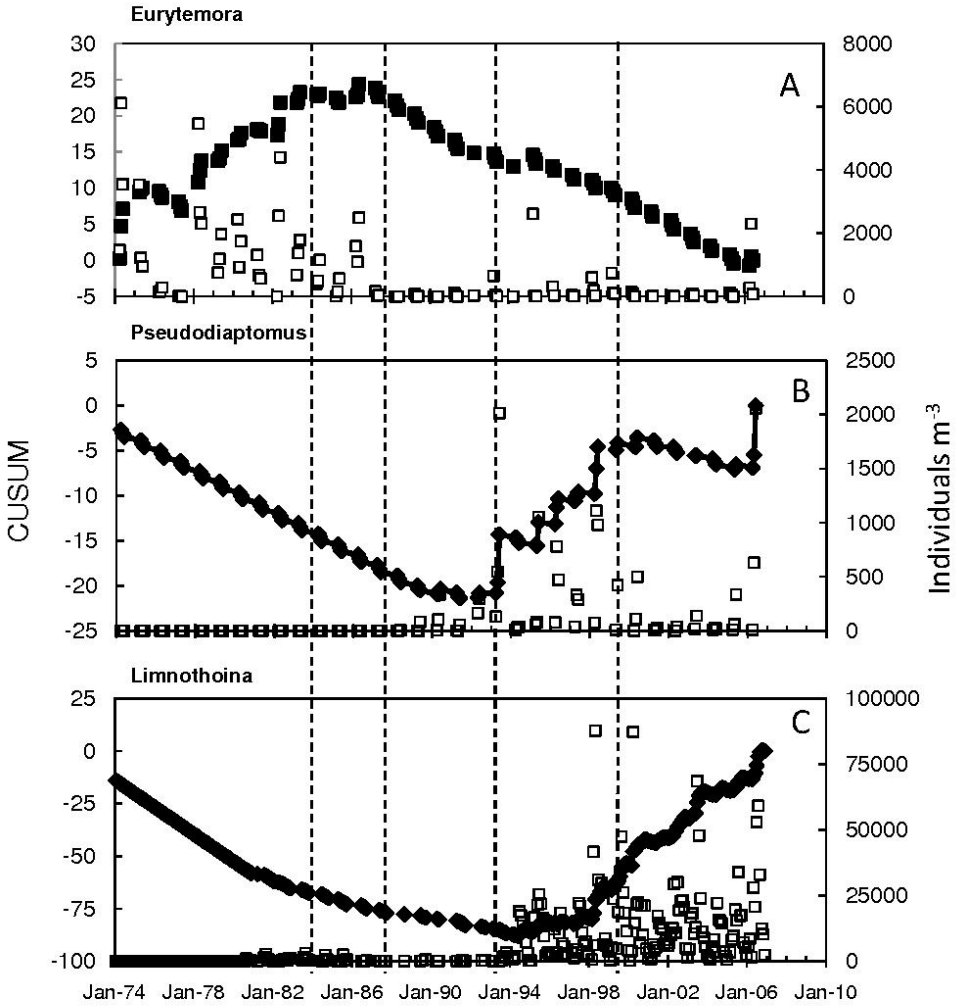

Fig. 11

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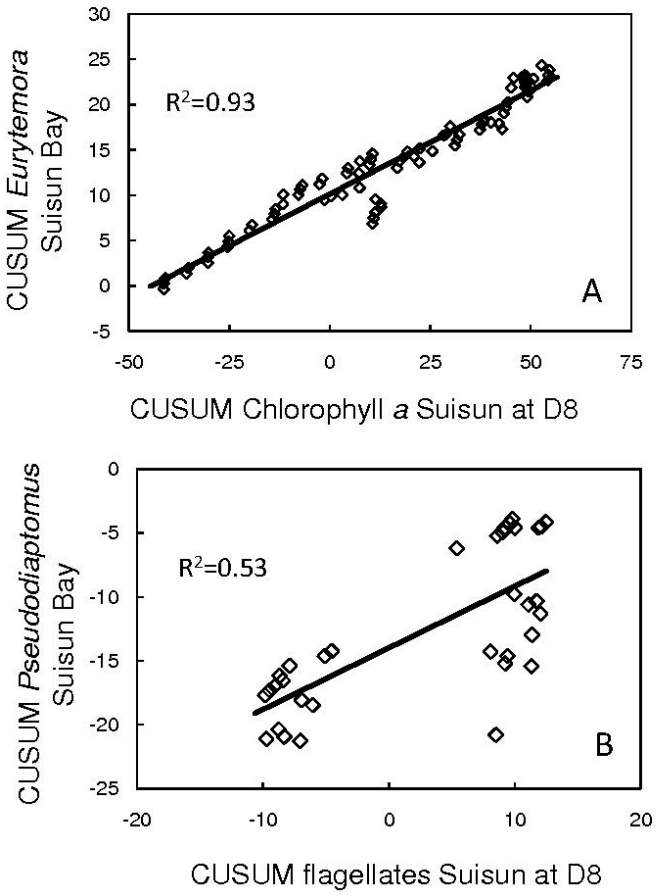

Fig. 12

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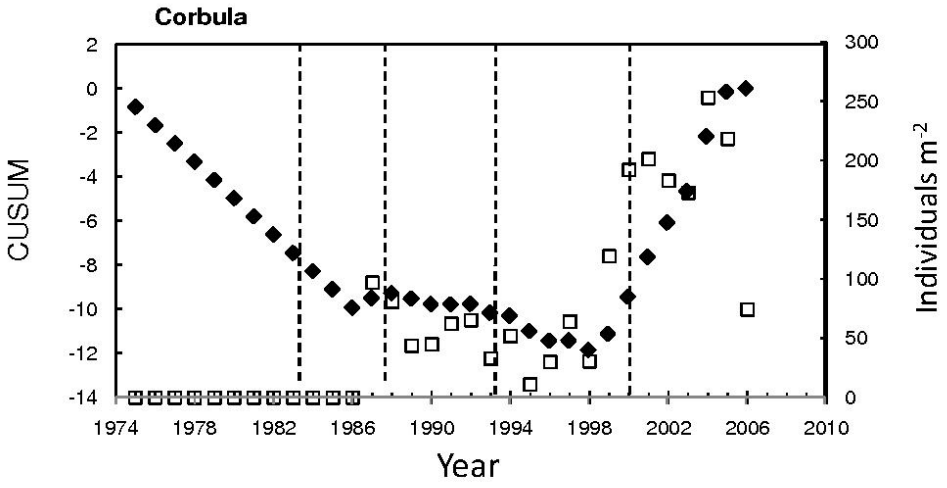

Fig. 13

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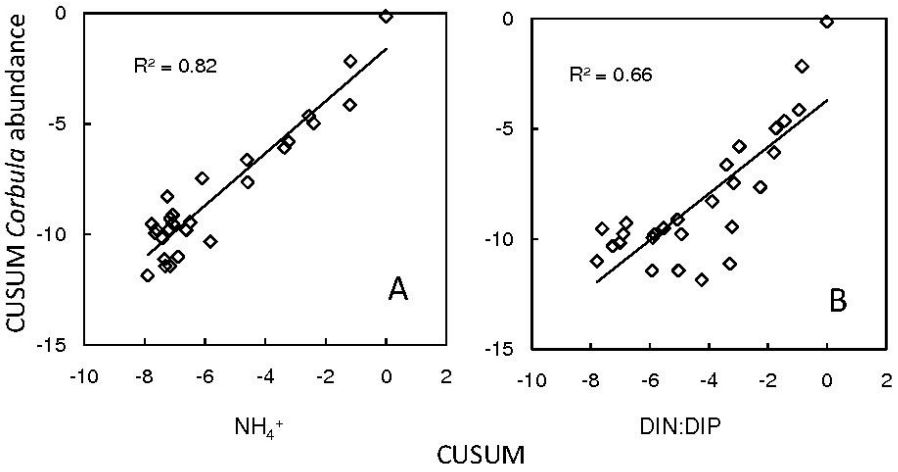

Fig. 14

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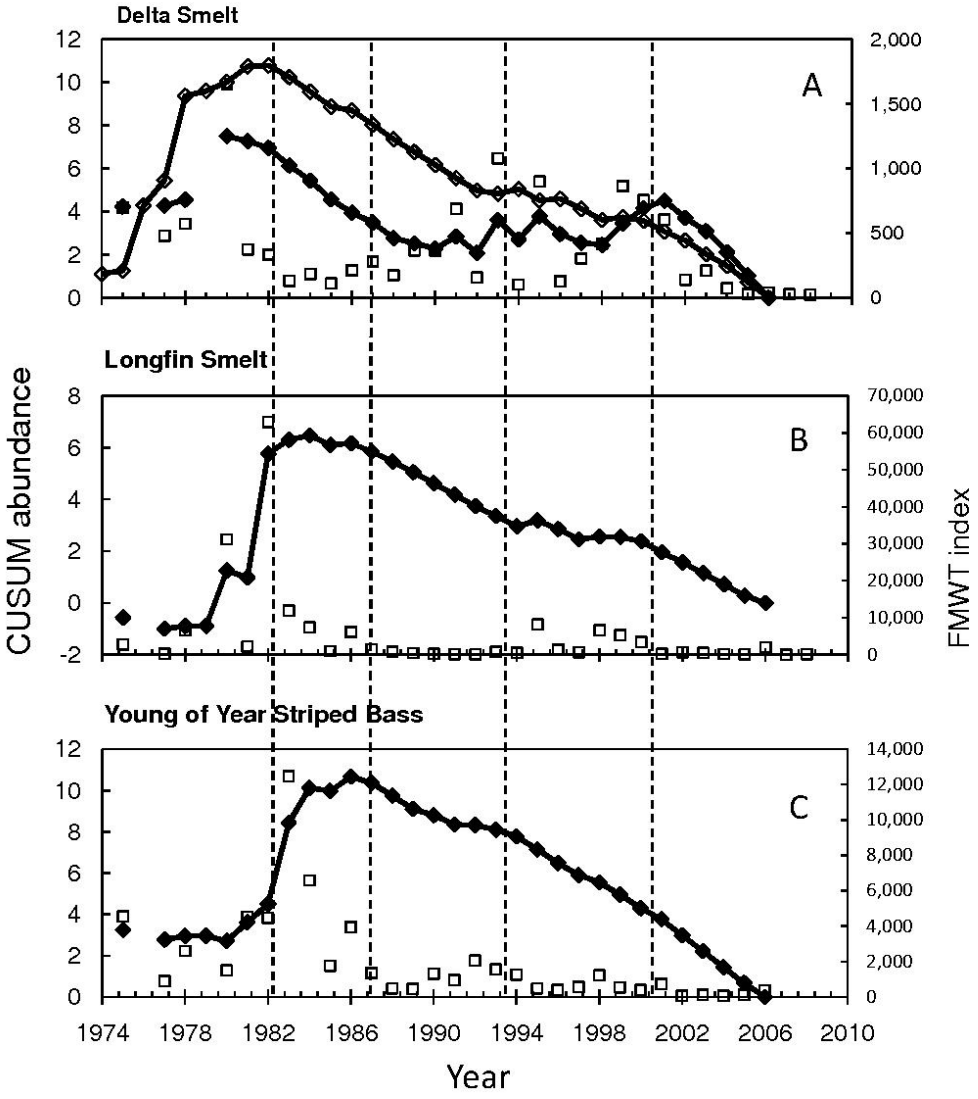

Fig. 15

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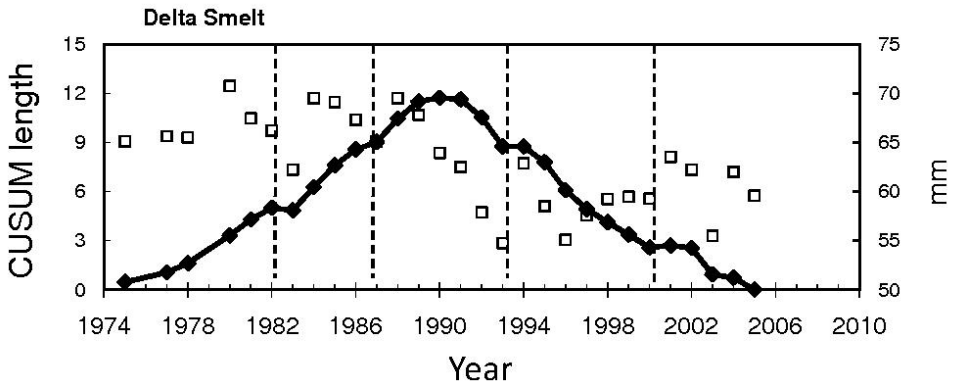

Fig. 16

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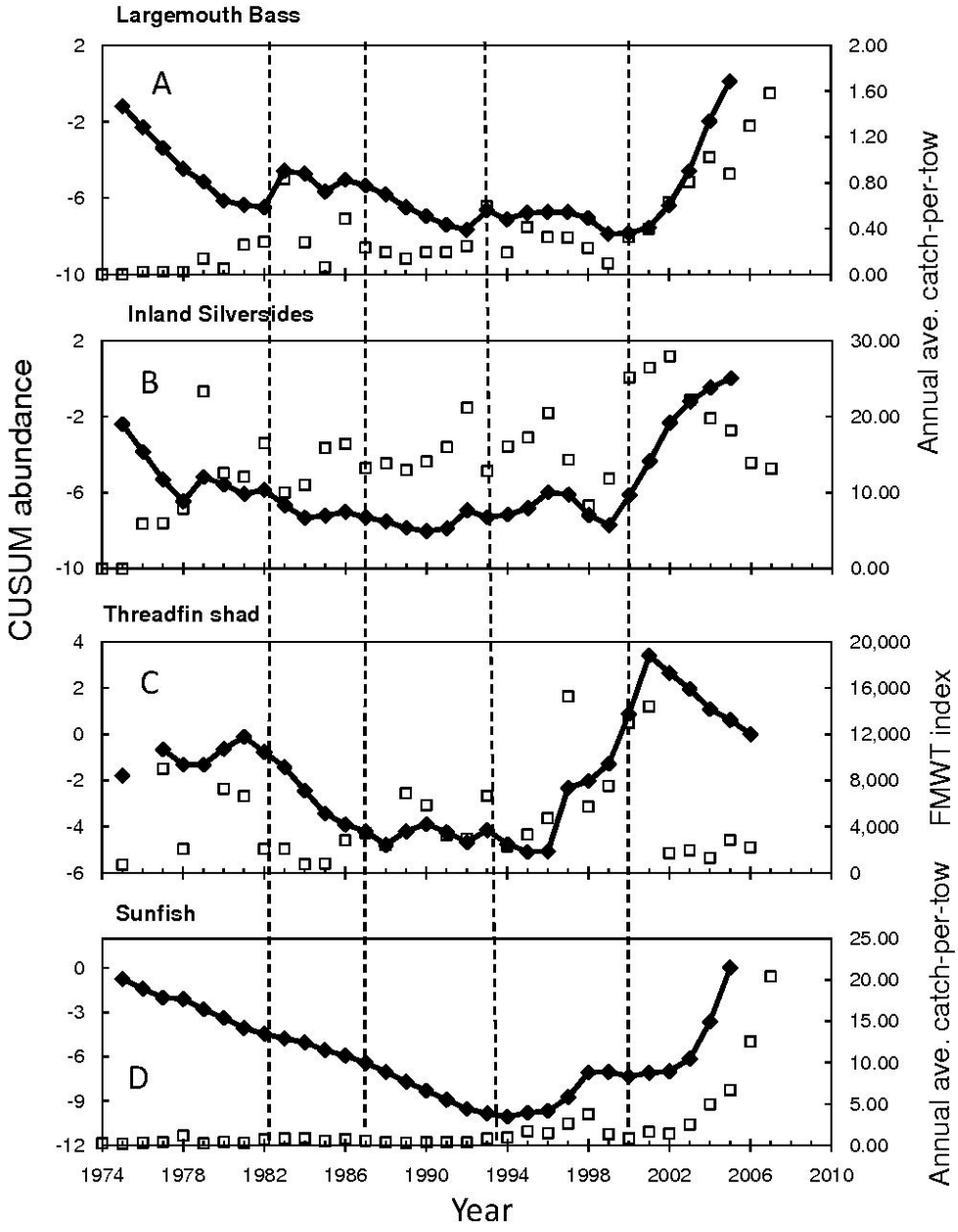

Fig. 17

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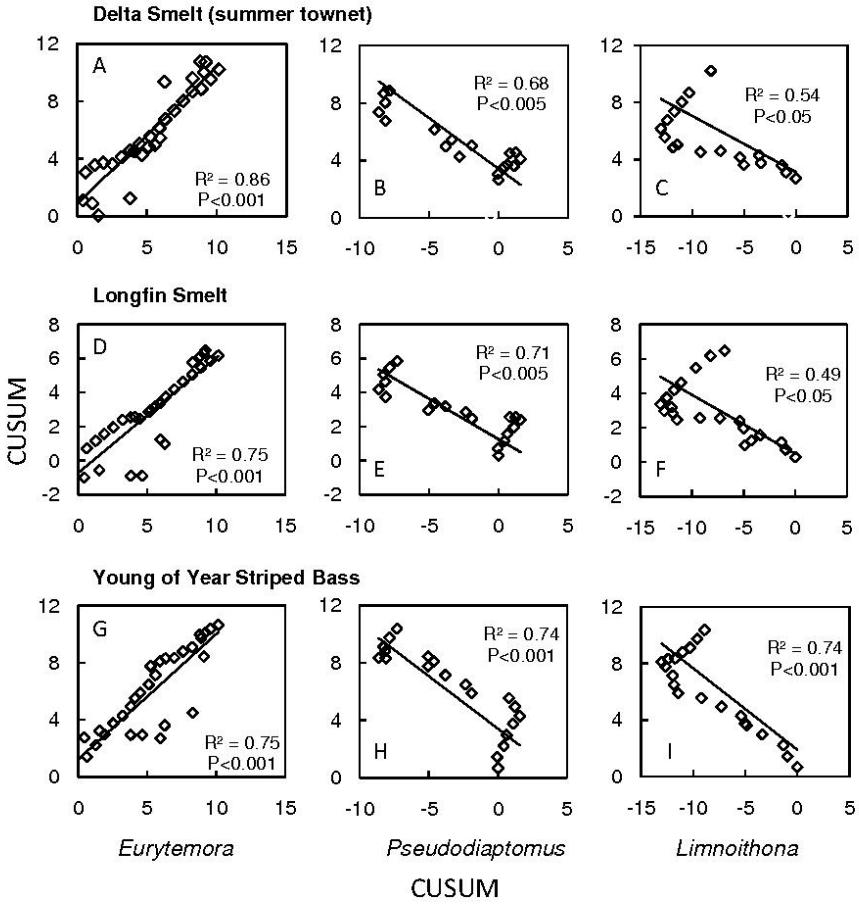

Fig. 18

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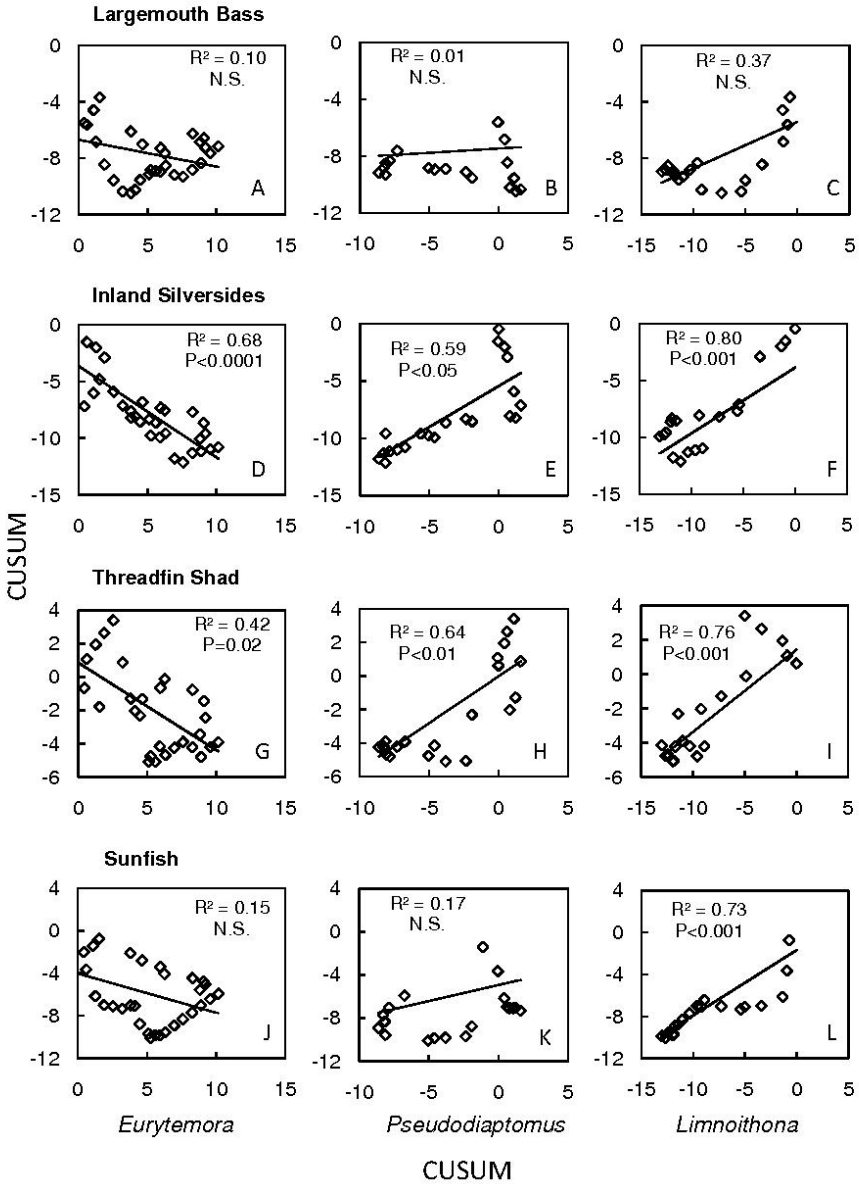

Fig. 19

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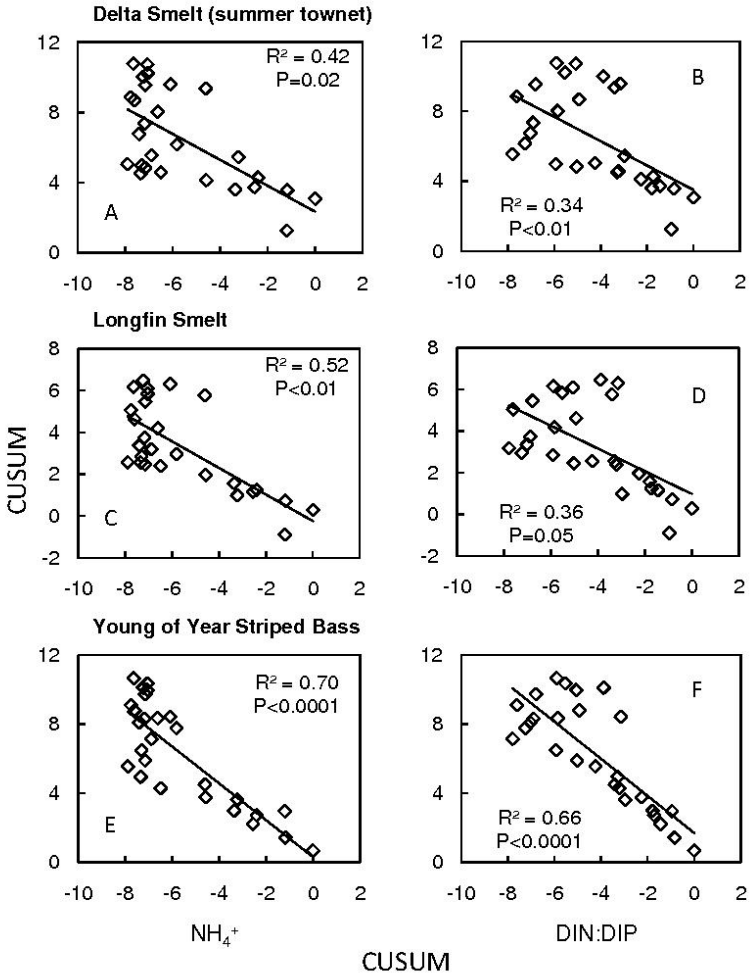

Fig. 20

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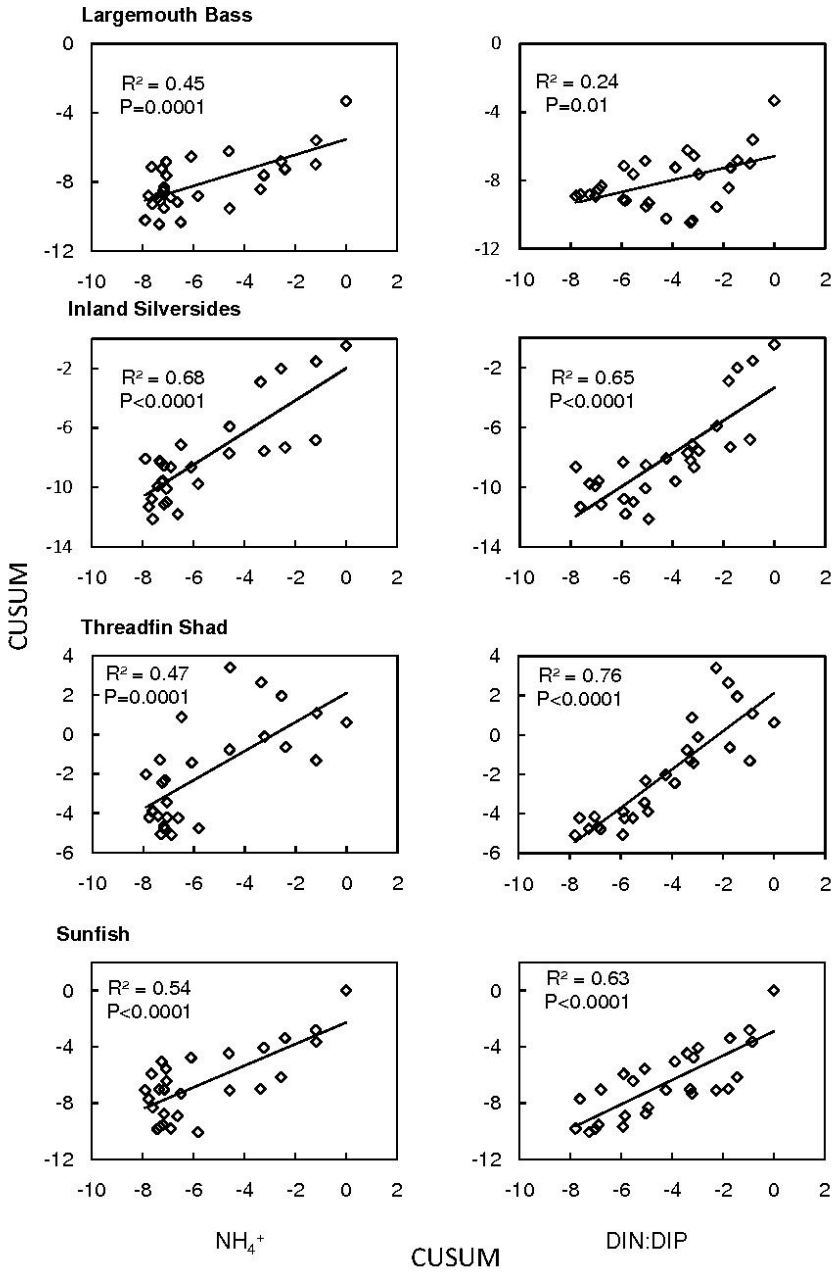

1125

1126 Fig. 21.

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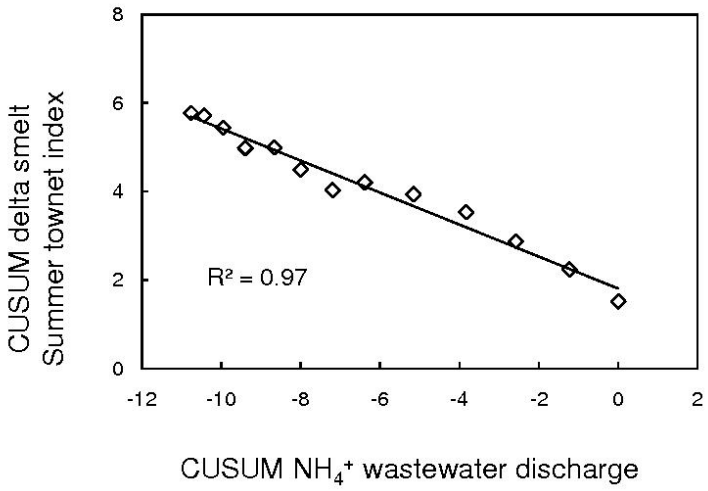

Fig. 22

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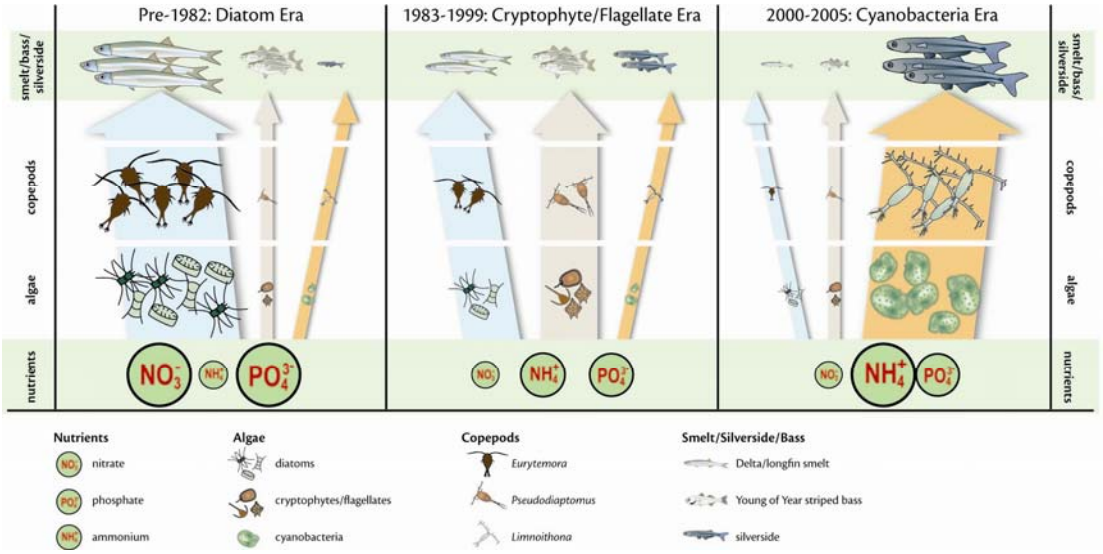

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Table 1. Correlations between CUSUM X2, the measured distance from the Golden Gate Bridge and the isohaline where salinity is 2, and CUSUM of the fish or clam species indicated. All fish data encompass the period from 1975-2005; the clam correlations encompass the period from 1987-2005. None of these relationships were significant.

Species	**R^2**
Delta smelt, *Hypomesus transpacificus* (summer townet index)	0.073
Delta smelt, *Hypomesus transpacificus* (fall midwater trawl index)	0.097
Longfin smelt, *Spirinchus thaleichthys*	0.167
Young-of-the-year striped bass, *Morone saxatilis*	0.037
Largemouth bass, *Micropterus salmoides*	0.089
Inland silversides, *Menidia beryllina*	0.004
Threadfin shad, *Dorosoma petenense*	0.051
Sunfish, *Lepomis* spp.	0.176

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Perils of correlating CUSUM-transformed variables to infer ecological relationships (Breton et al. 2006, Glibert 2010)

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We comment on a nonstandard statistical treatment of time-series data first published by Breton et al. (2006) in Limnology and Oceanography and, more recently, used by Glibert (2010) in Reviews in Fisheries Science. In both papers, the authors make strong inferences about the underlying causes of population variability based on correlations between cumulative sum (CUSUM) transformations of organism abundances and environmental variables. Breton et al. (2006) reported correlations between CUSUM-transformed values of diatom biomass in Belgian coastal waters and the North Atlantic Oscillation, and between meteorological and hydrological variables. Each correlation of CUSUM-transformed variables was judged to be statistically significant. On the basis of these correlations, Breton et al. (2006) developed "the first evidence of synergy between climate and human-induced river-based nitrate inputs with respect to their effects on the magnitude of spring *Phaeocystis* colony blooms and their dominance over diatoms."

Using the same approach, Glibert (2010) reported correlations between CUSUM-transformed abundances of organisms occupying many trophic levels and a range of environmental variables in the San Francisco Estuary, California. These correlations were reported to be statistically significant, and on this basis Glibert (2010) concluded that recent large population declines of diatoms, copepods and several species of fish were responses to a single factor – increased ammonium inputs from a municipal wastewater treatment plant. The study by Breton et al. (2006) is consistent with a large body of research demonstrating the importance of climate and human activity on phytoplankton communities in Belgian coastal waters (Lancelot 2007). However, Glibert's (2010) study piqued our curiosity about correlations between CUSUM-transformed variables because it contradicts the overwhelming weight of

evidence that population collapses of native fish (Sommer et al. 2007) and their supporting food webs in the San Francisco Estuary are responses to multiple stressors including landscape change, water diversions, introductions of exotic species, and changing turbidity (Bennett and Moyle 1996; Kimmerer et al. 2005; Cloern 2007; Jassby 2008; Mac Nally et al. 2010; Thomson et al. 2010). We ask here how CUSUM transformation leads to inferences about such cause-effect relationships when visual inspection of the data series (e.g., Fig. 1) shows no association between wastewater ammonium and fish abundance.

We emphasize an important distinction between the CUSUM chart and CUSUM transformation. The CUSUM chart is a well-established technique of quality assurance for industrial processes (Page 1954). The method involves keeping a running summation of the deviations of the quality of the quantity of interest (e.g., concentration of an industrial chemical) based on a sample of size *n*. If the quantity suddenly jumps, or gradually drifts from the specified tolerance, then a warning is raised and the process is stopped. The CUSUM chart has been used as a valuable off-line method in aquatic sciences to detect and resolve climatic (Breaker 2007) and ecological (Briceño and Boyer 2010) regime shifts, as well as departures of water-quality indicators from compliance conditions (Mac Nally and Hart 1997). In contrast, there appears to be no history for regression (or correlation) analyses on CUSUM-transformed variables prior to its use by Breton et al. (2006), and we have found no theoretical development or justification for the approach. We prove here that the CUSUM transformation, as used by Breton et al. (2006) and Glibert (2010), violates the assumptions underlying regression techniques. As a result, high correlations may appear where none are present in the untransformed data (e.g., Fig. 1). Regression analysis on CUSUM-transformed variables is, therefore, not a sound basis for making inferences about the drivers of ecological variability measured in monitoring programs.

This issue is sufficiently important to warrant exploration of the approach, which we present here.

The CUSUM function

The CUSUM function is a mathematical discrete operator that transforms an input time series (x_t) to an output time series (y_t) representing the running total of the input.

$$y_t = \sum_{i=1}^{t} x_i \tag{1}$$

The CUSUM function often is applied to time series of standardized residuals to detect changes in the mean of the time series (Zeileis et al. 2003; Breaker 2007). The CUSUM function changes the statistical properties of the input time series. If the standardized input time series consists of independent observations with zero mean ($E[x_t] = 0$) and variance σ^2 ($V[x_t] = \sigma^2$) then

$$E[y_t] = \sum_{i=1}^{t} E[x_i] = 0 \tag{2}$$

$$V[y_t] = \sum_{i=1}^{t} V[x_i] = t \cdot \sigma^2 \tag{3}$$

$$Cov[y_t, y_{t-1}] = Cov\left[\sum_{i=1}^{t} x_i, \sum_{i=1}^{t-1} x_i\right] = (t-1) \cdot \sigma^2 \tag{4}$$

$$Corr[y_t, y_{t-1}] = \frac{Cov[y_t, y_{t-1}]}{\sqrt{V[y_t] \cdot V[y_{t-1}]}} = \frac{(t-1) \cdot \sigma^2}{\sqrt{t \cdot \sigma^2 \cdot (t-1) \cdot \sigma^2}} = \frac{t-1}{\sqrt{t \cdot (t-1)}} \tag{5}$$

This means that the variance of the CUSUM-transformed variables and the autocovariance between two consecutive observations of the CUSUM-transformed variables both grow linearly with time and, consequently, the autocorrelation of the CUSUM-transformed variables quickly approaches 1.

Two key assumptions behind tests derived from standard regression analyses are that the observations comprising the sample are independently and identically distributed (IID). As shown above, both assumptions are violated when a random input variable is CUSUM-transformed because: the variance is not constant, so the transformed observations are not identically distributed; and the transformed observations are autocorrelated and therefore not independent of one another. Thus, applying statistical regression techniques to CUSUM-transformed time series violates the two most crucial assumptions for these tests.

CUSUM transformation inflates correlation

The CUSUM of a purely random process is a pure random walk, an example of a difference-stationary variable (because its first difference is stationary). Pfaff (2006) described the difficulty of using difference-stationary variables in regression and correlation: "In this case, the error term is often highly correlated and the *t* and *F* statistics are distorted such that the null hypothesis is rejected too often for a given critical value; hence the risk of a 'spurious regression' or 'nonsense regression' exists. Furthermore, such regressions are characterized by a high R^2." Regressions involving cumulative variables such as those produced by CUSUM transformation are classic examples of spurious regression and a well-known problem in econometrics (Hendry 1980).

To illustrate the problem more concretely, we conducted the following Monte Carlo experiment. We first generated two independent, standardized (mean 0, standard deviation 1), normal random processes of length 30, about the length of many annualized time series available from monitoring data (e.g., those analyzed by Glibert 2010). We then calculated the Pearson correlation between these two series and also between their CUSUM-transformed values. We

repeated the process 100,000 times, yielding two distributions of correlation coefficients from which we generated 95% confidence intervals (CI). The distribution of CUSUM correlations is very different from the distribution of correlations of the untransformed variables (Fig. 2). The 95% CI is (-0.36, 0.36) for the original variables (Fig. 2A), but (-0.71, 0.71) for the CUSUM-transformed variables (Fig. 2B). Thus, correlations must exceed 0.71 (instead of 0.36) for CUSUM-transformed variables to be considered significant at the $p < 0.05$ levels. This implies that the CUSUM transformation increases the probability of making a Type I error (incorrectly rejecting a null hypothesis of no correlation) from 5% to 42% when Pearson's statistics are applied. Therefore, on this basis alone, the *p*-values for correlations of CUSUM-transformed variables reported by Breton et al. (2006) and Glibert (2010) are incorrect.

The above experiment was based on independent random processes. Water resources data, however, commonly exhibit serial correlation (Helsel and Hirsch 2002). The introduction of serial correlation accentuates the problem by broadening the distribution of correlation coefficients even further than in the example above. To measure this effect, we repeated the simulations after introducing varying amounts of first-order serial correlation (r_1, r_2) into the paired series that otherwise represented random normal processes (using the *arima.sim* function of R; R Development Core Team 2010). This second experiment shows how the 95% CIs for the correlations broaden in proportion to the strength of serial correlation (Table 1, Fig. 2C). The presence of serial correlation thus increases the probability of making a Type I error further (53% when $r_1 = r_2 = 0.5$), making any conclusions from such correlations correspondingly less reliable. Even if a significance level of $p < 0.0001$ were used, the probability of making a Type I error (19% when $r_1 = r_2 = 0.5$) would still be much greater than 5%.

We showed that two CUSUM-transformed variables often have an apparent statistically significant correlation even if none exists between the original untransformed series. Moreover, even if a statistically significant relationship could be established between CUSUM-transformed variables, there is no proven basis for inferring relationships between the original variables. Given these difficulties, we wonder what purpose is served by CUSUM transformation for exploring relationships between two variables. As a real example, Glibert (2010) inferred a strong negative association between delta smelt abundance and wastewater ammonium from regression of CUSUM-transformed time series. However, the Pearson correlation (r = -0.096) between the time series (Fig. 1) is not significant, even under the naive IID assumptions (p = 0.68). In short, correlations between CUSUM-transformed variables should not be used as a substitute for analysis of the original untransformed variables.

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Table 1. Upper limits of the 95% CIs for correlation between two untransformed and CUSUM-transformed random variables with different combinations of serial correlation coefficients, r_1 and r_2.

r_1	r_2	Untransformed	CUSUM-transformed
0.0	0.0	0.36	0.71
0.1	0.1	0.36	0.73
0.1	0.5	0.38	0.77
0.1	0.9	0.39	0.82
0.5	0.5	0.44	0.81
0.5	0.9	0.51	0.86
0.9	0.9	0.71	0.92

Figure Legends

Figure 1. Annual (A) abundance index of delta smelt (*Hypomesus transpacificus*) in the San Francisco Estuary and (B) wastewater loadings of ammonium to the Sacramento River, 1985-2005. Treatment plant data were obtained from the Sacramento Regional County Sanitation District (S. Nebozuk pers. comm., 28 July 2006). Monthly loading was calculated from discharge-weighted ammonium concentrations using the methods described by Jassby and Van Nieuwenhuyse (2005). Delta-smelt abundance data were obtained from the California Department of Fish and Game (http://www.dfg.ca.gov/delta/data/townet/indices.asp?species=3).

Figure 2. (A) Frequency distribution of correlation coefficients for two independent random normal series of length 30 (n = 100,000). (B) Same as A after the samples are CUSUM-transformed. (C) Same as B, but with first-order serial correlation of 0.5 introduced into the otherwise random normal processes. Vertical dashed lines, 95% CI.

Fig 1

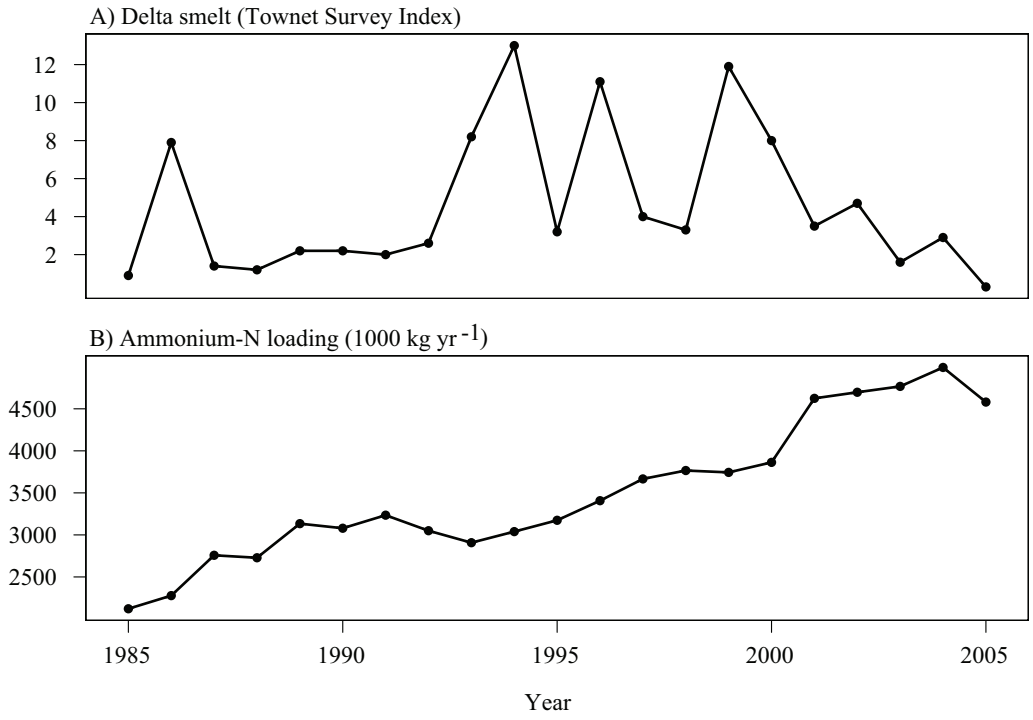
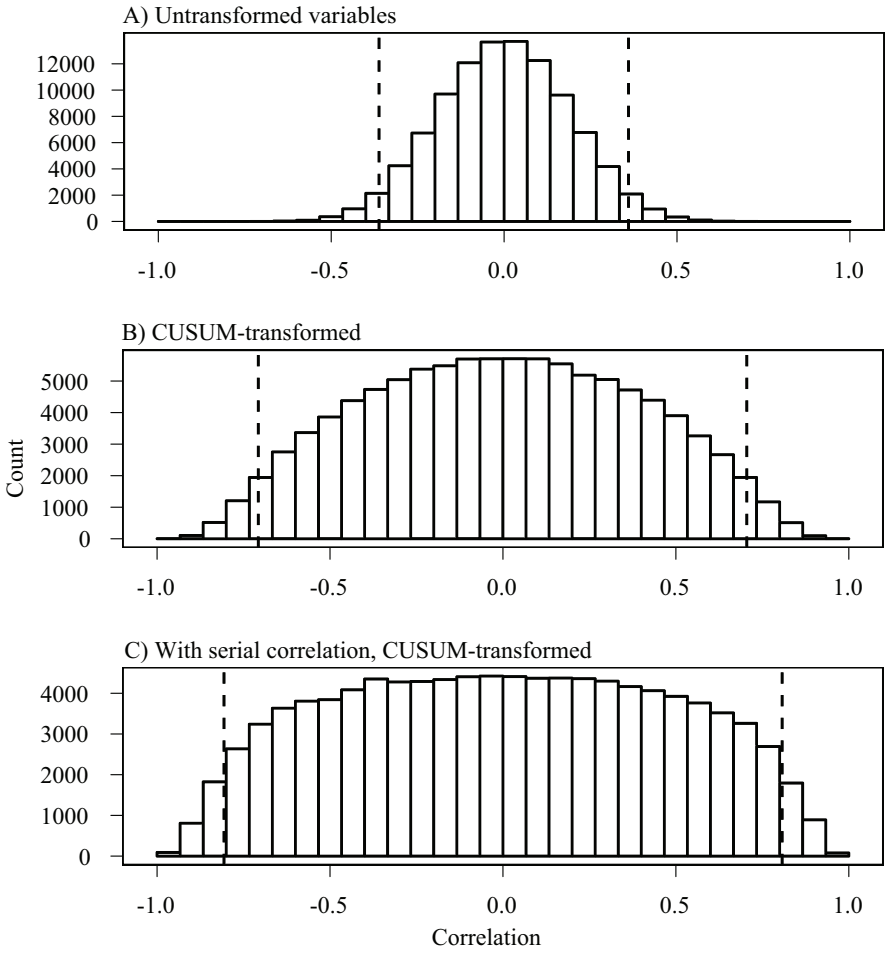

A) Delta smelt (Townet Survey Index)
12
10
8
6
4
2
B) Ammonium-N loading (1000 kg yr -1)
4500
4000
3500
3000
2500
1985
1990
1995
2000
2005
Year

Fig 2


A) Untransformed variables
12000
10000
8000
6000
4000
2000
0
-1.0
-0.5
0.0
0.5
1.0
B) CUSUM-transformed
Count
5000
4000
3000
2000
1000
0
-1.0
-0.5
0.0
0.5
1.0
C) With serial correlation, CUSUM-transformed
4000
3000
2000
1000
0
-1.0
-0.5
0.0
0.5
1.0
Correlation