Central Coast Long-term Environmental Assessment Network

REGIONAL MONITORING PROGRAM

Annual Report 2009–2010

January 31, 2011

Revised April 29, 2011

Lead Agency:

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2009–2010 Annual Report

Central Coast Long-term Environmental Assessment Network

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1.0 Executive Summary

The 2009-2010 Central Coast Long-term Environmental Assessment Network (CCLEAN) annual report incorporates the results from 2009-2010 with historic data. Major findings are as follows:

- Nearshore waters of Monterey Bay continue to exceed the California Ocean Plan (Ocean Plan) objective for the protection of human health for PCBs. Moreover, North Monterey Bay has exceeded the Ocean Plan objective for the protection of human health for Dieldrin in two out of 11 sampling periods. Although there have been samples of nearshore water that have approached or exceeded the Ocean Plan objectives for PAHs, there are not consistent patterns of exceedances for this POP that would warrant special concern.

  Recommendations: Sampling of nearshore waters should continue in order to document the effects on ocean waters caused by discharges from land.
- DDT concentrations in sediments at historic CCLEAN sites have been stable, except for spikes measured at two sites 2006. All DDT measurements at historic CCLEAN sites have exceeded the NOAA ERL. There have been significant declines in total organic carbon and percent fines at many CCLEAN sediment sites, which remain unexplained. CCLEAN results continue to suggest that POPs, principally DDTs, and river discharges appear to be having negative effects on benthic communities along the 80-meter contour in Monterey Bay. Concern for the effects of DDTs on a regional scale are supported by other studies showing that Monterey Bay is a primary source of DDTs distributed across the continental shelf and slope along the central California coast.

  Recommendations: Sediment sampling should continue in order to provide documentation of changing habitats and biological communities in Monterey Bay, as they are affected by POPs.
- While concentrations of Dieldrin in mussels along the northern shore of Monterey Bay often exceeded the U.S. EPA screening value for recreational fishers from 2002 to 2008, concentrations have declined since 2008. Several more years with Dieldrin concentrations consistently below the U.S. EPA screening value for recreational fishers would be a clear indication of reduced inputs to Monterey Bay.

  Recommendations: Mussel sampling should continue unchanged. The mussel data collected have been very valuable in documenting impairments of beneficial uses, as well as supporting assessments of contaminant sources.
- There have been no bacterial impairments to the water contact recreation beneficial use associated with discharges from any of the CCLEAN wastewater treatment plants. Only the Ocean Plan *Enterococcus* single sample objective for water contact recreation was exceeded once at the farfield receiving water monitoring station adjacent to the Santa Cruz discharge. Statistical analyses detected no differences between near-field and far-field samples at any wastewater discharge.

Recommendations: The CCLEAN participants have previously asserted their desire to continue monitoring receiving water bacteria, even in the absence of demonstrable effects of their discharges on beneficial uses. The results from the ongoing study of fecal pathogens by CCLEAN in collaboration with University of California at Davis and California Department of Fish and Game could inform future recommendations for more meaningful indicators of potential risks from pathogens.

- Estimated loads of most POPs remain much greater from rivers than from wastewater, although loads of PBDEs are higher from wastewater than from rivers. Nevertheless, high loads of PCBs and Dieldrin from rivers are apparently not solely responsible for exceedances of the Ocean Plan PCB objective for the protection of human health or U.S. EPA recreational fisher screening level for Dieldrin in mussels.

  PCBs in northern Monterey Bay were statistically linked to wastewater loads, while they were not statistically linked to loads from rivers. The correlation between wastewater discharges and PCBs in Monterey Bay was evident for total PCBs, as well as for the percentages of PCB homologues, although ocean waters had higher proportions of di- and trichlorobiphenyls than did wastewater discharges.

  Concentrations of Dieldrin in mussels along the northern shore of Monterey Bay were associated with loads from both rivers and wastewater, as well as to rainfall in some cases. These results suggest that rivers, wastewater and runoff from the land are all affecting Dieldrin concentrations in mussels. Partial correlations suggest that loads from rivers and wastewater have comparable effects, although the effect of rivers is slightly greater than that of wastewater.

  Recommendations: In the consideration of Monterey Bay's exceedances of the Ocean Plan objective for PCBs, it would be helpful to know the linkages between PCBs in Monterey Bay and human health. It is possible that this nexus could be provided by analyses of fish tissues performed by San Francisco Estuary Institute in the Monterey Bay in 2010. If fish and shellfish are not exhibiting elevated PCBs, the PCB water quality objective possibly could be raised through development of a Site Specific Objective.

  Water quality regulators should consider ways of reinitiating POP sampling in the Salinas River. As the source of the greatest historic loads of most POPs, data from the Salinas River would vastly improve the ability to more accurately partition causes of impairments among different sources. Moreover, the results PCB and Dieldrin source modeling were potentially affected by the unknown amounts of these POPs in the dissolved and particulate phases from each source, and data on the loads for each phase would improve source-tracking analyses.

  Development of a mass balance model for PCBs in Monterey Bay also could help to further discriminate the actual sources of impairments to water quality. Such a model would enable estimates of the mass of PCBs coming into Monterey Bay from many sources. This effort would undoubtedly require collection of samples from additional locations and depths than are currently sampled by CCLEAN.

- The assays of the effects of endocrine disrupting compounds (EDCs) on reproduction in fathead minnows, based on an EPA protocol, found no evidence that reproduction was adversely affected by the wastewater effluents at receiving water concentrations.

Nevertheless, several aspects of the assays reduced their ability to detect reproductive effects, such as high variability in fecundity among aquaria within and among treatments. An unknown portion of this variability could have been due to the use of fish from different sources, with attendant size differences and possible subtle physiological differences. The average age of the test fish used in the assays was higher than the recommended age in the EPA protocol, and this also may be significant in evaluating uncertainties in the results.

Several examples of female masculinization were observed in the effluent exposure aquaria and not in any control aquaria. Other studies of endocrine disruption in fish caused by historic discharges of DDTs and PCBs have found masculinzation of female fish thought due to binding of estrogen receptors by the synthetic organic chemicals (Spies and Thomas, 1997).

This study of EDC effects from municipal wastewater on reproduction in fathead minnows has been groundbreaking and the results and various problems encountered will be invaluable for improving the methods for future assays.

Recommendations: The availability of grant funding should be explored to further improve the method for routine application. Future assays should only use fish from a single source throughout the assay, which could require phased assays, such as testing effluent from one treatment facility each year. A statistical approach should be used to plan for the optimum number of replicates for minimizing within-test variability. A standardized EDC should be used as a reference toxicant. The use of other fish species, which might be more sensitive to EDCs should be explored. Reproductive hormone endpoints other than Vtg production, such as gene induction, should be investigated and tissues other than blood plasma for Vtg testing should be explored.

- In the Proposition 50-funded study of indicator bacteria (i.e., total coliform, fecal coliform and *Enterococcus*) and eight fecal pathogens (i.e., *Vibrio parahaemolyticus*, *V. cholerae*, *V. alginolyticus*, *Campylobacter*, *Salmonella*, *E. coli* O157, *Cryptosporidium* and *Giardia*), seven out of 11 microbes analyzed were detected at all wastewater treatment facilities. *Vibrio alginolyticus*, *Campylobacter* and *E. coli* O157 were not detected in any wastewater sample. Influent concentrations of *Enterococcus* and *Vibrio parahaemolyticus* varied significantly between sampling periods, as did effluent concentrations of *V. parahaemolyticus*. One wastewater discharge had significantly lower effluent concentrations of total coliform, fecal coliform and *Enterococcus*, while that site and another had significantly lower concentrations of *Giardia* than did either of the other two discharges.

  All wastewater treatment facilities appeared to remove more than 99% of the actual fecal pathogens measured. Across all sites, *V. cholerae*, *Giardia* and *Cryptosporidium* were most easily removed, with average removals exceeding 99.6%. *Salmonella* had the lowest average removal at 76.94%. One site averaged 99.99% over all microbes measured and 99.86% for fecal pathogens. The site with the lowest overall removal averaged 65.24%, although it removed 99.94% of fecal pathogens.

  Rough estimates of fecal pathogen loads from surface waters (i.e., streams and rivers), storm runoff and wastewater suggest that surface waters and storm runoff, when combined, exceed those from wastewater for every fecal pathogen, except *Giardia*. Surface waters were the largest source of *Vibrio cholerae*. The location and mode of

discharge from each source affect their respective probabilities of causing human and wildlife health concerns. Wastewater discharges are required to be located far from shore in deep water, such that high dilution of the discharge occurs before it reaches areas frequented by humans. Conversely, streams, rivers and storm drains discharge directly to nearshore regions resulting in very little dilution at the shoreline.

Recommendations: Future efforts to make nearshore waters cleaner should focus on the direct discharges to nearshore areas, such as streams, rivers and storm runoff. Concerted efforts should be made to generate more complete and accurate discharge flow and load estimates. Event-based sampling should be included to document flows and loads of fecal pathogens during high-flow events.

2.0 Program Background

The complexity of environmental issues affecting nearshore marine waters today have led to general agreement that their protection is only possible by implementing regional approaches to monitoring and resource management. Nearshore marine waters are affected by point-source discharges, storm runoff, rivers, discharges from ships, and aerial deposition. At the same time, many marine resources are diminishing under pressure from increasing usage. In the late 1990s, multiple agencies in the Monterey Bay area began working toward implementation of a regional approach to monitoring watersheds and marine waters.

CCLEAN is a long-term monitoring program that has been designed by program participants through a commitment to environmental stewardship in order to fulfill several regulatory objectives. CCLEAN is currently funded by the City of Santa Cruz, the City of Watsonville, Moss Landing Power Plant, Monterey Regional Water Pollution Control Agency (MRWPCA), and Carmel Area Wastewater District (CAWD), under the direction of the California Regional Water Quality Control Board, Central Coast Region (Water Board). CCLEAN fulfills a significant component of the subscribing agencies' compliance to their NPDES monitoring commitments, with an emphasis on receiving water monitoring. In addition, it represents a significant portion of their contributions to their communities' efforts at sustainability of their coastal environments. However, CCLEAN is also the current mechanism by which the Water Board fulfills part of its obligations under a monitoring framework developed to provide an ecosystem-based Water Quality Protection Program for the Monterey Bay National Marine Sanctuary. The monitoring framework evolved to fulfill the Water Board's obligations to the Management Plan for the Sanctuary. The Sanctuary's Management Plan includes a Memorandum of Agreement among eight federal, state, and regional agencies (including the Central Coast Regional Water Quality Control Board). The Water Board's framework for partial fulfillment of this Water Quality Protection Program is the Central Coast Ambient Monitoring Program (CCAMP). This multidisciplinary program includes sampling in watersheds that flow into coastal regions, in estuarine coastal confluences, and at coastal sites. The goal of CCAMP is to "collect, assess, and disseminate scientifically based water quality information to aid decision-makers and the public in maintaining, restoring, and enhancing water quality and associated beneficial uses." CCLEAN provides the initial nearshore component of CCAMP. CCLEAN has been underway since 2001 and its Quality Assurance Project Plan (QAPP) is being revised to incorporate recent program changes, and to retain consistency with the Water Board surface water ambient monitoring program (SWAMP) requirements for data compatibility.

Within the framework of CCAMP, the goal of the CCLEAN program is to assist stakeholders in maintaining, restoring, and enhancing nearshore water and sediment quality to support associated beneficial uses in the Central Coast Region, including recreation, wildlife habitat and biological communities. During the initial formation of CCLEAN, program participants decided that it should use high-quality data to address the following questions and objectives:

- What are the major sources of contaminants to nearshore waters?
- What are the effects of wastewater discharges in nearshore waters?
- Do nearshore waters and sediments comply with Ocean Plan?

- What are the status and long-term trends in the quality of nearshore waters, sediments, and associated beneficial uses?
- Develop a long-term database on trends in the quality of nearshore waters, sediments and associated beneficial uses.
- Ensure that the database is compatible with other regional monitoring efforts and regulatory requirements.
- Ensure that data are presented in ways that are understandable and relevant to the needs of stakeholders.

To answer these questions, CCLEAN uses various graphical and statistical approaches, as well as comparisons of data with numeric and narrative objectives, guidelines and alert levels from the Ocean Plan (State Water Resources Control Board 2005), Central Coast Basin Plan (RWQCB 1997), California State Mussel Watch Program (California State Mussel Watch Program 2003), California Office of Environmental Health Hazard Assessment (Office of Environmental Health Hazard Assessment 2003), and the National Oceanic and Atmospheric Administration (Long et al. 1998; Long et al. 2000).

3.0 Report Organization and Scope

This document incorporates the results from 2009-2010 in focused examinations designed to improve the efficiency of the CCLEAN program and guide management actions to reduce impairments of beneficial uses associated with discharges of persistent organic pollutants (POPs) to the ocean. Graphical and statistical presentations emphasize six POP groups that have either been associated with beneficial use impairments in previous CCLEAN reports or are pollutants of emerging concern that currently are not regulated by the California Ocean Plan (Ocean Plan) or NPDES waste discharge permits (Table 1). CCLEAN also measures more compounds in several of these groups than are regulated by the Ocean Plan (Table 1). Whenever data are compared to Ocean Plan water quality objectives in this report, the values presented include only compounds regulated by the Ocean Plan.

The sampling of discharges from rivers ceased to be a formal part of the CCLEAN program in 2007. Nevertheless, the City of Watsonville continues to support measurement of POPs in the Pajaro River and those data have been utilized at various places in this report with the City's permission.

Program monitoring activities during 2009-2010 (program year = July 1, 2009 – June 30, 2010) and their relationship to program objectives are shown in Table 2. Sampling sites are shown in Figure 1 and the dates of sampling are shown in Table 3. All sampling methods have been described in previous CCLEAN reports (CCLEAN 2007). In 2009–2010, CCLEAN implemented a one-year pilot program to examine whether wastewater discharges could be causing endocrine disruption in natural populations using a fathead minnow assay developed by the U.S. Environmental Protection Agency. Also in 2009–2010, the four sediment sampling sites that were located near the mouths of the Salinas and Pajaro rivers in 2008–2009 in an effort to more definitively document those rivers as the major sources of contaminated sediments entering Monterey Bay, were abandoned. The effort allocated to these four sites was transferred back to four sites that have historically been sampled along the 80-meter contour. Also in this year's report, CCLEAN continues the conservative approach to calculating sums of analytes (e.g.,

PAHs, DDTs, PCBs, etc.), in which individual analytes whose reported values have received the SWAMP QA code of "JA" (i.e., peak detected, but did not meet quantification criteria, result reported represents the estimated maximum possible concentration) are excluded from summed concentrations.

Table 1. POP groups emphasized in this report.

POP Group	Names of Compounds Included
PAHs	Polynuclear aromatic hydrocarbons: Biphenyl, Naphthalene, 1-methylnapthalene, 2-methylnapthalene, 2,6-dimethylnapthalene, 2,3,5-trimethylnapthalene, Acenaphthene, Acenaphthylene, Anthracene, Dibenzothiophene, Fluorene, Phenanthrene, 1-methylphenanthrene, Benz(a)anthracene, Chrysene, Fluoranthene, Pyrene, Benzo(a)pyrene, Benzo(e)pyrene, Benzo(b)fluoranthene, Benzo(k)fluoranthene, Dibenz(a,h)anthracene, Perylene, Benzo(ghi)perylene, Indeno(1,2,3-cd)pyrene
DDTs	o,p'-DDT, p,p'-DDT = (1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane) o,p'-DDD, p,p'-DDD = (1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethane) o,p'-DDE, p,p'-DDE = (1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene)
Dieldrin	1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4α,5,6,7,8,8α-octahydro-1,4-endo,exo-5,8-dimethanonaphthalene
Chlordanes	trans-Chlordane, cis-Chlordane, trans-Nonachlor, cis-Nonachlor, Oxychlordane, Heptachlor, Heptachlor epoxide
PCBs	Polychlorinated biphenyls: congener numbers = 5, 8, 18, 20, 21, 28, 31, 33, 43, 44, 49, 52, 56, 60, 61, 66, 69, 70, 73, 74, 76, 80, 86, 87, 89, 90, 93, 95, 97, 99, 101, 105, 106, 110, 111, 113, 115, 116, 117, 118, 127, 128, 132, 138, 139, 141, 147, 149, 151, 153, 156, 158, 160, 163, 164, 168, 170, 174, 177, 180, 181, 182, 183, 187, 190, 194, 195, 196, 201, 203
PBDEs[1]	Polybrominated diphenyl ethers: congener numbers = 7, 8, 10, 11, 12, 13, 15, 17, 25, 28, 30, 32, 33, 35, 37, 47, 49, 51, 66, 71, 75, 77, 79, 85, 99, 100, 105, 116, 119, 120, 126, 128, 138, 140, 153, 154, 155, 166, 181, 183, 190, 203, 206, 207, 208, 209
PFCs[1]	Perfluorinated Compounds: PFOA (Perfluorooctanoic acid), PFOS (Perfluorooctane sulfonic acid), PFOSA (Perfluorooctanesulfonamide), PFDoA (Perfluorododecanoic acid), PFDeA (Perfluorodecanoic acid), PFBA (Perfluorobutyric acid), PFHpA (Perfluoroheptanoic acid), PFNA (Perfluorononanoic acid), PFUnA (Perfluoroundecanoic acid), PFHxS (Perfluorohexane sulfonic acid), PFBuS (Perfluorobutane sulfonic acid), PFHxA (Perfluorinatedhexanoic acid), PFPeA (Perfluoropentanoic acid)

[1] = Currently not regulated by the California Ocean Plan.

Figure 1. Locations of CCLEAN sampling sites for receiving water, sediment, mussels, and rivers.

Table 2. Sampling sites, parameters sampled, frequency of sampling, applicable water-quality stressors, and relevant program objectives for CCLEAN during the 2009–2010 program period.

Sampling Sites	Parameters Sampled at Each Site	Frequency of Sampling	Applicable Water-quality Stressors and Program Objectives
Water Sampling Four wastewater discharges (Santa Cruz, Watsonville, MRWPCA, CAWD) in effluent and one river (Pajaro)	30-day flow proportioned samples using automated pumping equipment, solid-phase-extraction techniques for persistent organic pollutants (POPs).	Twice per year (wet season and dry season)	Sources, loads, trends, effects and permit compliance for: POPs
	Grabs of effluent for ammonia and nitrate, turbidity, temperature, conductivity, pH, urea, orthophosphate, dissolved silica and total suspended solids	Monthly	Sources, loads, trends and permit compliance for: Nutrients
	Evaluate satellite imagery for algal blooms	Periodically	Effects of: Nutrients
	Reproductive success and behavior in fathead minnows	Twice per year (wet season and dry season)	Effects of: Endocrine disrupting compounds
30-ft contour sites for Santa Cruz, Watsonville and MRWPCA	Grabs for total and fecal coliform, *enterococcus*	At least monthly	Sources, trends, effects and permit compliance for: Pathogen indicators
Two nearshore background sites	30-day time-integrated samples using automated pumping equipment and solid-phase-extraction techniques for: POPs, nitrate, ammonia, urea, orthophosphate and dissolved silica, total suspended solids, temperature, conductivity, pH, total and fecal coliform, *enterococcus*	Twice per year (wet season and dry season)	California Ocean Plan compliance for: POPs Nutrients Pathogen indicators
Mussel Sampling Five rocky intertidal sites	One composite of 30-40 mussels for POPs, total and fecal coliform, and *enterococcus*	Annually in the wet season	Status, trends, effects and alert level comparisons for: POPs Pathogen indicators
Sediment Sampling Six sites along the 80-meter contour	POPs, sediment grain size and total organic carbon, benthic infauna	Annually in the fall	Status, trends, effects and alert level comparisons for POPs

Table 3. Dates, volumes and numbers of samples collected for CCLEAN in 2009–2010.

Matrix & Season	Site	Start Date	Ending Date	
Effluent				Number of Liters
Dry	Santa Cruz	August 24, 2009	October 12, 2009	264
	Watsonville	August 24, 2009	September 22, 2009	278
	MRWPCA	August 24, 2009	September 22, 2008	258
	CAWD	NS[1]	NS[1]	NS[1]
Wet	Santa Cruz	January 28, 2010	March 11, 2010	253
	Watsonville Influent	January 28, 2010	March 11, 2010	269
	Watsonville Effluent	January 28, 2010	March 11, 2010	428
	MRWPCA	January 28, 2010	March 3, 2010	302
	CAWD	January 28, 2010	March 3, 2010	500
River Sampling				Number of Liters
Dry	Pajaro River	August 24, 2009	October 2, 2009	261
Wet	Pajaro River	February 2, 2010	March 2, 2010	550
Nearshore Sampling				Number of Liters
Dry	North	August 27, 2009	September 25, 2009	250
	South	August 27, 2009	September 25, 2009	250
Wet	North	February 18, 2010	March 23, 2010	250
	South	February 18, 2010	March 23, 2010	250
Sediment Sampling				Samples for Chemistry/Benthos
	SedRef 2		October 30, 2010	1/1
	SedRef 3		October 30, 2010	1/1
	SedRef 4		October 30, 2010	1/1
	SedDep 1		October 30, 2010	1/1
	SedDep 2		October 30, 2010	1/1
	SedDep 3		October 30, 2010	1/1
Mussel Sampling				Mussels for POPs/Bacteria
	Scott Creek		March 24, 2010	46/30
	Laguna Creek		March 24, 2010	51/30
	The Hook		March 24, 2010	51/30
	Fanshell Overlook		March 24, 2010	45/30
	Monterey Creek		March 24, 2010	46/30
	Carmel River Beach		March 24, 2010	44/30

[1] – NS, not sampled. All effluent was being reclaimed and there was no discharge to the ocean.

4.0 Results for Program Objectives

4.1 What are the status and long-term trends in the quality of nearshore waters, sediments, and associated beneficial uses?

To give a more complete evaluation of POP loads to the ocean, CCLEAN measures more POPs than are regulated by the Ocean Plan or limited in NPDES discharge permits (see Table 1). Nevertheless, comparisons between Ocean Plan water quality objectives and concentrations measured in Monterey Bay are all based on only those POPs that are listed in Table B of the Ocean Plan (Table 4). While some of the PCB congeners reported by CCLEAN were not detected in commercial Aroclor mixtures regulated by the Ocean Plan (Frame et al. 1996), these congeners co-elute with other more widely detected congeners and are included here in ocean PCB concentrations. Moreover, as CCLEAN measures only 70 out of a possible 209 PCB congeners, the concentrations of PCBs reported here are probably conservative.

Table 4. Ocean Plan POPs emphasized in this report.

POP Group	Names of Compounds Included
Ocean Plan PAHs	acenaphthylene, anthracene,1,2-benzanthracene (Benz(a)anthracene), 3,4-benzofluoranthene (Benzo(b)fluoranthene), benzo[k]fluoranthene, 1,12-benzoperylene (Benzo(ghi)perylene), benzo[a]pyrene, chrysene, dibenzo[ah]anthracene, fluorene, indeno[1,2,3-cd]pyrene, phenanthrene and pyrene
Dieldrin	1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4α,5,6,7,8,8α-octahydro-1,4-endo,exo-5,8-dimethanonaphthalene
DDTs	o,p'-DDT, p,p'-DDT = (1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane) o,p'-DDD, p,p'-DDD = (1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethane) o,p'-DDE, p,p'-DDE = (1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene)
Ocean Plan PCBs	The sum of chlorinated biphenyls whose analytical characteristics resemble those of Aroclor-1016, Aroclor-1221, Aroclor-1232, Aroclor-1242, Aroclor-1248, Aroclor-1254 and Aroclor-1260 (All CCLEAN congeners are included)

Several elements of the CCLEAN program provide data that enable assessment of both the status and trends in the quality of nearshore waters, sediments and associated beneficial uses (see Table 1). These include sampling of nearshore waters for POPs, nutrients and bacteria; sediments for POPs and benthic infauna; and mussels for POPs and bacteria. Analysis of status involves comparisons with the objectives, guidelines and alert levels described in Section 2. Describing status also documents compliance with the Ocean Plan and other applicable regulatory guidelines, which can also indicate contaminant effects. Analysis of trends involves statistical tests to determine whether measured parameters are changing over time.

The analyses in this section are focused on particular contaminants that previously have been associated in CCLEAN reports either with a status that indicated impairment to beneficial uses or were very close to impairments. These include the following:

- PCBs, PAHs and Dieldrin in nearshore waters (i.e., exceeded 30-day average in California Ocean Plan [Ocean Plan] for the protection of human health)
- DDTs in sediments (i.e., exceeded NOAA ERL)

- Dieldrin in mussels (i.e., exceeded US EPA alert level for recreational fishers).

All of these impairments were discussed in previous reports (CCLEAN 2008; CCLEAN 2009). In addition to these previously noted impairments to beneficial uses, this consideration of status and trends also includes analysis of bacteria monitoring in ocean waters by each wastewater treatment plant. The following sections discuss the current status and trends of these contaminants in the associated matrices.

4.1.1 Status and Trends of PCBs, PAHs and Dieldrin in Nearshore Waters

Nearshore waters in Monterey Bay frequently exceed the Ocean Plan objective for 30-day average concentrations of PCBs (Figure 2). Six out of ten samples from South Monterey Bay and seven out of ten samples from North Monterey Bay have exceeded the Ocean Plan objective for the protection of human health (i.e., 0.019 ng/L), with the March 2009 sample from North Monterey Bay at 518% of this objective. There have been no consistent differences between sites or between wet-season and dry-season samples.

With the exception of the March 2006 sample from the North Monterey Bay site, PAHs have been below the Ocean Plan objective for the protection of human health (i.e., 8.8 ng/L) (Figure 3). Both sites exhibited relatively high concentrations of PAHs in March 2006, but displayed no substantial differences between sites or between wet-season and dry-season samples.

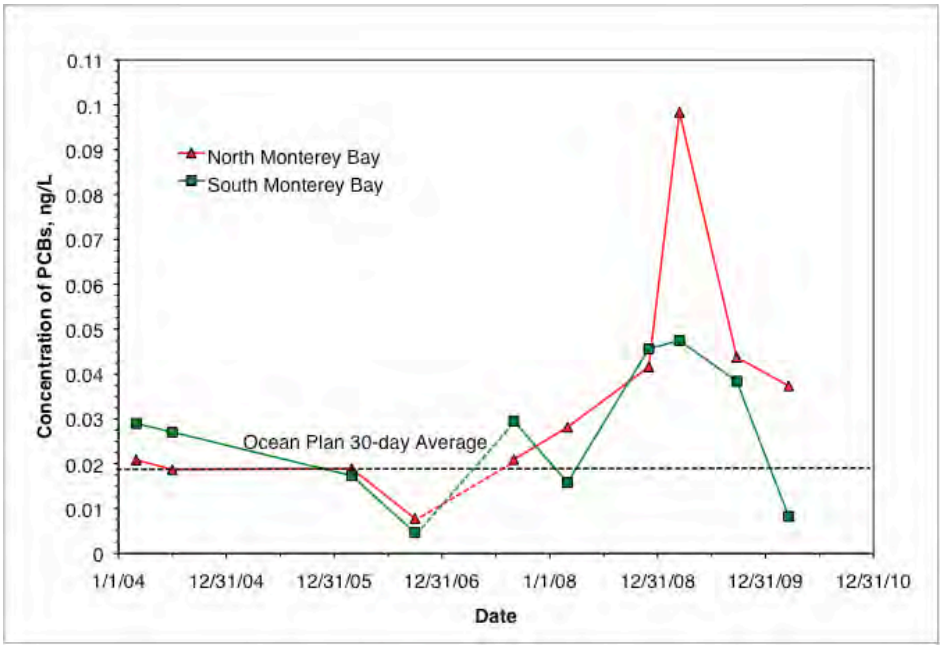


Figure 2. Concentrations of PCBs in nearshore waters at two CCLEAN sites in Monterey Bay.

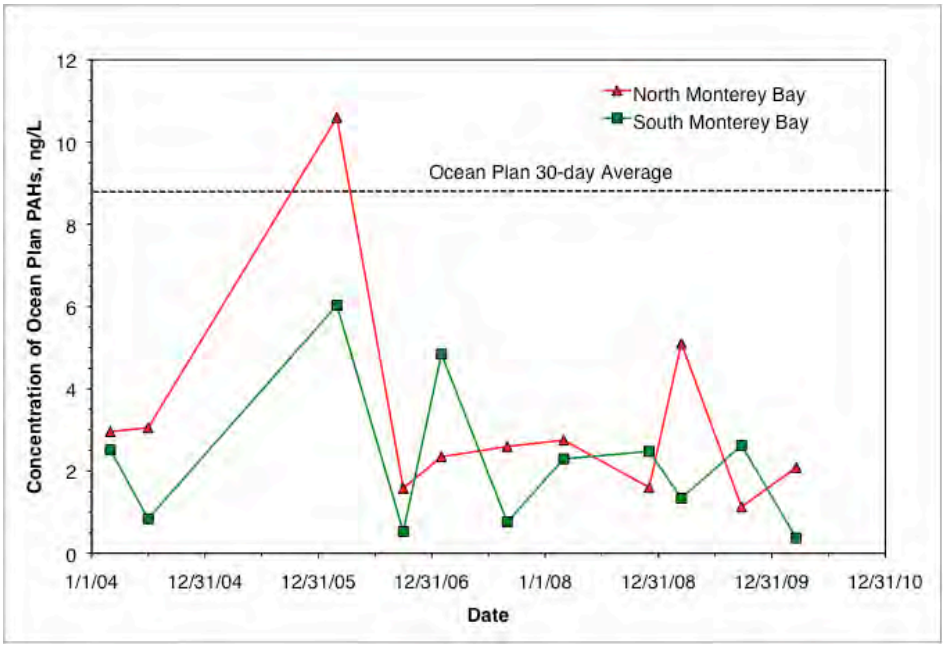


Figure 3. Concentrations of Ocean Plan PAHs in nearshore waters at two CCLEAN sites in Monterey Bay.

Dieldrin in the March 2004 and March 2010 samples from North Monterey Bay exceeded the Ocean Plan objective for the protection of human health (i.e., 0.04 ng/L) (Figure 4). The concentrations have been generally similar between sites, with wet-season samples usually exhibiting higher Dieldrin concentrations than corresponding dry-season samples.

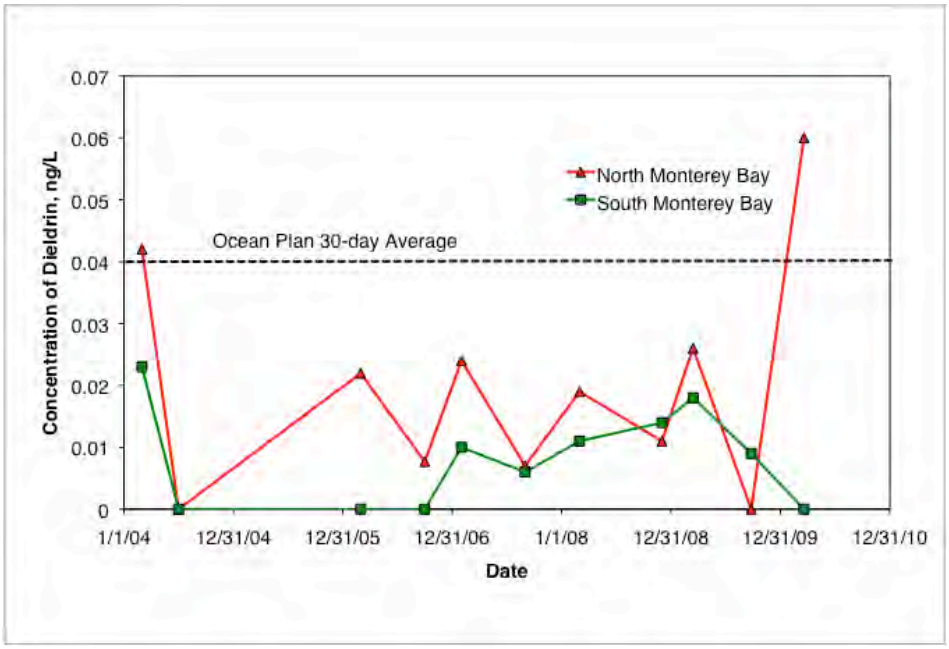


Figure 4. Concentrations of Dieldrin in nearshore waters at two CCLEAN sites in Monterey Bay.

The State of California has established a policy for designating impaired water bodies (State Water Resources Control Board 2004). This policy specified statistical criteria for the number of exceedances in the total sample size needed to place a water segment on the 303d list. For 2 – 24 samples, two exceedances are the minimum number needed to designate impairment. According to this policy, both northern and southern Monterey Bay are impaired for PCBs. Northern Monterey Bay is also impaired for Dieldrin. In fact, the 11 exceedances for PCBs in Monterey Bay would satisfy the criteria for listing even if the sample size were 94.

There were few statistically significant trends in concentrations of PCBs, PAHs or Dieldrin. When wet- and dry-season samples were considered separately, only dry-season Dieldrin exhibited a significant increase over time ($r^2 = 0.76$, $p = 0.0010$). The increase in dry-season PCBs was marginally non-significant ($r^2 = 0.386$, $p = 0.0596$). There were no significant trends in wet-season concentrations. When data for both seasons were combined, only PCBs revealed a marginally non-significant increase over time ($r^2 = 0.16$, $p = 0.0618$), which was probably due to the single high value in 2009.

4.1.1.2 Conclusions

Nearshore waters of Monterey Bay continue to exceed the Ocean Plan objective for PCBs for the protection of human health. Moreover, North Monterey Bay also exceeds the Ocean Plan objective for Dieldrin for the protection of human health. Although there have been samples of nearshore water that have approached or exceeded the Ocean Plan objectives for PAHs, there are not consistent patterns of exceedances for this POP that would warrant special concern.

4.1.1.3. Recommendations

Sampling of nearshore waters should continue in order to document the effects on ocean waters caused by discharges from land.

4.1.2 Status, Trends and Effects of DDTs in Sediments

DDTs in sediments at sites along the 80-meter contour have consistently exceeded the concentration above which 10% of samples nationwide exhibited toxicity in 1,513 laboratory bioassays in an analysis conducted by the National Oceanic and Atmospheric Administration (Long et al. 2000). DDT concentrations have historically been similar among CCLEAN sites and have not varied substantially among years, except for very high concentrations measured at sites SedRef 02 and SedDep 01 in 2006 (Figure 5). These two sites are the two CCLEAN sediment sites that have been consistently sampled throughout the program.

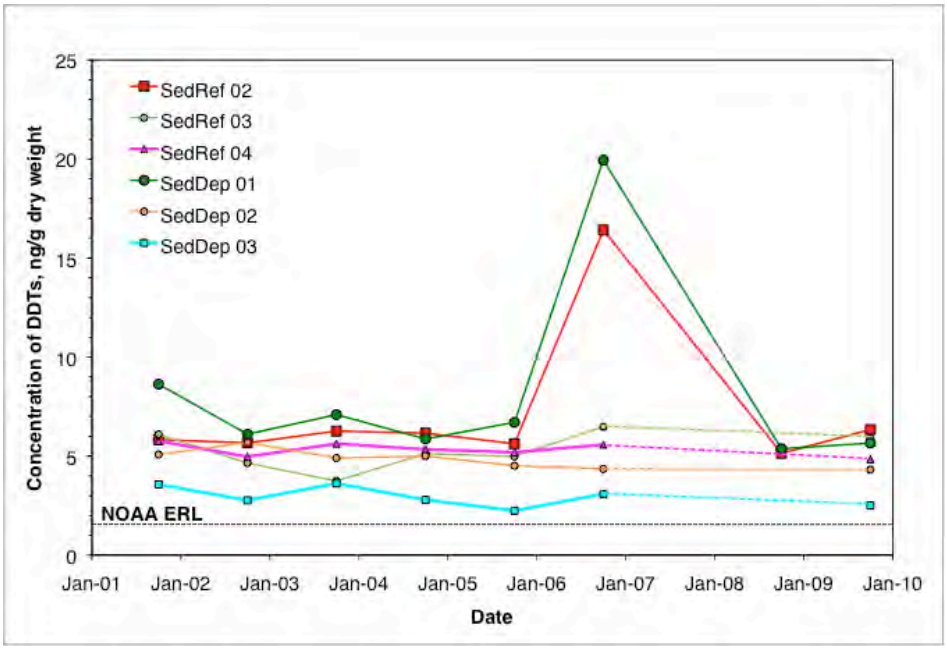


Figure 5. DDTs measured in sediments from historic CCLEAN sites in Monterey Bay.

During the first few years of the CCLEAN program, statistical analyses of sediment data suggested that high DDT concentrations in sediments along the 80-meter contour were adversely affecting some species of benthic infauna (CCLEAN 2004; CCLEAN 2006). With the greater statistical power afforded now by 60 samples collected over eight years, the effects of DDT and sediment characteristics were again examined in detail.

Four species of polychaetes and several benthic infaunal community indicators, total polychaetes, total crustaceans, total organisms, and total taxa, were selected for closer examination. *Cossura pygodactylata*, *Levinsenia gracilis*, *Nephtys cornuta* and *Pholoe glabra* were chosen because they were identified in samples each year and were among the species with the highest overall average densities. Each of these species has exhibited different temporal patterns since 2001, with *C. pygodactylata* declining across all sites from 2001 to 2009 and *L. gracilis* and *N. cornuta* fluctuating substantially from year to year (Figure 6). Several of these species exhibited peaks at most sites in 2004 with much lower densities in 2003 and 2005. Total numbers of organisms have consistently been dominated by polychaetes and both total polychaetes and total organisms fluctuated similarly to *L. gracilis* and *N. cornuta* (Figure 7).

There also have been changes in sediment characteristics at CCLEAN sampling sites over time. Total organic carbon and sediment silt + clay (fines) have declined at many sites since 2001. Total organic carbon concentrations were lower in 2009 at all sites than in any other year (Figure 8a). The percentages of fines in sediments also generally trended downward (Figure 8b). Linear regressions revealed statistically significant declines in total organic carbon at all sites, with probabilities ranging from 0.0255 to 0.0005. The percentage of fines in sediments also declined significantly at all sites except SedRef 4 and SedDep 2, with probabilities between 0.0045 and 0.0450. DDTs normalized to total organic carbon trended slightly upward (Figure 10c), probably because DDT concentrations exhibited no obvious trends (Figure 6) while total organic carbon

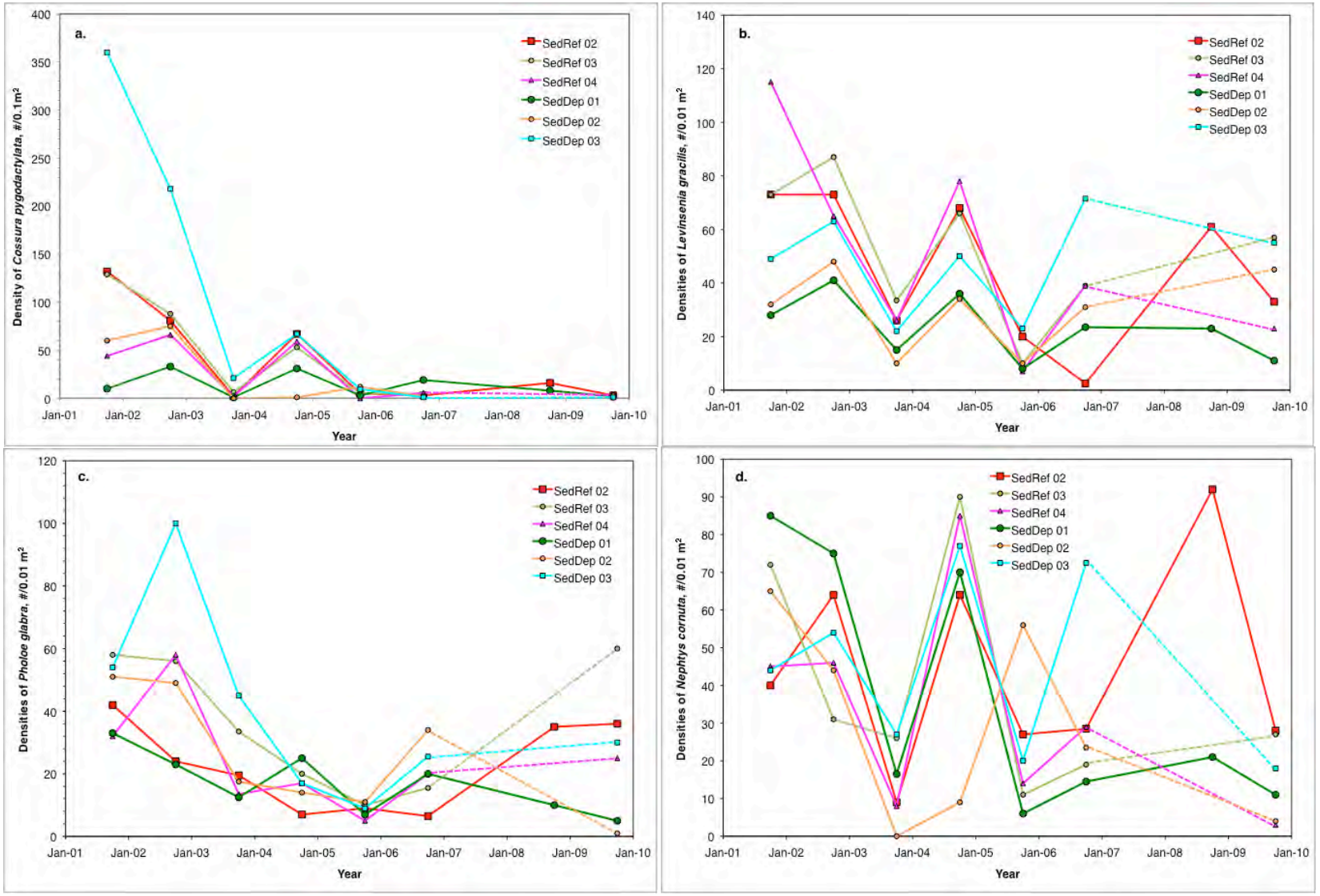


Figure 6. Temporal patterns of four polychaete species at six CCLEAN sediment sites along the 80-meter contour in northern Monterey Bay.

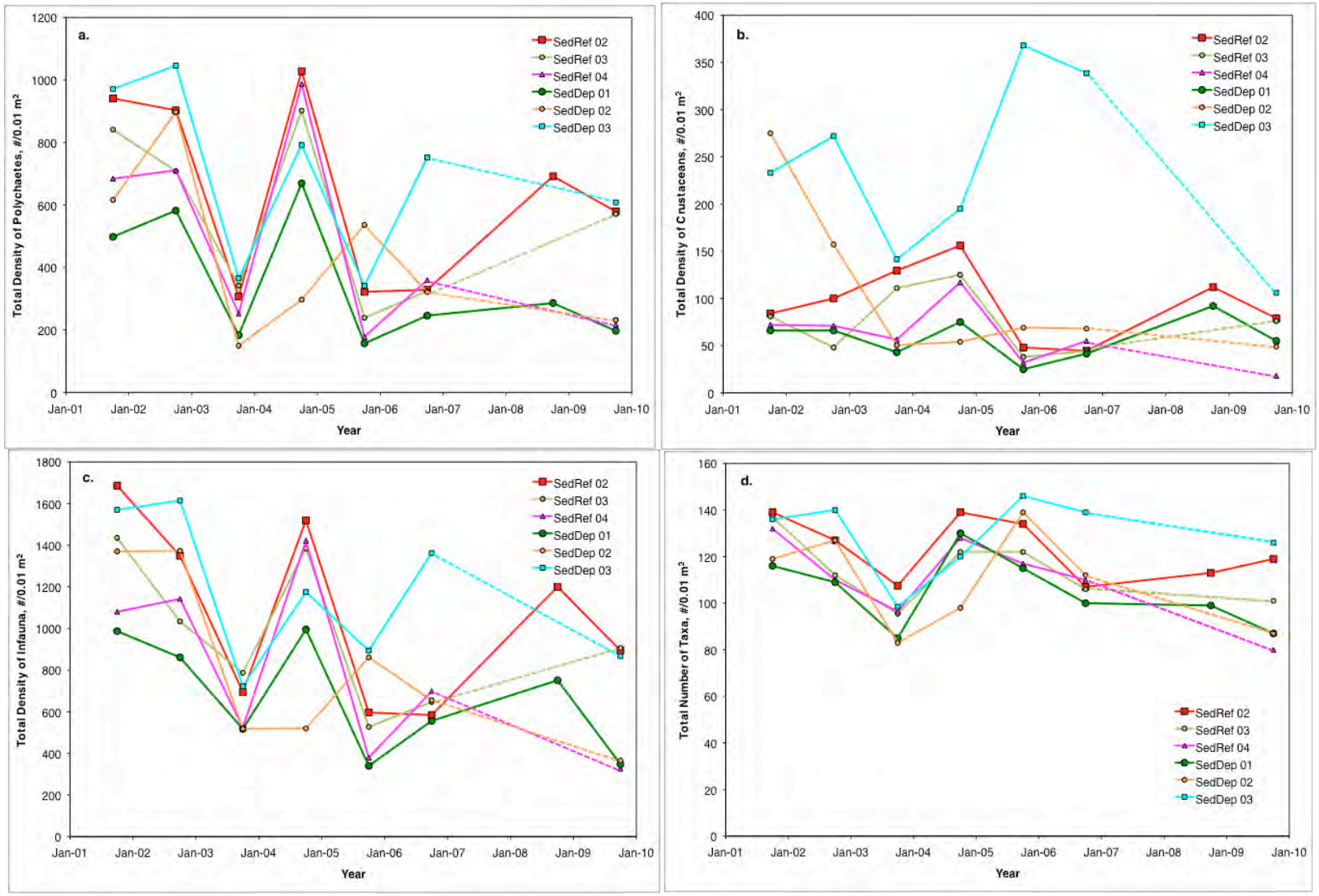


Figure 7. Temporal patterns of total polychaetes, total crustaceans, total abundance and total taxa at six CCLEAN sediment sites along the 80-meter contour in northern Monterey Bay.

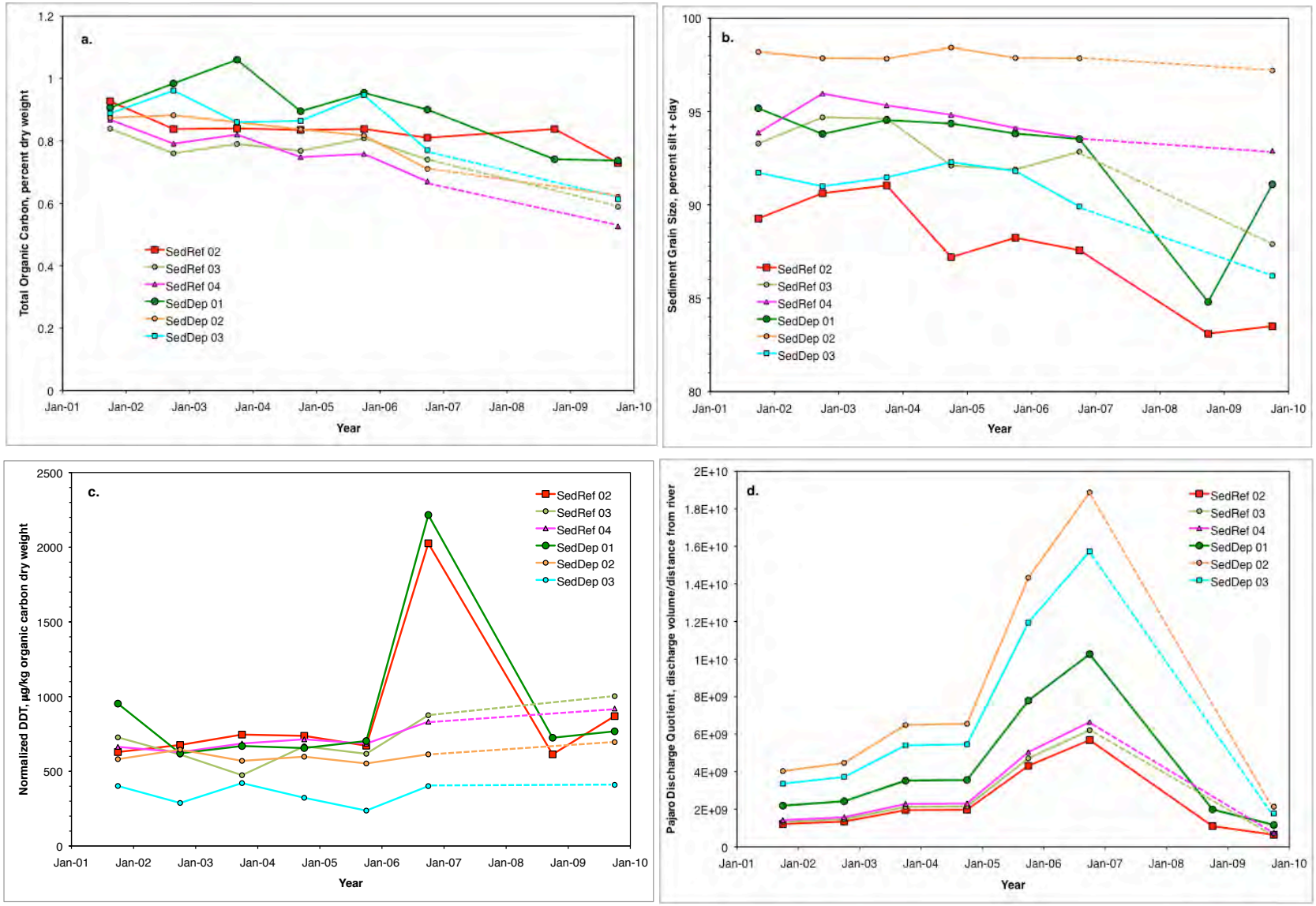


Figure 8. Temporal patterns of sediment total organic carbon, silt + clay, DDTs normalized to organic carbon and the Pajaro discharge quotient at six CCLEAN sediment sites along the 80-meter contour in northern Monterey Bay.

concentrations were declining. While the reasons for these changes in sediment characteristics remain unknown, total organic carbon and sediment fines are often primary factors in the distribution of infaunal species (Muniz and Pires 1999; Dalto and Albuquerque 2000) and declines in both would be expected to affect infaunal species composition, complicating attempts to discern POP effects.

Given the inconsistent temporal patterns among polychaetes and community indicators, and trends in sediment characteristics, additional statistical procedures were employed to discern whether DDTs, other sediment characteristics and discharges from the Pajaro River might have affected benthic organisms. Stepwise multiple regressions are useful for determining how numerous environmental (independent) variables are affecting biological (dependent) variables. In these regressions, models are developed from independent variables that explain significant variation in the dependent variable. Results are often obtained that contradict simple bivariate regressions because the multiple regression models account for the combined effects of all significant independent variables together. Backward regressions were performed, in which all independent variables were included and those that did not significantly explain variance in the dependent variable were sequentially removed until only significant independent variables remained.

The multiple regressions were performed using the four polychaetes, total numbers of polychaetes, total numbers of crustaceans, total numbers of organisms and total numbers of taxa as dependent variables and sediment DDT concentrations, total organic carbon, percent fines and discharges from the Pajaro River as independent variables. Time also was tested as an independent variable to determine whether trends over time were present, independent of sediment characteristics and annual discharges from the Pajaro River. While previous stepwise multiple regressions had shown that annual loads of suspended sediments from the Pajaro River and Salinas River were both important independent variables, we used only the volume of water discharged by the Pajaro in the current analyses because of uncertainties about basing estimates of annual sediment loads from the rivers from monthly samples and because monitoring of the Salinas River was discontinued in 2006. Because DDT can bind tightly to organic matter in sediments, some investigators prefer to infer toxic effects of DDT from concentrations normalized to organic carbon (Swartz et al. 1994) and DDT concentrations were normalized to total organic carbon for these analyses. The effects of discharges from the Pajaro River were estimated by using the total annual volume discharged ÷ distance of each site from the river Figure 10d). To improve conformance with the assumptions of parametric statistical procedures, data were transformed using the natural log transformation for counts and concentrations and the arcsine transformation for percentages (Sokal and Rohlf 1995).

The stepwise multiple regressions confirmed previous findings from the early years of CCLEAN (CCLEAN 2004; CCLEAN 2006) that DDTs and/or discharges from rivers have negatively affected infaunal abundances. The latest results suggest that densities of *C. pygodactylata*, *L. gracilis* and *P. glabra*, as well as total polychaetes, total crustaceans, total density of organisms and total number of taxa were all negatively affected by DDT and discharges from the Pajaro River (Table 5). Nevertheless, substantial unexplained variation remains for each of these species and community indicators. The amount of variation explained by the model was only 8% (i.e., r^2 = 0.0813) for *N. cornuta* and the other models explained from 25%–43% of variation. Partial

correlations indicated that DDTs were the most influential variable for *C. pygodactylata*, total arthropods and total abundance, whereas it was the second most influential variable for total annelids and total taxa. Discharges from the Pajaro River were the second most influential variable for *L. gracilis* and *P. glabra*.

While these regression and partial correlation results do not establish causality between the independent variables and dependent variables, they do suggest that DDTs and discharges from the Pajaro River may be having negative effects on benthic organisms along the 80-meter contour. Moreover, these statistical analyses are not comprehensive for the possible effects of pesticides and other organic chemicals on macrobenthos. There are other pesticides and synthetic organic chemicals, as well as multiple natural factors, such as temperature, oxygen concentration and interspecific interactions that were not included in the analyses. Nevertheless, the regression analyses and partial correlations continue to suggest that human activities on land could be affecting biological communities in Monterey Bay.

Although the sediment concentrations of DDTs normalized to total organic carbon at CCLEAN sites are two orders of magnitude below the threshold for acute toxicity found in San Francisco Bay (e.g., 100 micrograms DDT per gram of organic carbon, Swartz 1994), subtle, nonlethal effects could contribute to changes in infaunal organism abundances. Long-term studies in relatively stable environments, such as performed by CCLEAN, afford the opportunity to look for such subtle effects of POPs.

Table 5. Results of stepwise multiple regressions for the effects of total organic carbon (TOC), silt + clay (fines), concentrations of TOC-normalized DDTs, and a discharge quotient for the Pajaro River (total annual volume discharged ÷ distance from the river), and time on infaunal densities (number per 0.1m^2) in 60 sediment samples collected over eight years. All data were log transformed (natural log).

Taxon	r^2	p	Model
Cossura pygodactylata	0.2517	0.0016	$y = 33.59 - 5.71^{-9}$ Time – 1.16 DDT – Pajaro
Levinsenia gracilis	0.4051	<0.0001	y = 10.21 – 0.60 DDT – 0.42 Pajaro + 0.05 Fines
Nephtys cornuta	0.0813	0.0331	$y = 14.66 - 3.55^{-9}$ Time
Pholoe glabra	0.2985	<0.0001	$y = 25.68 - 5.4^{-9}$ Time – 0.25 Pajaro
Total Polychaetes	0.2889	0.0005	$y = 19.96 - 2.48^{-9}$ Time – 0.35 DDT – 0.24 Pajaro
Total Crustaceans	0.3489	<0.0001	$y = 6.21 - 9.7^{-10}$ Time – 0.30 DDT – 0.0.01 Fines
Total number of organisms	0.3589	<0.0001	$y = 19.01 - 2.68^{-9}$ Time – 0.28 DDT – 0.15 Pajaro
Total number of taxa	0.4300	<0.0001	$y = 6.54 - 5.9^{-10}$ Time – 0.12 DDT – 0.01 Fines + 0.04 Pajaro

4.1.2.1 Conclusions

DDT concentrations in sediments at historic CCLEAN sites have been stable, except for spikes measured at two sites 2006. All DDT measurements at historic CCLEAN sites have exceeded the NOAA ERL. There have been significant declines in total organic carbon and percent fines at many CCLEAN sediment sites, which remain unexplained. CCLEAN results continue to suggest that POPs, principally DDTs, and river discharges appear to be having negative effects on benthic communities along the 80-meter contour in Monterey Bay. Concern for the effects of DDTs on a regional scale are supported by other studies showing that Monterey Bay is a primary

source of DDTs distributed across the continental shelf and slope along the central California coast (Hartwell 2008).

4.1.2.2 Recommendations

Sediment sampling should continue in order to provide documentation of changing habitats and biological communities in Monterey Bay, as they are affected by POPs.

4.1.3 Status and Trends of Dieldrin in Mussels

CCLEAN has previously reported concentrations of Dieldrin above a human health alert level published by the California Office of Environmental Health Hazard Assessment (OEHHA) (Office of Environmental Health Hazard Assessment 2003). OEHHA has recently published advisory tissue levels (ATLs), which consider the health benefits of consuming fish and have substantially increased the concentrations of contaminants that will trigger alerts. The prior screening level published by OEHHA for Dieldrin in fish was 2.0 ng/g, whereas the new ATL for dieldrin is ≤15 ng/g for three 8-ounce servings of fish per week. The U.S. Environmental Protection Agency has published screening values for contaminants to protect recreational fishers, above which additional study is warranted to determine actual human health risks from consumption (U.S. Environmental Protection Agency 2000). In order to take a conservative approach for the protection of human and wildlife health, we have replaced comparisons of historic mussel data to the OEHHA screening value with the U.S. EPA screening level for recreational fishers (Figure 9). In March 2010, CCLEAN mussel measurements of Dieldrin remained below the U.S. EPA screening value at all sites for the second year in a row. Overall downward trends, due largely to the recent low concentrations, were not statistically significant.

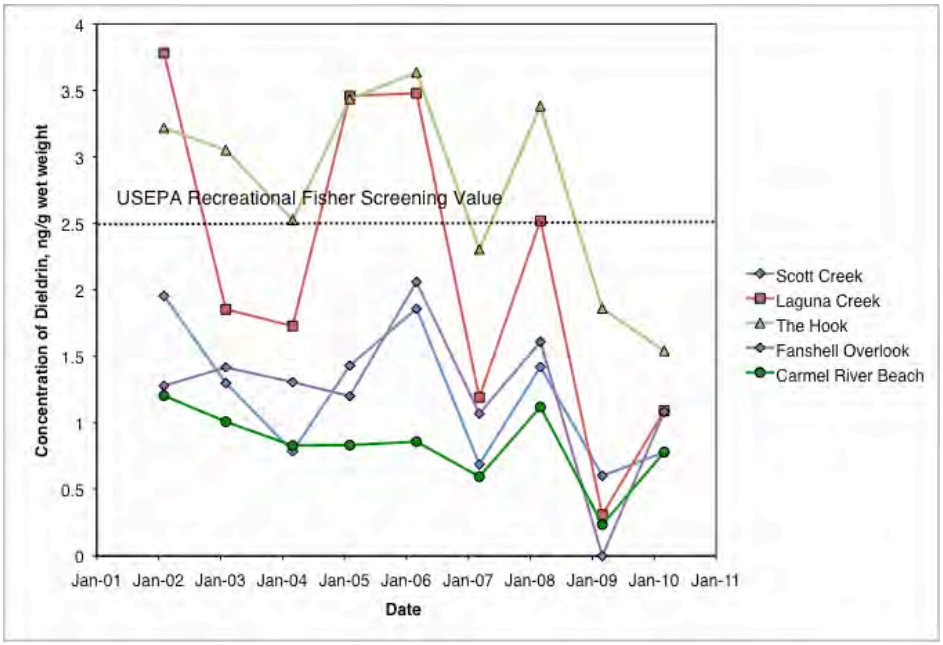


Figure 9. Dieldrin measured in mussels during the wet season from five CCLEAN sites in the Monterey Bay area.

4.1.3.1 Conclusions

Concentrations of Dieldrin in mussels along the northern shore of Monterey Bay were lower in 2010 than they were in 2008, although it is too early to know whether this is a long-term trend of improvement. Several more years with Dieldrin concentrations consistently below the U.S. EPA screening level would be a clear indication of reduced inputs to Monterey Bay.

4.1.3.2 Recommendations

Mussel sampling should continue unchanged. The mussel data collected have been very valuable in documenting impairments of beneficial uses, as well as supporting assessments of contaminant sources.

4.1.4 Status, Trends and Effects of Bacteria in Receiving Waters

The Ocean Plan limits the bacterial concentrations in ocean waters designated for use in water-contact recreation. These objectives are as follow:

30-day Geometric Mean:
i. Total coliform density shall not exceed 1,000 per 100 ml;
ii. Fecal coliform density shall not exceed 200 per 100 ml; and
iii. Enterococcus density shall not exceed 35 per 100ml.
Single Sample Maximum:
i. Total coliform density shall not exceed 10,000 per 100 ml;
ii. Fecal coliform density shall not exceed 400 per 100ml;
iii. Enterococcus density shall not exceed 104 per 100 ml; and
iv. Total coliform density shall not exceed 1,000 per 100 ml when the fecal coliform/total coliform ratio exceeds 0.1.

Receiving water data collected by the City of Santa Cruz, City of Watsonville and MRWPCA from July 2009 through June 2010 were compared with these objectives to assess the status of ocean waters for water contact recreation and the potential effects of wastewater on bacterial concentrations. CAWD is required to sample receiving water bacteria only if the total coliform concentration in their wastewater exceeds 2,400 MPN/100ml three or more times in a 30-day period, which has not occurred during the existence of the CCLEAN program. Because only Santa Cruz samples bacteria in receiving waters more than once per month, 30-day geometric means could not be calculated for most data and geometric means were, instead, calculated over the entire year.

There are no indications that the wastewater discharges are causing impairments to water contact recreation due to bacterial concentrations (Table 6). Only the Ocean Plan *Enterococcus* single sample objective for water contact recreation was exceeded at the far-field receiving water monitoring station associated with the Santa Cruz discharge in February 2009 and no exceedances in 2009–2010 (Figure 12). Moreover, in all cases except the geometric means for total and fecal coliform at Monterey Regional and the single sample maximum for fecal coliform and Enterococcus at Monterey Regional and Santa Cruz, respectively, the sites nearest the discharges had lower values than the sites farther from the discharges.

There were few temporal trends in bacteria concentrations near the CCLEAN wastewater discharges. Bacteria concentrations near each discharge tended to be greatest during the winter

and spring months and lower in the summer and fall months (Figure 10, Figure 11 and Figure 12).

4.1.4.1 Conclusions

There have been no bacterial impairments to the water contact recreation beneficial use associated with discharges from any of the CCLEAN wastewater treatment plants. Only the Ocean Plan *Enterococcus* single sample objective for water contact recreation was exceeded once at the farfield receiving water monitoring station adjacent to the Santa Cruz discharge. Statistical analyses detected no differences between near-field and far-field samples.

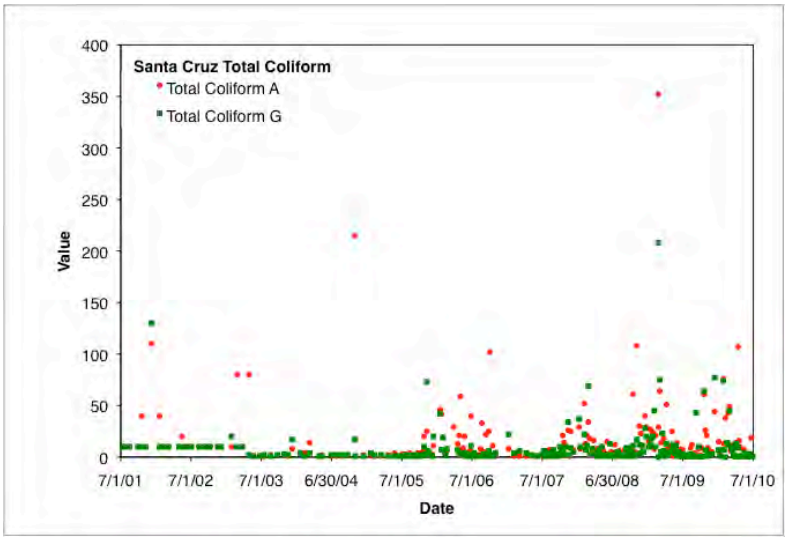
4.1.4.2 Recommendations

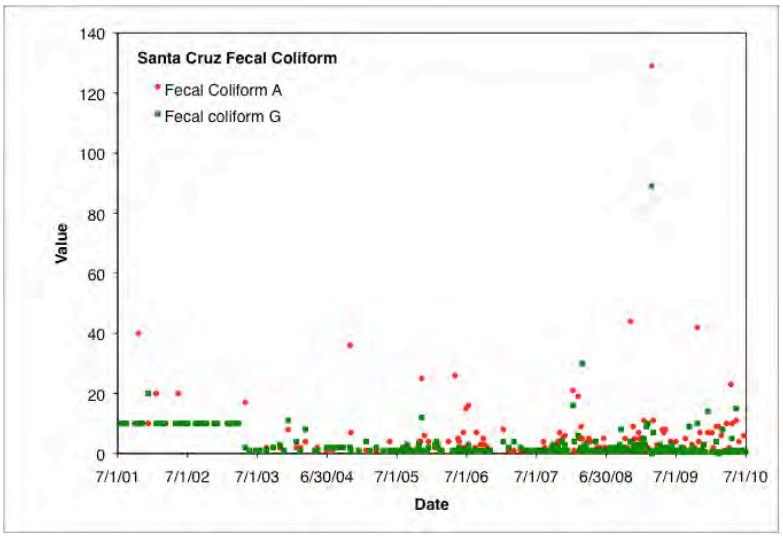
The CCLEAN participants have previously asserted their desire to continue monitoring receiving water bacteria, even in the absence of demonstrable effects of their discharges on beneficial uses. The results from the ongoing study of fecal pathogens by CCLEAN in collaboration with University of California at Davis and California Department of Fish and Game could inform future recommendations for more meaningful indicators of potential risks from pathogens.

Table 6. Ocean Plan objectives, geometric means and single sample maxima for indicator bacteria in receiving waters adjacent to ocean outfalls for three CCLEAN wastewater dischargers from July 2009 through June 2010.

Agency	Site	MPN/100 ml Total Coliform	MPN/100 ml Fecal Coliform	MPN/100 ml Enterococcus
		Geometric Means		
Ocean Plan Objective		1,000	200	35
Santa Cruz[1]	A (far)	22.47	6.95	3.86
	G (near)	10.84	1.71	2.38
Watsonville	A (far)	2.81	2.18	1.68
	D (near)	2.40	1.58	1.66
MRWPCA	A (far)	1.87	1.07	1.21
	B (near)	2.24	1.62	1.07
		Single Sample Maxima		
Ocean Plan Objective		10,000	400	104
Santa Cruz	A (far)	107	42	89
	G (near)	77	15	91
Watsonville	A (far)	80	11	9
	D (near)	17	4	7
MRWPCA	A (far)	30	2	2
	B (near)	17	13	2

[1] = Maximum 30-day geometric mean from July 1, 2009 through June 30, 2010 shown for Santa Cruz.





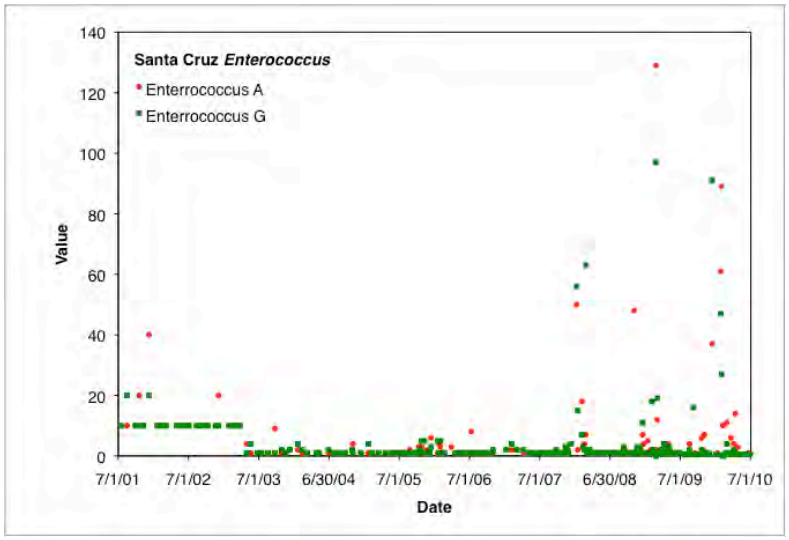


Figure 10. Receiving water bacteria measured at two stations near and far from the Santa Cruz wastewater discharge between July 2001 and June 2010.

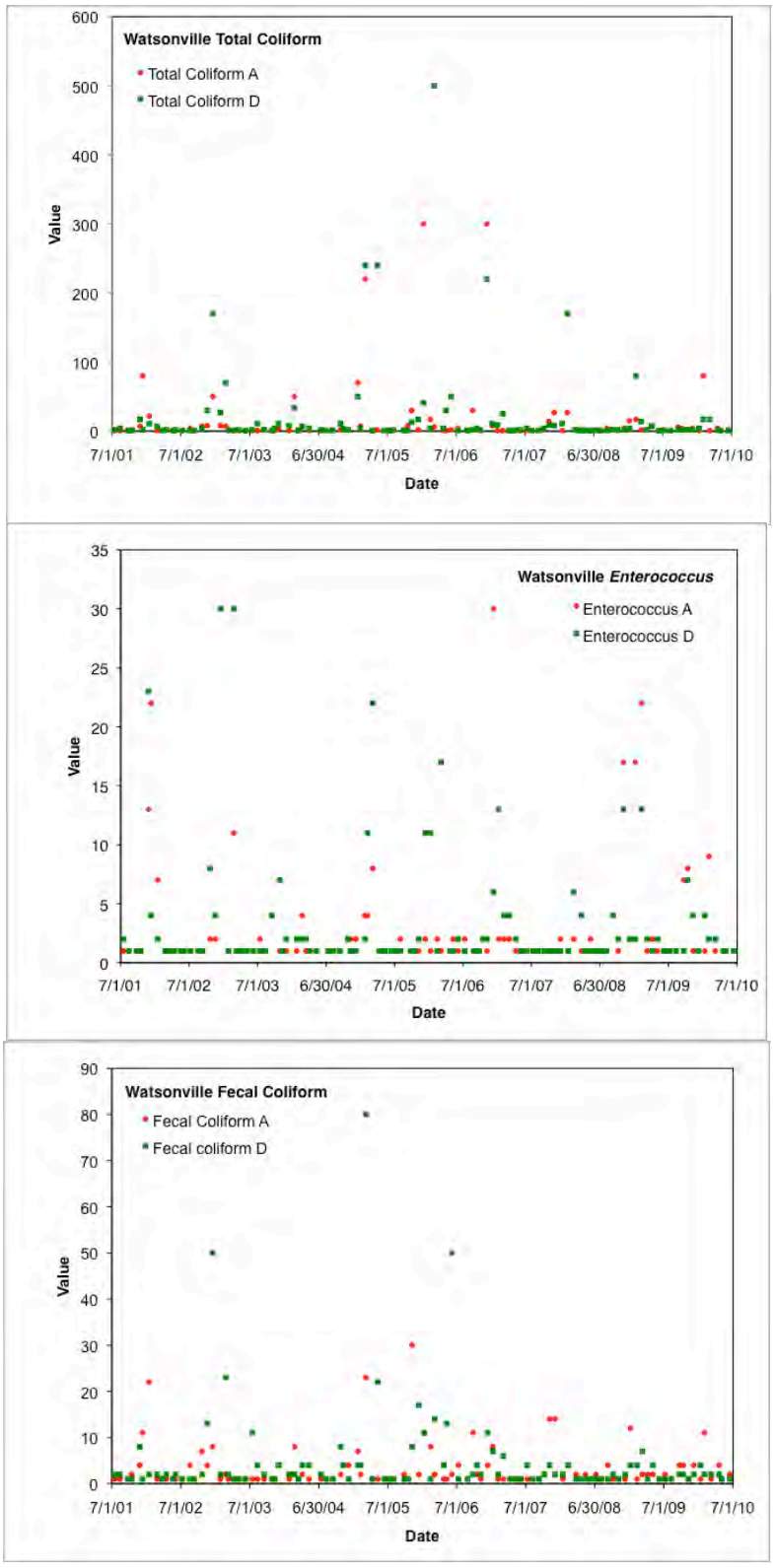


Figure 11. Receiving water bacteria measured at two stations near and far from the Watsonville wastewater discharge between July 2001 and June 2010.

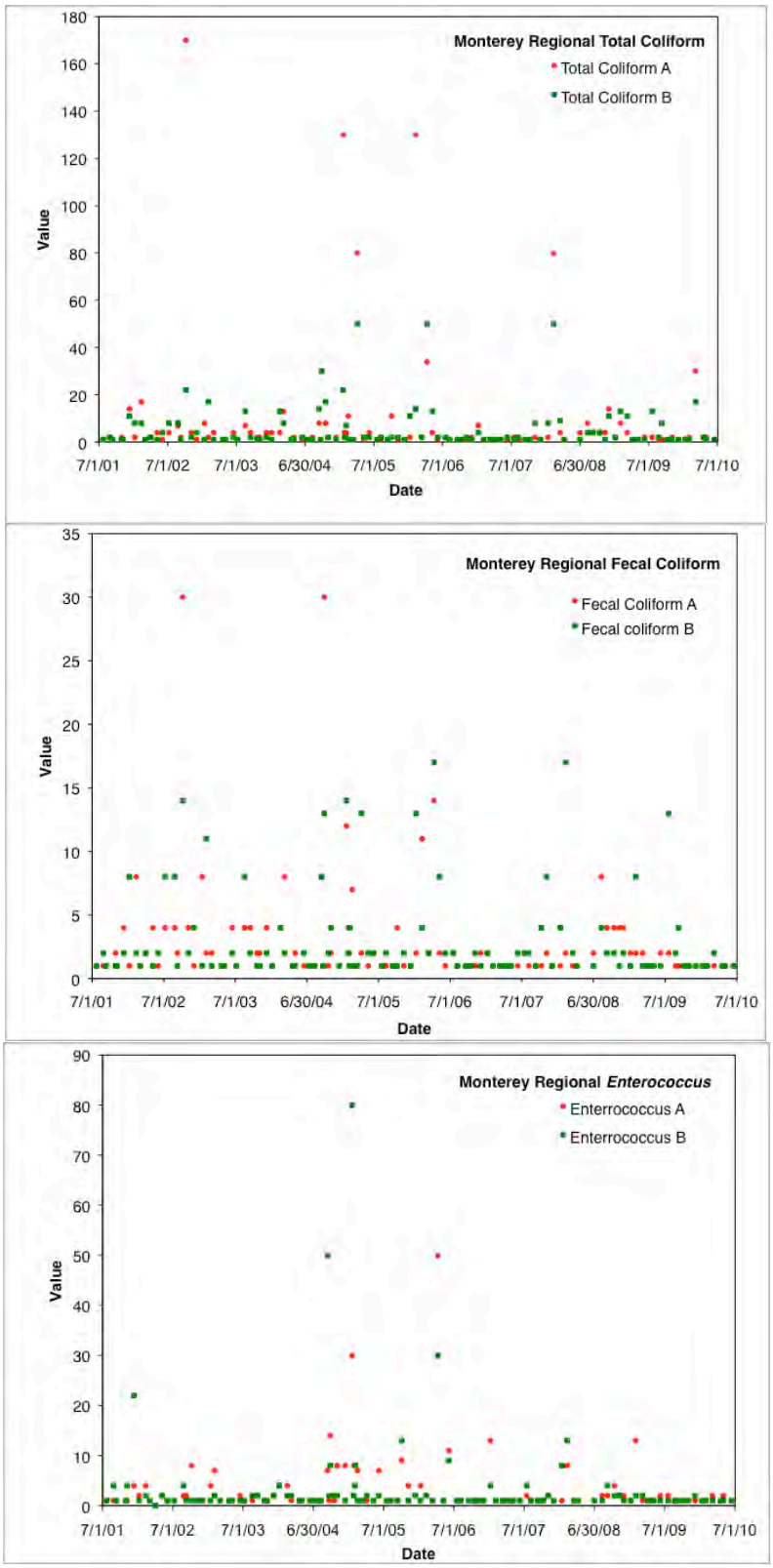


Figure 12. Receiving water bacteria measured at two stations near and far from the MRWPCA wastewater discharge between July 2001 and June 2010.

4.2 What are the major sources of contaminants to nearshore waters?

4.2.1 Loads of POPs

CCLEAN continues to measure POPs in wastewater and, as mentioned in Section 3.0, the City of Watsonville continues to support POP sampling in the Pajaro River. POP concentrations in wastewater are usually much below limits established in each discharger's NPDES permit (Table 7). There were no permit exceedances noted in 2009–2010. In the category of currently unregulated pollutants, Carmel had lower PBDE concentrations than the other wastewater discharges, but near the highest wet-season PFC concentrations (Table 8). Concentrations of PCBs, DDTs, Chlordanes and Dieldrin in the Pajaro River exceeded the California Toxics Rule concentrations in one or both samples in 2009–2010 (Table 7). Wet-season concentrations of POPs were much higher in the Pajaro River than in most of the wastewater discharges, except for PBDEs and PFCs, which had consistently higher concentrations in wastewater.

CCLEAN has previously documented that discharges from rivers contain the greatest loads of contaminants to ocean waters from the sources measured in the Monterey Bay area (CCLEAN 2007). For most POPs, cumulative loads from rivers constitute most of the total mass of contaminant discharged into ocean waters between 2002 and 2007. Since the cessation of CCLEAN monitoring in all rivers in 2007, total river POP loads have been estimated using average ratios of Pajaro River loads to those from the other rivers. While cumulative loads of PAHs, DDTs, Dieldrin and PCBs from rivers have varied substantially through time, mostly in response to storm flows, wastewater loads have been consistently low (Figures 13, 14, 15 and 16). In years with lower river discharges (e.g., 2002–2003 and 2006–2007) loads of many POPs from rivers and wastewater are similar.

Loads of some POPs have been higher from wastewater than from rivers. CCLEAN began measuring the PBDEs, used primarily as flame retardant in cushions, mattresses and plastic electronics cases, in the 2006–2007 program year. Loads from wastewater were more than twice those from rivers (CCLEAN 2007). Continued sampling in the Pajaro River provides a reference for continued comparison between river and wastewater loads of PBDEs that suggests wastewater loads still exceed those from rivers (Figure 17).

4.2.2 Relationships Between POP Sources and Water Quality Impairments

Much higher loads of PCBs, DDTs and Dieldrin from rivers than from wastewater suggest that river discharges are the primary sources of impairments described in Section 4.1. To test this assumption, the effects of different POP sources and environmental variables on the water quality impairments caused by PCBs and Dieldrin were investigated using statistical procedures documented in the following sections. This exercise also supports potential management decisions regarding actions needed to reduce water quality impairments by helping discern which sources are most closely associated with impairments and which, therefore, should be the focus of corrective efforts.

Table 7. Concentrations of regulated (Ocean Plan) pollutants in CCLEAN wastewater discharges (with NPDES permit limits for each discharger) and the Pajaro River (with California Toxics Rule numeric criteria). Note different column headings for the Pajaro River.

Source & Date	PCBs ng/L	PAHs ng/L	DDTs ng/L	HCHs ng/L	Chlordanes ng/L	Dieldrin ng/L	Endosulfans ng/L	Dioxin/Furan TEQs
Lab Blank								
Sep-09	0.001155	0	0	0	0	0	0	0.0000021
Feb-10	0.001579	0.514	0	0	0	0	0	0.0000117
Equip. Blank								
Sep-09	0.0357	0.9069	0	0.036	0	0	0	0.0000051
Mar-10	0.0237	0.7134	0.008	0	0	0	0	0.0000050
Santa Cruz								
Permit	2.66	1200	23.8	560	3.2	5.6	1260	0.00055
Sep-09	0.40	20.50	0.20	0.98	0.37	0.25	0.09	0.000056
Feb-10	0.54	15.46	0.37	0	1.49	0.98	2.80	0.000079
Watsonville								
Permit	1.62	748	14.45	340	1.96	3.4	770	0.00033
Sep-09	0.72	19.13	0.69	0.48	0.49	0.30	0.14	0.000046
Feb-10	0.79	12.76	0.86	0	0.73	0.61	0.22	0.000076
MRWPCA								
Permit	2.774	1284.8	24.82	292	3.358	5.84	1314	0.00057
Sep-09	0.33	20.69	0.75	0.07	0.68	0.33	0.22	0.000044
Feb-10	0.4000	19.40	0.54	0	0.45	0.30	0.20	0.000087
Carmel								
Permit	2.318	1073.6	20.74	488	2.806	4.88	1098	0.00048
Sep-09	NS	NS	NS	NS	NS	NS	NS	NS
Feb-10	0.40	28.20	0.35	0.06	0.96	1.41	0.28	0.000055

Pajaro River	PCBs ng/L	4,4'-DDT ng/L	4,4'-DDE ng/L	4,4'-DDD ng/L	Chlordanes ng/L	Dieldrin ng/L	Benzo(b)fluoranthene ng/L
CTR	0.17	0.59	0.59	0.83	0.57	0.14	4.4
Sep-09	0.12	0.51	1.01*	0.61	0.20	0.44*	0.36
Feb-10	0.59*	7.85*	16.5*	2.1*	1.29*	1.36*	1.75

NA = Not analyzed
NS = Not sampled

Table 8. Concentrations of unregulated pollutants in CCLEAN lab blanks, equipment blanks, wastewater discharges and the Pajaro River.

Source & Date	PBDEs, ng/L	PFCs, ng/L
Lab Blank		
Sep-09	0.00122	0
Feb-10	0.00635	0
Equip. Blank		
Sep-09	0.0187	NA
Feb-10	0.1048	NA
Santa Cruz		
Sep-09	11.44	53.74
Feb-10	16.10	165.69
Watsonville		
Sep-09	13.56	32.02
Feb-10	19.02	48.02
MRWPCA		
Sep-09	16.86	30.88
Feb-10	15.60	70.27
Carmel		
Sep-09	NS	NS
Feb-10	5.99	161.5
Pajaro River		
Sep-09	0.58	11.87
Feb-10	0.59	9.73

NA = Not analyzed
NS = Not sampled

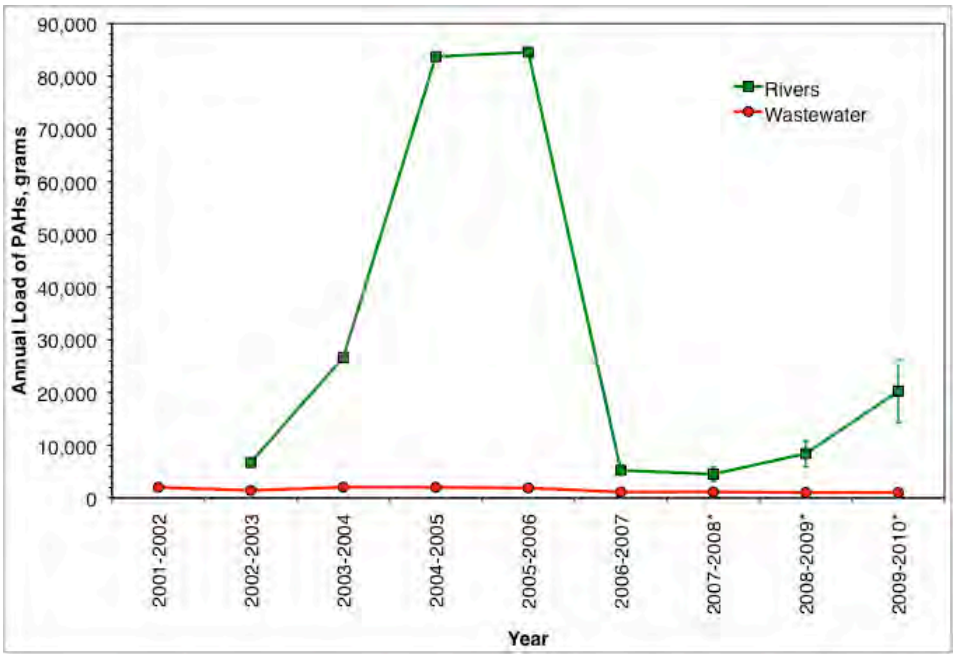


Figure 13. Loads of PAHs from all rivers combined and all wastewater discharges combined into the ocean in the Monterey Bay area. Sampling of all rivers but the San Lorenzo and Pajaro ceased in 2007. * = Last three years are estimated from historic ratios of Pajaro River to other rivers. Standard error indicated.

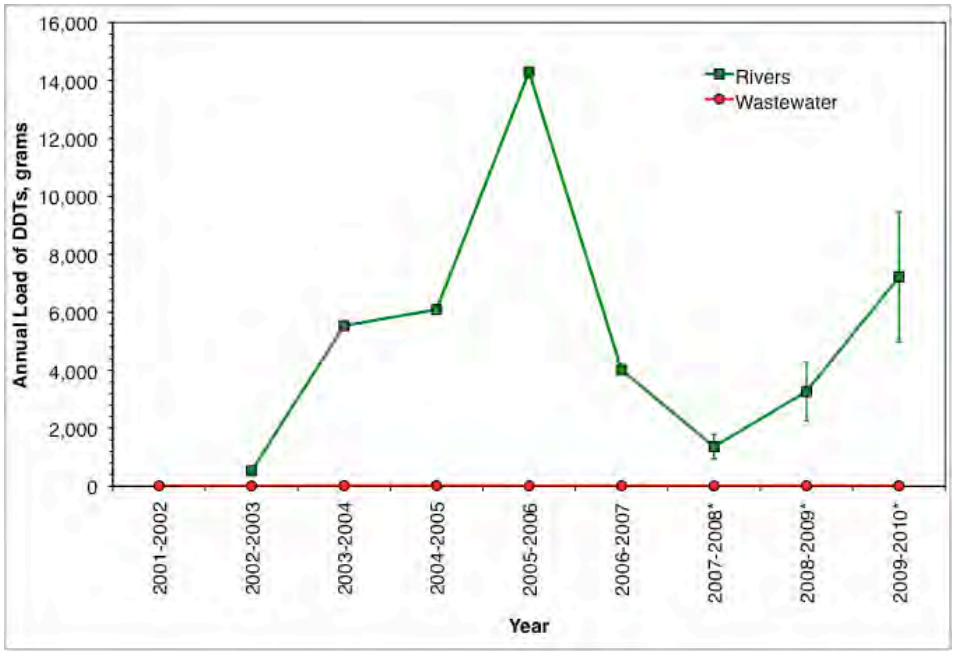


Figure 14. Loads of DDTs from all rivers combined and all wastewater discharges combined into the ocean in the Monterey Bay area. Sampling of all rivers but the San Lorenzo and Pajaro ceased in 2007. * = Last three years are estimated from historic ratios of Pajaro River to other rivers. Standard error indicated.

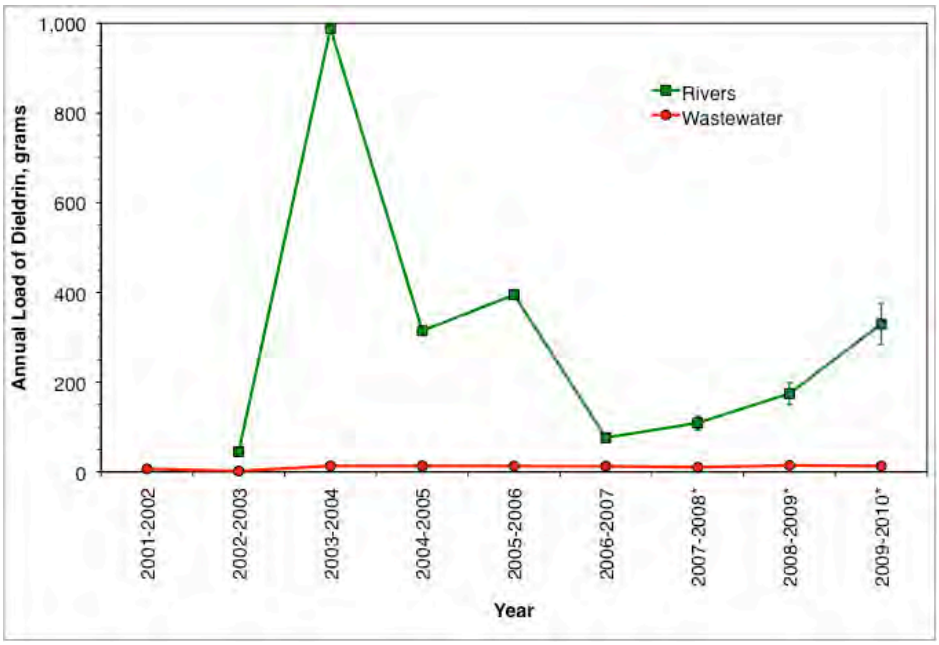


Figure 15. Loads of Dieldrin from all rivers combined and all wastewater discharges combined into the ocean in the Monterey Bay area. Sampling of all rivers but the San Lorenzo and Pajaro ceased in 2007. * = Last three years are estimated from historic ratios of Pajaro River to other rivers. Standard error indicated.

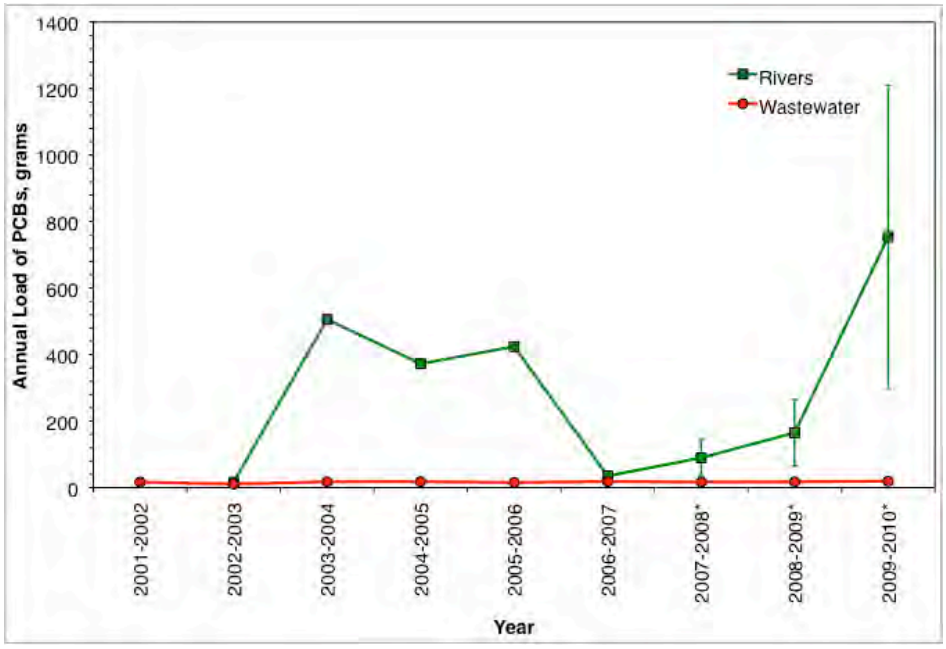


Figure 16. Loads of PCBs from all rivers combined and all wastewater discharges combined into the ocean in the Monterey Bay area. Sampling of all rivers but the San Lorenzo and Pajaro ceased in 2007. * = Last three years are estimated from historic ratios of Pajaro River to other rivers. Standard error indicated.

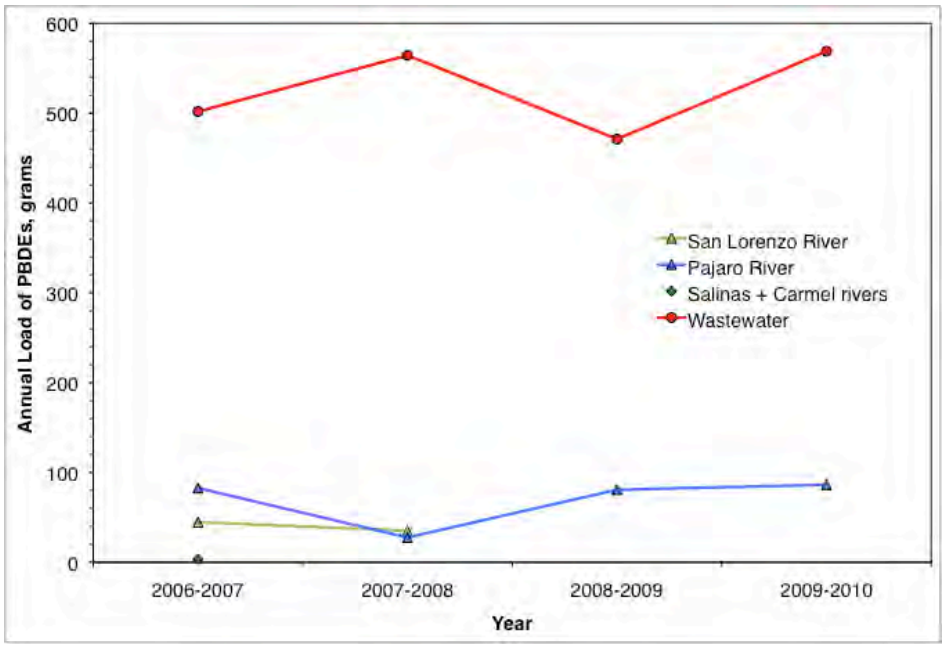


Figure 17. Loads of PBDEs from two rivers and all wastewater discharges combined into the ocean in the Monterey Bay area. Sampling of all rivers but the San Lorenzo and Pajaro ceased in 2007.

4.2.2.1 PCBs in Ocean Water

The examination of relationships between concentrations of PCBs in Monterey Bay and sources involved several model-building exercises using stepwise multiple regressions. These exercises examined relationships between total PCBs and the percentages of different PCB compounds as the dependent variables, and PCB concentrations in wastewater and river sources as independent variables. To account for dilution effects on PCB sources, indices of source effects were calculated based on loads of wastewater aggregated from the two discharges immediately upstream of the North Monterey Bay site in the prevailing current (i.e., Watsonville and Monterey Regional) and the Pajaro River with and without the Salinas River. As CCLEAN monitoring of the Salinas River ceased in October 2006, we do not have data from both rivers for the entire period of monitoring at North Monterey Bay. Consequently, some regressions used data from both rivers for a subset of data available from North Monterey Bay and some regressions used data from only the Pajaro River for the entire set of data from North Monterey Bay. The indices of the loads from wastewater and rivers were calculated to approximate an exponential dilution with increasing distance from the discharges (i.e., effect at North Monterey Bay = PCB load/2^x, where x = the number of kilometers from the discharge to North Monterey Bay). All load data were first log-transformed (natural log) for better conformance with the assumptions of parametric statistical tests.

Initial stepwise multiple regressions examined whether total PCB concentrations at North Monterey Bay could be statistically modeled with loads from wastewater, the Pajaro River and both rivers combined. Neither the calculated index for the Pajaro River, by itself, nor that for both rivers combined were significant variables for explaining the variability of total PCBs at

North Monterey Bay. In the model that included only discharges from the Pajaro River, only the index of wastewater loads was significantly associated with concentrations of PCBs at North Monterey Bay, explaining 71% of the variation in ocean concentrations (Table 9).

Table 9. Results of stepwise multiple regressions for the effects of PCB loads from nearby wastewater discharges and rivers on PCB concentrations at North Monterey Bay. Load indices were weighted to approximate an exponential dilution with increasing distance from the discharges (i.e., effect at North Monterey Bay = PCB load/2^x, where x = the number of kilometers from the discharge to North Monterey Bay). All data were transformed (natural log).

Independent Variables	*N*	r^2	*p*	Model (Significant Variables)
Total PCB load indices from wastewater and the Pajaro River	10	0.7123	0.0021	y = 2662.8 Wastewater Load Index – 0.0272
Total PCB load indices from wastewater and both rivers (Pajaro and Salinas)	4	0.2107	0.8884	None

The apparent influence of wastewater discharges on concentrations of PCBs in ocean waters at North Monterey Bay suggested by the stepwise multiple regressions were surprising given the much larger annual loads of PCBs from rivers (Figure 16). These results prompted more detailed source tracking procedures, for which PCBs are especially amenable because of their chemical properties. PCBs include up to 209 different congeners, which differ from each other in the number and placement of chlorine atoms on the biphenyl rings. All congeners with the same number of chlorine atoms are called homologues. PCBs from different sources often have different homologue profiles (i.e., total PCBs distributed among homologues in different percentages). Consequently, similar homologue profiles between a source and the ocean would support a link between impairments in the ocean and the source. Conversely, very different homologue profiles between the wastewater or rivers and the ocean samples would cast doubt on the causality of the associations between the sources and ocean samples.

Comparison of homologue profiles between ocean water from North Monterey Bay, the aggregate discharges from the Pajaro and Salinas rivers, the three Monterey Bay wastewater discharges and ocean samples collected in the summers of 2004, 2006, 2007 and 2009 just outside the Golden Gate (San Francisco Estuary Institute 2011) show very different homologue distributions. Samples from North Monterey Bay have higher percentages of homologues with fewer chlorine atoms (e.g., di- and trichlorobiphenyls) and lower percentages of penta- and hexachlorobiphenyls than the other sources (Figure 18). Nearly 34% of total PCBs in North Monterey Bay samples were trichlorobiphenyls, while all other sources were dominated by pentachlorobiphenyls. Regardless of higher percentages of di- and trichlorobiphenyl at North Monterey Bay, overall concentrations of these homologues were generally lower at North Monterey Bay than in wastewater or the Pajaro River (Figure 19).

Despite the differences in average percentages of homologues at North Monterey Bay and wastewater and rivers, a more detailed comparison of homologue profiles between samples within each sampling period is necessary to investigate how variations in ocean concentrations might be responding to sources. A statistical model-building procedure was performed using

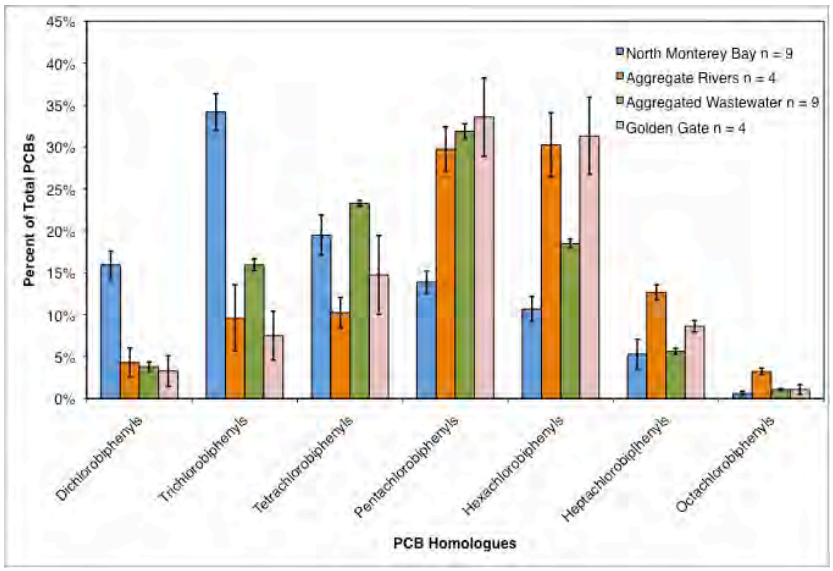


Figure 18. Average distribution of PCBs among homologues from different sources. Standard errors are indicated. Golden Gate data from the summers of 2004, 2006, 2007 and 2009 (San Francisco Estuary Institute 2011).

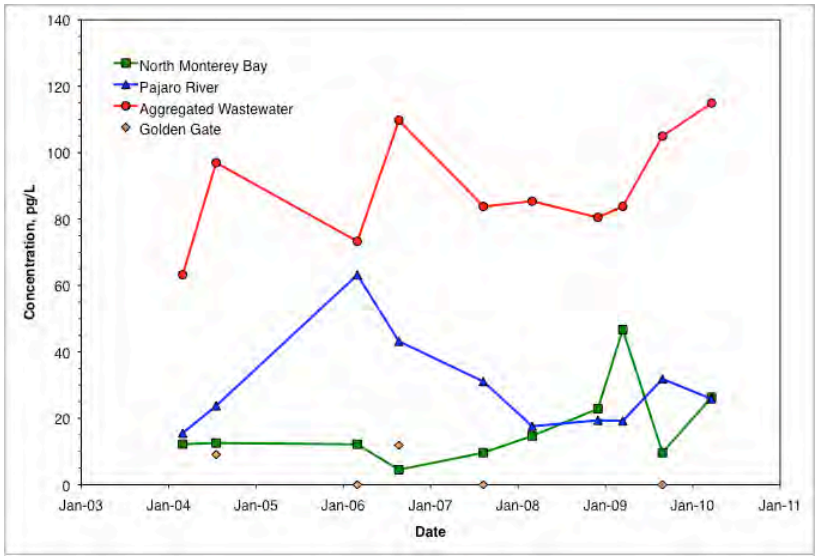


Figure 19. Combined concentrations of di- and trichlorobiphenyl homologues in PCBs from several sources. Golden Gate data from the summers of 2004, 2006, 2007 and 2009 (San Francisco Estuary Institute 2011).

stepwise multiple regressions, as for total PCBs, to determine how homologue concentrations (i.e., homologue profiles) in North Monterey Bay samples were associated with homologue loads from wastewater and rivers. In this exercise, homologue concentrations from North Monterey Bay were the dependent variables in multiple stepwise regressions and loads from the two wastewater discharges closest to the North Monterey Bay site combined (i.e., Watsonville and Monterey Regional; Figure 1) and the Pajaro River were the independent variables. In some

tests, the limited data from the Salinas River were also included with the Pajaro River to represent aggregate river loads. Homologue concentrations at the Golden Gate were also used as independent variables to examine whether waters from offshore a heavily urbanized estuary could be affecting Monterey Bay.

This model-building procedure found that homologue concentrations at North Monterey Bay were significantly associated with the index of aggregate wastewater loads, but were not significantly associated with the index of loads from either the Pajaro River or aggregate river loads (Table 10). Golden Gate homologue concentrations exhibited significant negative associations with North Monterey Bay and, as no logical connection could be conceived for such a relationship, data from the Golden Gate were not considered in the final modeling exercises. The wastewater loads accounted for 29% of the variation in ocean homologue concentrations. Support for this significant relationship between wastewater loads and ocean concentrations, in the absence of a significant relationship between ocean concentrations and river discharges, is provided by examining bivariate plots of homologue concentrations at North Monterey Bay and load indices from wastewater and the Pajaro River (Figure 20a and b). Comparisons between North Monterey Bay and loads from wastewater revealed that values for octachlorobiphenyls, heptachlorobiphenyls, hexachlorobiphenyls, pentachlorobiphenys and tetrachlorobiphenyls clustered fairly tightly. Both sets of samples had low values for octachlorobiphenyls and higher values for tetra- and pentachlorobiphenyls with intermediate values for hexa- and heptachlorobiphenyls. Values for di- and trichlorobiphenyls were relatively much higher in ocean water than in wastewater loads (Figure 20a). Comparisons between North Monterey Bay and the Pajaro River revealed much less within-homologue consistency than between ocean water and wastewater discharges due to high variability in the Pajaro River (Figure 20b).

The primary differences between PCBs in ocean waters at North Monterey Bay and loads from wastewater and the Pajaro River were the high percentages of di- and trichlorobiphenyl homologues in ocean water. When di-and trichlorobiphenyls were removed from the analysis, the amount of variation in ocean PCBs due to loads from wastewater and the Pajaro River increased to 65% and 13%, respectively (Figure 21). Stepwise multiple regressions using homologue data without di- and trichlorobiphenyls found that both wastewater and the Pajaro River were significantly associated with ocean concentrations (Table 10).

Because an important objective of source-tracking activities is to determine which sources might account for the exceedances of water quality objectives (e.g., if exceedances result from increased concentrations of certain homologues that are more typical of some sources than of others), another series of analyses compared data for the four samples from North Monterey Bay with the highest PCB concentrations, and data for the four samples with the lowest PCB concentrations (see Figure 2). Initially, a comparison of these two sets of North Monterey Bay samples revealed that the highest-PCB samples had higher concentrations of every homologue (Figure 22a). Nevertheless, only slightly higher percentages of dichlorobiphenyls and slightly lower percentages of tetra- and pentachlorobiphenyls in the high-PCB samples than in the low-PCB samples suggest these two sets of samples were composed from similar sources (Figure 22b) and the modeling exercises revealed that only wastewater loads were significantly associated with homologue concentrations at North Monterey Bay in both sets of samples. The weaker association with wastewater loads in the four highest-PCB samples than in the four

lowest-PCB samples (Table 9) suggests a decreasing importance of wastewater loads with increasing ocean PCB concentrations.

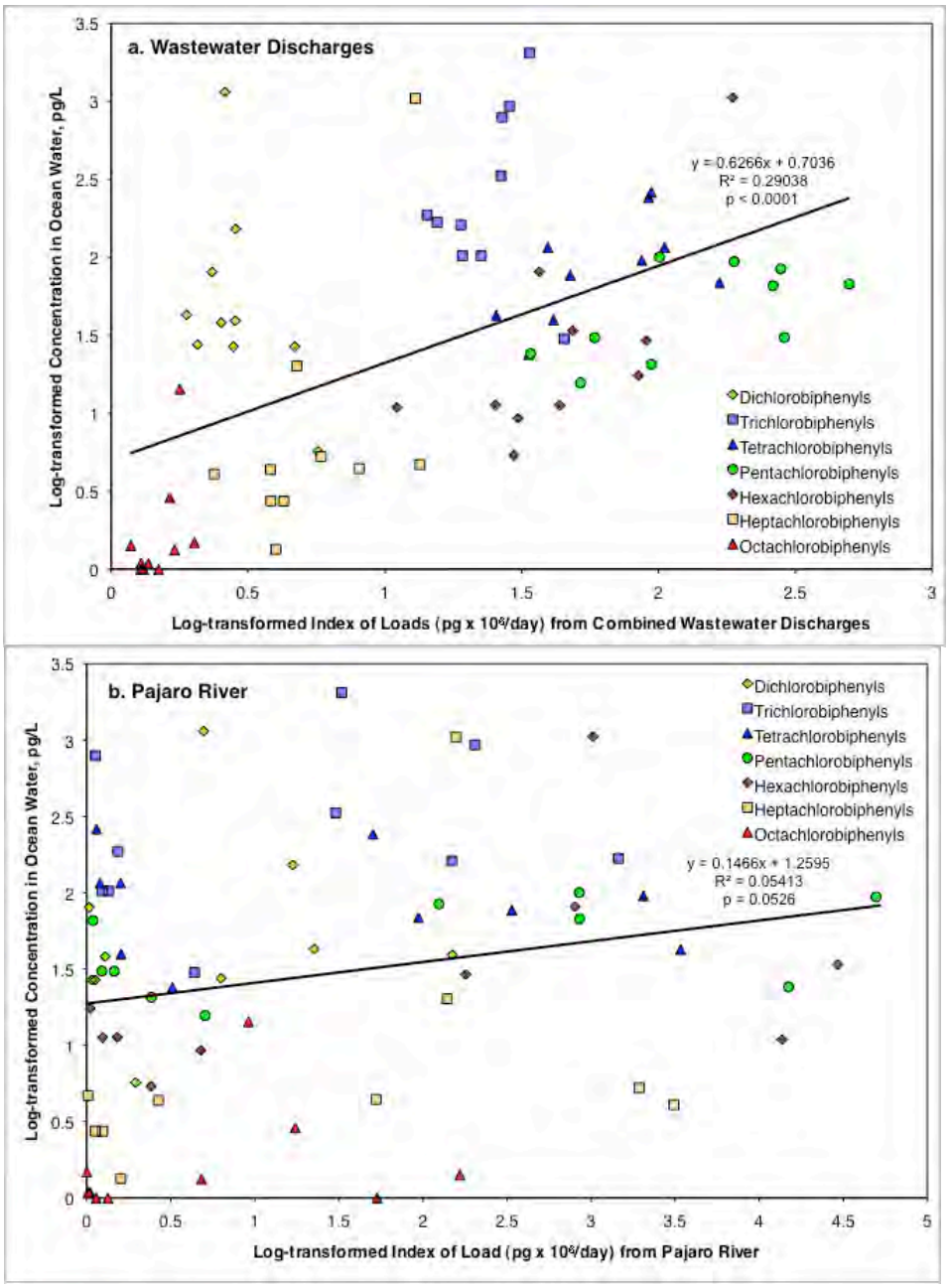


Figure 20. Comparisons between weighted loads of PCB homologues from wastewater and the Pajaro River and concentrations at North Monterey Bay.

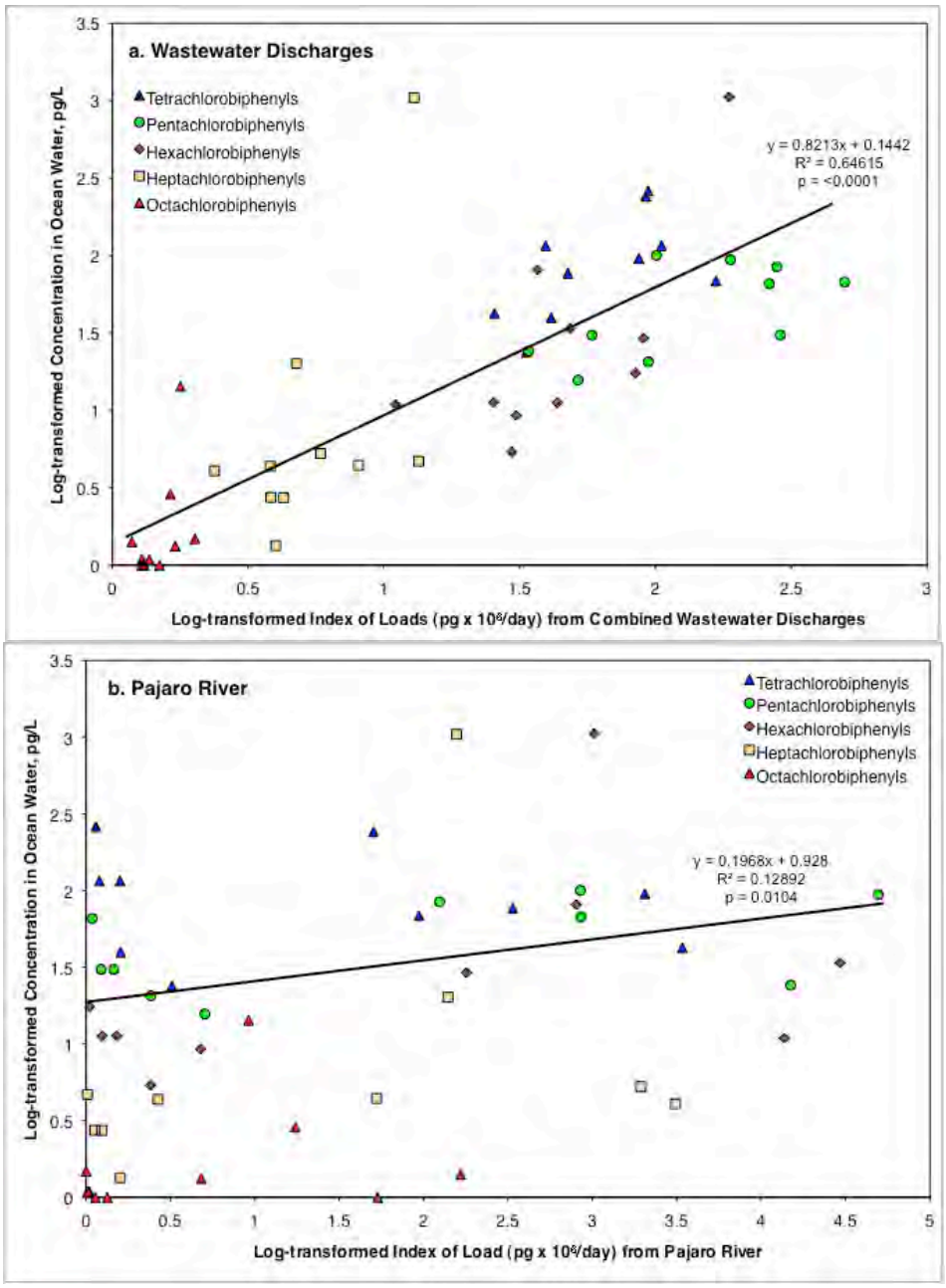


Figure 21. Comparisons between weighted loads of selected PCB homologues (i.e., no di- or trichlorobiphenyls) from wastewater and the Pajaro River and concentrations at North Monterey Bay.

The apparent relationship between wastewater PCB loads and North Monterey Bay concentrations could change if full sets of data were available for the Golden Gate and Salinas River covering all the sampling periods for which North Monterey Bay samples were analyzed. Although data from the Golden Gate and aggregate loads from both rivers for a subset of all samples were either negatively or not significantly associated with North Monterey Bay concentrations (Table 10), it is possible that data from the Golden Gate and Salinas River for the

recent period of high PCB concentrations at North Monterey Bay would have revealed different relationships between the Golden Gate, river loads and North Monterey Bay concentrations.

Table 10. Results of stepwise multiple regressions for the effects of PCB homologue loads from nearby wastewater discharges and rivers on PCB concentrations at North Monterey Bay. Load indices were weighted to approximate an exponential dilution with increasing distance from the discharges (i.e., effect at North Monterey Bay = PCB load/2^x, where x = the number of kilometers from the discharge to North Monterey Bay). All data were log transformed (natural log).

Independent Variables	*N*	r^2	*p*	Model
Homologue loads from wastewater and the Pajaro River and from wastewater and both rivers	70	0.2904	<0.0001	y = 0.7036 + 0.6266 Wastewater Load Index
Homologue loads from wastewater and the Pajaro River without di- and trichlorobiphenyls	50	0.6822	<0.0001	y = 0.0482 + 0.7783 Wastewater Load Index + 0.1066 Pajaro River Load Index
Homologue loads from wastewater and the Pajaro River, four samples with highest total PCBs	28	0.1641	0.0325	y = 1.0787 + 0.4788 Wastewater Load Index
Homologue loads from wastewater and the Pajaro River, four samples with lowest total PCBs	28	0.3581	0.0008	y = 0.5367 + 0.6579 Wastewater Load Index

While these results suggest that PCB concentrations at North Monterey Bay are varying in response to wastewater discharges, the significant relationship between wastewater discharges and PCB concentrations in Monterey Bay may not be causal. The same caveat applies to the results of these regressions as applied to the regressions performed to identify important variables for densities of benthic organisms; not all possible variables have been considered. We do not know the loads of PCBs that are discharged from urban landscapes in storm runoff. Most importantly, PCB concentrations in the ocean offshore of Monterey Bay are unknown, precluding a definition of true background conditions against which concentrations at North Monterey Bay can be compared.

Physicochemical factors at work in both ocean water and wastewater also could partly explain the relationship. In particular, homologues with fewer chlorine atoms are known to be more soluble in water and less likely to adhere to sediment particles (Jarman et al. 1997; Agency for Toxic Substances and Disease Registry 2000; McKee et al. 2006). Moreover, degradation of PCBs in water surface waters occurs primarily through photolysis, which acts more quickly on homologues with greater numbers of chlorine atoms; the half-lives of homologues with fewer chlorine atoms are much greater in water than are those of homologues with greater numbers of chlorine atoms (Agency for Toxic Substances and Disease Registry 2000). The higher concentrations of di- and trichlorobiphenyls in ocean waters could be due to their longer half-lives in surface waters compared to other homologues. Consequently, because ocean waters and highly treated wastewater both have very low concentrations of suspended sediments and because lower chlorinated homologues persist longer in surface waters, both ocean water and wastewater would be expected to be relatively more dominated by homologues with fewer chlorine atoms than would rivers and streams. The domination of samples from near the Golden

Gate by penta- and hexachlorobiphenyls (Figure 20) is more reminiscent of the Pajaro River, which is not surprising because of the Golden Gate's proximity to a large urbanized estuary that is impaired by PCBs.

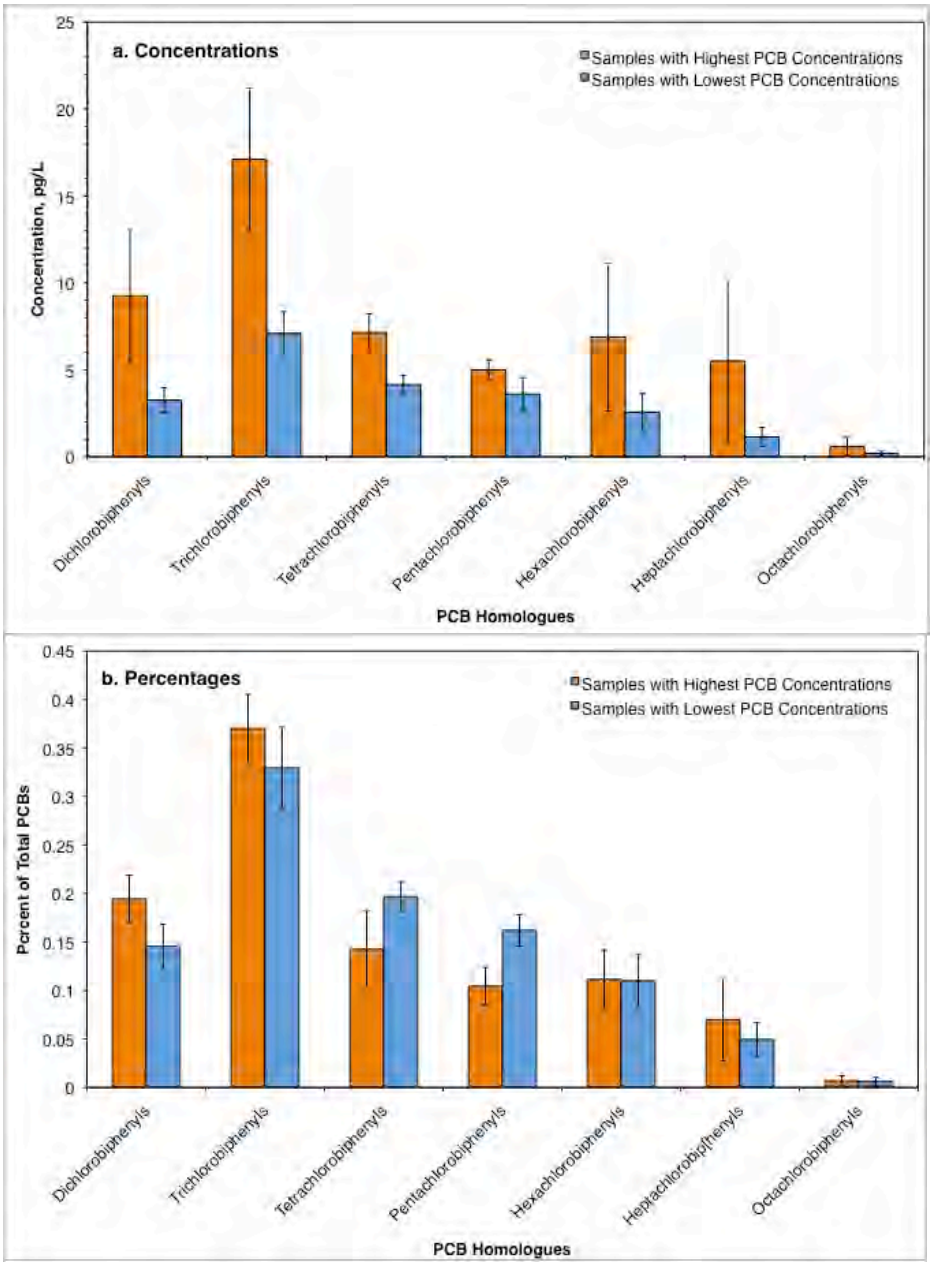


Figure 22. Comparison of the concentrations and percentages of PCB homologues in the four samples with the highest and lowest PCB concentrations at North Monterey Bay. Standard errors are indicated.

Various factors could affect the relationship between PCBs in wastewater and ocean water at North Monterey Bay. The natural partitioning of PCB homologues between dissolved and particulate phases, the unknown ocean background concentration of PCBs and more complete data for river loads all would aid development of a more complete picture for the relationship between wastewater and ocean water. It is also likely that higher loads of PCBs from river discharges are associated with suspended sediments that eventually settle to the seafloor, making it very difficult to establish a link between river loads and ocean concentrations of PCBs. If only dissolved phases were analyzed in all samples, homologue profiles in rivers, wastewater and ocean water could be similar, perhaps with the exception of higher di- and trichlorobiphenyls in ocean waters.

Although the apparent association between wastewater loads and ocean PCB concentrations might not be causal, these findings raise questions about the relative effects of rivers and wastewater discharges on impairments of beneficial uses in Monterey Bay. These questions are controversial not only because of the much higher loads of PCBs discharged by rivers than by wastewater, but especially because none of the wastewater dischargers has exceeded their effluent permit limits for PCBs. Effluent permit limits are set by applying a dilution factor that corresponds to the diffuser characteristics for each discharge, such that the Ocean Plan objective will not be exceeded. A basic assumption of effluent permit limits is that concentrations of regulated chemical pollutants in the receiving water are zero, which is not the case for PCBs in Monterey Bay.

4.2.2.2 Dieldrin in Mussels

Previous analyses have shown that concentrations of Dieldrin in mussels were correlated with rainfall and river discharges (CCLEAN 2007; Hardin et al. 2007). As with the assessment of PCB sources in Section 4.2.2.1, additional data gathered in recent years permits a more detailed analysis than previously was reported.

This analysis was similar to that performed for PCBs. Indices of exposure to each Dieldrin source were calculated for each mussel site to approximate an exponential dilution with increasing distance from the discharges (i.e., effect on Dieldrin in mussels = Dieldrin load from each source/2^x, where x = the number of kilometers from the source discharge to the mussel site). This index was calculated for both rivers and wastewater discharges covering two time scales, the year immediately before the mussel measurements and the cumulative period extending back two years before the mussel measurements. Estimates of exposures to wastewater sources were restricted to the one or two discharges most immediately up current from the sites (i.e., Scott Creek and Laguna Creek = Santa Cruz; The Hook = Watsonville and Monterey Regional; Fanshell Overlook and Carmel River Beach = Carmel Area). Based upon proximity and historic records of Dieldrin discharges, exposures of mussel sites to river sources were restricted to the Pajaro and/or Salinas rivers for northern Monterey Bay sites (i.e., Scott Creek, Laguna Creek and The Hook) and the Carmel River for Fanshell Overlook and Carmel River Beach. The wastewater and river exposure indices and annual rainfall for the year prior to mussel sampling were used as independent variables in a stepwise multiple regression to determine whether any of them explained significant variation in wet-season concentrations of Dieldrin in mussels.

In order to provide greater focus on the source of impairments in the northern bight of Monterey Bay, where exceedances of Dieldrin in mussels have been most prevalent, Scott Creek, Laguna

Creek and The Hook were combined in one set of analyses and Fanshell Overlook and Carmel River Beach were combined in another set of analyses. Analysis of the full time period of mussel data required that only the Pajaro River be included as the river source for Monterey Bay. Nevertheless, previous CCLEAN results have shown that the Salinas River has contributed 60% of the Dieldrin load from rivers and wastewater into Monterey Bay (CCLEAN 2007), so another analysis was based on the subset of mussel samples for which Salinas River data could be combined with Pajaro River data to provide an aggregate river load of Dieldrin.

Analysis of all mussel data using loads from wastewater and the Pajaro River to Monterey Bay revealed that the indices of Dieldrin loads from wastewater in the year prior to mussel sampling and the indices of river loads for the two-year period prior to sampling explained 45% of the variation in mussel Dieldrin concentrations (Table 11). When the subset of mussel data was analyzed for data available from both the Pajaro and Salinas rivers, it was found that annual rainfall, the one-year wastewater index and the two-year river index explained 87% of the variation in mussel Dieldrin concentrations. Partial correlations between Dieldrin concentrations in mussels, rainfall, and load indices for rivers (i.e., using loads from both the Pajaro and Salinas rivers) and wastewater revealed that river discharge was the most important independent variable explaining Dieldrin concentrations in mussels, and wastewater loads are more important than rainfall (Table 12). There were no significant associations between mussel Dieldrin concentrations, rainfall and river or wastewater indices for Fanshell Overlook and Carmel River Beach.

Table 11. Results of stepwise multiple regressions for the effects of rainfall, river and wastewater discharges on concentrations of Dieldrin in mussels along the northern shore of Monterey Bay. Load indices were weighted to approximate an exponential dilution with increasing distance from the discharges (i.e., effect at mussel site = Dieldrin load/2^x, where x = the number of kilometers from the discharge to mussel site). All data were transformed (natural log).

Independent Variables	*N*	r^2	*p*	Model
Dieldrin loads from wastewater and the Pajaro River	21	0.4499	0.0046	y = 0.7026 + 59.77 Wastewater 1-year Load Index + 652.7 Pajaro two-year Load Index
Dieldrin loads from wastewater and both rivers	11	0.8708	0.0006	y = 0.3356 + 0.1878 Rainfall + 68.55 Wastewater 1-year Load Index + 591.6 Aggregate River 2-year Load Index

Table 12. Partial correlations between concentrations of Dieldrin in mussels and significant independent variables from stepwise multiple regressions in samples with and without data from the Salinas River.

Dependent Variable	Significant Independent Variables
Wet-weight Dieldrin, all years	Pajaro two-year Load Index = 0.6610 Wastewater 1-year Load Index = 0.4927
Wet-weight Dieldrin, years with Salinas River data	Aggregate River 2-year Load Index = 0.8948 Wastewater 1-year Load Index = 0.8444 Rainfall = 0.7445

As was done in the analysis of PCB sources, the relationships between Dieldrin in mussels and independent variables were examined for the sampling periods in which concentrations in mussels exceeded the U.S. EPA screening value for recreational fishers at one or more sites to see whether the relative importance of river and wastewater loads were different from what was seen when the entire data set was analyzed. Analyses were performed using the three sampling periods with the highest and the three sampling periods with the lowest overall Dieldrin concentrations. Significant independent variables when mussels had high Dieldrin concentrations were local rainfall, river and wastewater discharges (Table 13), which was very similar to the model based on years with data from both rivers (Table 11). Wastewater loads were not a significant independent variable in samples with low concentrations of Dieldrin, suggesting wastewater loads have a greater influence on high than on low Dieldrin concentrations in mussels. Partial correlations based upon samples with high Dieldrin showed the same order of importance among independent variables as for the entire data set (i.e., river loads exert a greater influence than do wastewater loads), although two-year loads from the Pajaro and one-year loads from wastewater had nearly identical partial correlations (Table 14).

Table 13. Results of stepwise multiple regressions for the effects of rainfall, river and wastewater discharges on concentrations of Dieldrin in mussels along the northern shore of Monterey Bay during sampling events in which Dieldrin exceeded the U.S. EPA screening value for recreational fishers at one or more sites. Load indices were weighted to approximate an exponential dilution with increasing distance from the discharges (i.e., effect at mussel site = Dieldrin load/2^x, where x = the number of kilometers from the discharge to mussel site). All data were transformed (natural log).

Independent Variables	*N*	r^2	*p*	Model
Dieldrin loads from wastewater and the Pajaro River, three years with highest Dieldrin	6	0.8843	0.0394	y = 0.9980 + 61.53 Wastewater 1-year Load Index + 444.8 Pajaro two-year Load Index
Dieldrin loads from wastewater and the Pajaro River, three years with lowest Dieldrin	9	0.6477	0.0089	y = 0.5734 + 627.2 Pajaro two-year Load Index

Table 14. Partial correlations between concentrations of Dieldrin in mussels and significant independent variables from stepwise multiple regressions in the three samples with the highest concentrations of Dieldrin.

Dependent Variable	Significant Independent Variables
Wet-weight Dieldrin, years with high Dieldrin	Pajaro two-year Load Index = 0.9243 Wastewater 1-year Load Index = 0.9188

4.2.3 Conclusions

Estimated loads of most POPs remain much greater from rivers than from wastewater, although loads of PBDEs are higher from wastewater than from rivers. Despite higher loads of PCBs and

Dieldrin from rivers than from wastewater, rivers are apparently not solely responsible for impairments of ocean waters due to PCBs or mussels due to Dieldrin.

Elevated concentrations of PCBs in ocean waters in northern Monterey Bay were statistically linked to wastewater loads, while they were not statistically linked to loads from rivers. The correlation between wastewater discharges and PCBs in Monterey Bay was evident for total PCBs, as well as for the percentages of PCB homologues, although ocean waters had higher proportions of di- and trichlorobiphenyls than did wastewater discharges. These relationships were consistent in analyses using all samples, as well as only those samples that exceeded the Ocean Plan objective for PCBs, which suggests that conditions leading to exceedances of the Ocean Plan do not differ fundamentally from those that generally influence concentrations of PCBs in Monterey Bay.

Concentrations of Dieldrin in mussels along the northern shore of Monterey Bay were associated with loads from both rivers and wastewater, as well as with rainfall in some cases. These results suggest that rivers, wastewater and runoff from the land are all affecting Dieldrin concentrations in mussels. Partial correlations suggest that loads from rivers and wastewater have comparable effects, although the effect of rivers is slightly greater than that of wastewater. The effects of discharges from rivers and wastewater appear to operate on different time scales, with mussels reflecting cumulative two-year river loads and one-year wastewater loads. This difference in time scale suggests that there could be differences in the rate of bioaccumulation by mussels of materials from wastewater and rivers, perhaps related to the proportions of Dieldrin in the dissolved and particulate phases from each source. It is assumed that much more of the Dieldrin is adsorbed to particulate matter in river discharges, compared to wastewater.

The modeling results are highly dependent on the independent variables that are included in the stepwise linear regressions. Incomplete data for one significant independent variable automatically restricts model results to those periods covered by the incomplete data. Results that have implicated wastewater discharges in water quality impairments could have been different if data for other variables and a more complete set of river data were available.

4.2.4 Recommendations

In the consideration of Monterey Bay's exceedances of the Ocean Plan objective for PCBs to protect human health, it would be helpful to know the linkages between PCBs in Monterey Bay and human health. It is possible that this nexus could be provided by analyses of fish tissues performed by San Francisco Estuary Institute in the Monterey Bay in 2010. If fish and shellfish are not exhibiting elevated PCBs, the PCB water quality objective could possibly be raised through development of a Site Specific Objective.

Water quality regulators should consider ways of reinitiating POP sampling in the Salinas River. As the source of the greatest historic loads of most POPs, data from the Salinas River would vastly improve the ability to more accurately partition causes of impairments among different sources. Moreover, the results PCB and Dieldrin source modeling were potentially affected by the unknown amounts of these POPs in the dissolved and particulate phases from each source, and data on the loads for each phase would improve source-tracking analyses.

Development of a mass balance model for PCBs in Monterey Bay also could help to further discriminate the actual sources of impairments to water quality. Such a model would enable estimates of the mass of PCBs coming into Monterey Bay from many sources. This effort would undoubtedly require collection of samples from additional locations and depths than are currently sampled by CCLEAN.

4.3 What are the Effects of Wastewater Discharges on Nearshore Waters?

4.3.1 Assay of Endocrine Disruption using Fathead Minnows

4.3.1.1 Background

CCLEAN, in collaboration with California State Waterboard (Region 3), sponsored a two-season study (approximating wet-season and dry-season periods) to screen for specific effects of endocrine disrupting compounds (EDCs) in the effluents of dischargers. The study was commissioned to satisfy NPDES permit requirements and to inform and assist CCLEAN members on future possible program options and responses to published reports and concerns about the effects of EDC compounds in effluent-influenced waters (Woodling et.al., 2006; Jobling et.al., 2002; Snyder 2008). Additionally, studies in marine systems have documented endocrine disruption effects linked to sewage effluent in marine flatfish in California (Schlenk et al., 2005), Japan (Hashimoto et al., 2000) and the United Kingdom (Lye et al., 1997). The effects of EDCs on reproduction and development of wildlife as well as other public health concerns have placed EDCs on the U.S. EPA's high priority research area list (USEPA, 1996).

Under contract to CCLEAN, the Marine Pollution Studies Laboratory (MPSL) operated by University of California Davis at Granite Canyon performed this study (Appendix A) using methods adapted from *A Short-term Test Method for Assessing the Reproductive Toxicity of Endocrine-Disrupting Chemicals Using the Fathead Minnow (Pimephales promelas) EPA/600/R-01/067* (USEPA, 2008). During a 21-day exposure period, the effluent from each wastewater treatment facility was diluted to approximate initial dilutions provided by the respective outfall diffusers and pumped through four replicate aquaria for each effluent. A 14-day pre-exposure period preceded the effluent exposures. Four replicate control aquaria were exposed to fresh well water during both pre-exposure and exposure periods. During both pre-exposure and exposure periods, the number of eggs laid, the percentage of eggs fertilized and hatching success were measured on a daily basis. At the conclusion of the test, fish were sacrificed and body weight, gonad weight, gonadosomatic index (GSI = gonad weight ÷ body weight), and enzyme-linked immunosorbent assay (ELISA) analysis of plasma vitellogenin (Vtg) were measured on individual fish. Vitellogenin measurements were based on readings of serial dilutions of blood plasma to ensure a value within the linear range of the calibration curve. Readings of each dilution were duplicated and the relative percent difference was considered in selecting the dilution from which to report the Vtg concentration. Observations at the conclusion of tests also included secondary male sexual characteristics [presence of nuptial tubercles, measurement of dorsal fat pad index (FPI = fat pat weight ÷ body weight), tubercles and coloration].

4.3.1.2 Results of EDC Assay using Fathead Minnows

Results of the MPSL assays were reported to CCLEAN in a final report delivered on February 11, 2011 (Appendix A). The Summary and Conclusions from that report are as follows:

> The overall fecundity of the fathead minnow populations in both the wet season and dry season tests were quite variable among replicates and treatments, and between the tests themselves. Wet season fecundity ranged from 75 to 133 eggs/day, whereas the dry season fecundity ranged from 14 to 46 eggs/day. Standard deviations among replicates were sometimes equivalent to the mean values indicating extremely high variability and reduced statistical power. Low replication in the EPA protocol design (4 replicates per treatment) may not be sufficient to adequately capture and account for variability within treatments. Even if individual concentrations varied considerably between fish, it might be possible to determine trends per treatment if replication was higher.
>
> The dry season effluent from Santa Cruz was the only treatment that had significantly lower fecundity than the control. The average fecundity in the exposure was 10 eggs/female/day, which was lower than the control test acceptability criterion of 15. However, this result was not significantly different from the fecundity in the Santa Cruz pre-exposure aquaria. There were no statistically significant differences in egg production between the pre-exposure and exposure periods in any of the effluents. Mean fecundity in the Santa Cruz treatment was greatly influenced by the lack of egg production in the first replicate. The other three replicates had egg production that ranged from 7 to 23 eggs/female/day. One female in the third replicate of the dry season Santa Cruz treatment also exhibited territorial behavior, and although this could be a sign of endocrine disruption, this replicate had the second highest egg production. The reduced reproductive output observed in the dry season Santa Cruz effluent exposure was most likely due to low reproductive capacity of the fish. In the wet season test, fat pads were observed on two Santa Cruz females. This could also be a sign of endocrine disruption, but egg production in this replicate was also high. Although some masculinization was observed in replicates from both of the tests with Santa Cruz effluents, the preponderance of evidence does not suggest this effluent is causing endocrine disruption.
>
> There were several other minor observations and significant differences in the other three effluents, but only one might be considered a result of endocrine disruption. The only other incidence of masculinization occurred in the wet season effluent from Monterey. A dark and banded territorial female was observed in a replicate that contained two males, but the presence of this female did not impact egg production in this aquarium. There were a number of males with light coloration and no banding observed in Watsonville, Monterey and Carmel treatments, but these fish did not appear to be feminized, and the egg production of these replicates was not impacted. There were also several observations of significantly larger gonads. These occurred in the wet season males from the Monterey treatment, the dry season males from Santa Cruz and Carmel, and the dry season females from Watsonville. Because the gonads from these effluents were significantly larger than the controls, it was assumed they were not impacted by the effluents.

The vitellogenin analysis produced abnormally high results in several male control fish, and in many other male fish in the effluents of both tests. Similar, though less dramatic results were observed in the female fish analyzed for the dry season test. The high vitellogenin concentrations were variable throughout the tests and were not linked to any reproductive effects in the effluents. After many detailed discussions with the ELISA kit manufacturer and other researchers, we were still unable to determine the cause of the elevated and variable concentrations, although it is likely there was some interfering factor in the plasma samples themselves. Because of the difficulties encountered in the analysis of vitellogenin, these results were largely inconclusive and considered unreliable, and are not included in the summary tables below.

The results suggest that there was likely no impact on fathead minnow reproduction in any of the treatment plant effluents at the concentrations tested. While some parameters measured in the test are possible indicators of endocrine disruption due to effluent exposure, only the Santa Cruz treatment had significantly lower fecundity, the primary endpoint indicating reproductive impairment. Based on the fact that the pre-exposure egg production in the Santa Cruz aquaria did not significantly differ from the effluent exposure egg production, it was concluded that reduced egg production in the Santa Cruz effluent was not caused by endocrine disruption.

Because of the complex nature of the EDC assays and the problems encountered by MPSL, CCLEAN commissioned an external peer review with the following objectives:

- Analyses of the technical information, including the USEPA (2002a) testing protocol and related documents and the MPSL proposal to complete the study as requested by CCLEAN, and the correspondence between the study objective (*i.e.,* to assess the endocrine disrupting activity of each participant's wastewater at real-world concentrations using the fathead minnow) and study design, including whether study results could address other ancillary objectives;
- Review of the laboratory data, narratives and analyses from the study, for completeness and data quality assessment;
- Review of the study conclusions relative to study objectives in relation to standard endocrine effects, including but not limited to fathead minnow fecundity;
- Review and quality checks of the results and conclusions, including checking of the calculations used for statistical analyses; and,
- Recommendations for improving the linkage between study design, objectives and execution.

Golder Associates (Golder) was selected to perform this peer review and submitted their findings on March 30, 2011. It is included in this report as Appendix B.

Golder identified a number of deficiencies in the performance of the assays, including the following:

1. The use of test fish of different sizes and ages, which were obtained from different sources, in each test violated EPA recommendations and introduced uncontrolled variability in test results.

2. While pre-exposure periods lasted more than the minimum 14 days required by the EPA protocol, pre-exposure data only included 9 and 10 days of data for the wet and dry seasons, respectively.
3. There were several cases in which low pre-exposure fecundities occurred in replicate aquaria, which should have required their replacement. These low fecundity aquaria were, instead, included in the exposure period.

MPSL was unable to obtain sufficient numbers of fish from a single supplier for either test, as noted above. In the wet-season test, fish from Osage Catfisheries (Osage Beach, MO) and Aquatic Bio Systems (Fort Collins, CO) (ABS) were used in the controls and effluent treatments, whereas fish from Aquatic Research Organisms (Hampton, NH) (ARO) were used in the reference toxicant test. In the wet-season test, all fish of the same sex in each replicate were from the same supplier, although the sources of males and females sometimes differed and replicates sometimes differed in the sources of their fish. In the dry-season test, fish of the same sex from Osage and ARO were generally combined in replicates, although some replicates contained males only from Osage. The use of fish from different suppliers contributed a potential uncontrolled source of variation. Consequently, CCLEAN performed an analysis to determine whether test results were affected by the source of the fish. Because the source of individual fish could not be determined at the conclusion of each test, it was necessary to base these analyses on replicates in which all fish of a given gender were known to be from a single supplier.

All the statistical tests performed in these analyses assume normally distributed data and data in which there is not a positive correlation between the magnitude of data values and variation among data. To improve adherence to these assumptions, all data were transformed; data from direct measurements were treated with the log transformation [log(x+1)] and percent data were treated with the arcsine transformation [arcsine(square root of x)].

4.3.1.2.1 Effects of Fish Source

A series of one-way ANOVAs were performed to answer the following questions from wet-season data:

1. Does plasma vitellogenin differ in male fathead minnows from different sources?
2. Does the final calculated concentration of plasma Vtg differ depending on the dilution used for the reported value?
3. Does the dilution factor for the final reported Vtg differ in male fathead minnows from different sources?
4. Does the weight of each gender differ from different sources?
5. Does the gonad weight of each gender differ from different sources?
6. Does the %GSI of each gender differ from different sources?

The ANOVA results revealed that the answers to all the above questions were “yes” except for female gonad weight and male %GSI (Table 15). These results expand the areas into which problems have been documented due to use of fish from different sources. The significant differences between males from different sources in reported Vtg concentrations, different reported Vtg concentrations among plasma dilution factors, and fish from different sources systematically having different dilution factors selected all highlight the complexities of the ELISA methods.

Table 15. Results of one-way ANOVAs for effects of fish source and plasma dilution on measurements in fathead minnows from the wet-season assay. Transformed data [log(x+1) or arcsine(square root x)] used.

		Model Fit		
Measurement	Main Effect	r^2	*p*	*A posteriori*
Male plasma Vtg	Fish Source	0.1424	0.0333*	Osage>ABS
Dilution of plasma for male Vtg analysis	Fish source	0.2598	0.0029*	Osage>ABS
Male plasma Vtg	Plasma Dilution	0.7781	<0.0001*	50000>5000>50
Female weight	Fish Source	0.1978	<0.0001*	Osage>ABS
Male weight	Fish Source	0.3687	<0.0001*	Osage>ABS
Female Gonad weight	Fish Source	0.0059	0.4996	Osage=ABS
Male Gonad weight	Fish Source	0.1887	0.0081*	Osage>ABS
Female %GSI	Fish source	0.0521	0.0431*	Osage>ABS
Male %GSI	Fish source	0.0046	0.6940	Osage=ABS

* = Statistically significant difference.

4.3.1.3 Conclusions

A number of conclusions can be drawn from the assays, as conducted. Firstly, both MPSL and Golder concluded that there was no evidence from the assays that reproduction of the fathead minnows was adversely affected by the wastewater effluents at receiving water concentrations. Nevertheless, both MPSL and Golder acknowledged that several aspects of the assays reduced their ability to detect reproductive effects. In this regard, an important feature of the assay results was the high variability in fecundity among aquaria within and among treatments. An unknown portion of this variability could have been due to the use of fish from different sources, with attendant size differences and possible subtle physiological differences. The average age of the test fish used in the assays was higher than the recommended age in the EPA protocol, and this also may be significant in evaluating uncertainties in MPSL's results.

While MPSL satisfied the EPA protocol by running a sodium-chloride reference toxicant positive control, we question the use of a mortality end-point in controls for an assay of subtle reproductive effects. Use of a standardized EDC as a reference toxicant would have documented the ability of the test populations to respond to EDCs in the wastewater treatments.

One area of conflict in the conclusions drawn by MPSL and Golder involves the designation of female masculinization as potential endocrine disruption. While Golder states that development of male secondary sexual characteristics "can occur infrequently in unexposed female fish," it is worth noting that such characteristics were observed in the current assays only in effluent exposure aquaria and not in any control aquaria. Moreover, other studies of endocrine disruption in fish caused by historic discharges of DDTs and PCBs have found masculinzation of female fish thought due to binding of estrogen receptors by the synthetic organic chemicals (Spies and Thomas, 1997).

Golder offered critiques of the assay design including the fact that effluent testing was performed only at a single effluent concentration that approximated the initial dilution provided by each

treatment facility's outfall diffuser. Golder recommended exposing fathead minnows to various effluent concentrations, including levels that would exceed those higher than the calculated concentrations following initial dilution in the ocean. However, CCLEAN's objective in conducting this study was to answer the question "What are the effects of wastewater discharges in nearshore waters?" Consequently, testing effluent to see whether ANY effect can be detected, regardless of concentration, would be a departure from this specific study objective. Nevertheless, as more wastewater treatment agencies are moving toward water reclamation for agricultural and horticultural applications, such questions of the effects of concentrated effluent become more appropriate.

Golder also recommended that the previous experience of the laboratory conducting the assay should be a consideration in awarding contracts for such assays. In general, we agree that previous experience is an excellent criterion for awarding contracts. An important logistical consideration in the current assays was the desire to provide daily effluent samples to the assay laboratory and perform the assays within tight budget constraints. These logistic and budgetary issues required use of one of the two local laboratories, neither of which had previous experience with the EPA EDC assay. If EDC assays are performed in the future, the same logistic and budgetary constraints are likely to apply, unless other laboratory options emerge in the interim.

This study of EDC effects from municipal wastewater on reproduction in fathead minnows has been groundbreaking. The EPA protocol that formed the basis for the assays was designed to measure endocrine disruption caused by single compounds to which fish are exposed at various dilutions, in order to estimate EC_{50} concentrations. Instead, we have applied the protocol to test for effects of a complex mixture of organic chemicals at the concentrations anticipated after initial dilution of municipal wastewater discharges in the ocean. As a groundbreaking effort, the results of this study and the various problems encountered will be invaluable for improving the methods for future assays. The recommendations that follow have been based on the experience gained with this study.

4.3.1.4 Recommendations

If effluent assays for reproductive effects are performed in the future, several recommendations should be considered, as follows:

- Explore the availability of grant funding to further improve the method for routine application.
- Ensure that only fish from a single source are used throughout the assay. This could require phased assays, such as testing effluent from one treatment facility each year, such that each facility is tested once in each permit cycle.
- Use a statistical approach to plan for the optimum number of replicates for minimizing within-test variability.
- Use a standardized EDC as a reference toxicant.
- Explore uses of other fish species. While use of test species other than the fathead minnow would depart from the EPA protocol, other species, such as the zebra fish, could be more sensitive to estrogen mimics, although potentially less desirable in other ways, such as having a smaller body size that would provide less blood for Vtg measurements.
- Explore reproductive hormone endpoints other than Vtg production, such as gene induction, and tissues other than blood plasma for Vtg testing. This recommendation is

directed toward providing more sensitive and repeatable results, although it could involve collaboration in basic research with no assurance of a positive outcome.

4.3.2 Proposition 50-funded Study of Indicator Bacteria and Fecal Pathogens

4.3.2.1 Background

In 2005, CCLEAN became aware of recent studies that reported a correlation between proximity to coastal freshwater discharges and an increased risk of *Toxoplasma gondii* infection in southern sea otters (*Enhydra lutris nereis*) (Miller et al., 2002). Because of the wide public interest in this pathogen at the time, CCLEAN participants decided to seek grant funding under Proposition 50 to study sources of fecal pathogens along the central California coast. Following exploratory discussions with researchers in the field of marine wildlife pathogens, a team was formed with scientists at University of California at Davis, California Department of Fish and Game Marine Wildlife Veterinary Care and Research Center and Applied Marine Sciences, Inc. to apply for a Proposition 50 grant in the 2005–2006 application cycle. Based upon discussions with State Water Board staff, the conceptual proposal was considered under a special segment of the Proposition 50 funds designated for ocean protection, for which applications received expedited review. On February 9, 2006, a concept proposal was submitted and on March 14, 2006 the conceptual proposal was approved for submittal of a detailed proposal. A full proposal was submitted on May 9, 2006 and on June 21, 2006, following a recommendation from the California Ocean Protection Council, the California State Water Resources Control Board approved a resolution to fund the project.

This project was designed to answer the following questions:

1. What are the spatial and temporal patterns in fecal indicator bacteria and pathogens along the central coast, and what is the relationship between concentrations of fecal indicator bacteria and fecal pathogen detection in: wastewater influent and effluent; rivers and streams; ocean and mussels; stormwater; and wetlands?
2. How can loading of fecal pathogens into nearshore ecosystems be compared across types of surface water samples along the central coast?
3. What is the relationship between exceedences of water quality objectives for fecal indicator bacteria and fecal pathogen detection in surface water samples?
4. Are mussels better indicators of ocean microbial water quality than direct testing of seawater?
5. Which of three microbial source tracking methods is most promising and what can be learned about trends in human versus animal sources of fecal pollution?
6. What are the patterns and risk factors for fecal pathogen shedding from central coast animals, and are the same types of fecal pathogens detected in sea otters as are detected in other marine and terrestrial animals?
7. Are wetlands effective in reducing loads of fecal pathogens and, if so, what characteristics of wetlands are most important for reducing loads of fecal pathogens in surface waters?

Various sources of surface waters, storm runoff and wastewater were sampled for indicator bacteria (i.e., total coliform, fecal coliform and *Enterococcus*) and eight fecal pathogens (i.e., *Vibrio parahaemolyticus*, *V. cholerae*, *V. alginolyticus*, *Campylobacter*, *Salmonella*, *E. coli*

O157, *Cryptosporidium* and *Giardia*). The draft final report for this study has been submitted to the State Water Resources Control Board for review and the final report will be submitted by May 31, 2011.

4.3.2.2 Wastewater Results

Sampling at CCLEAN wastewater treatment facilities involved collection of 10 paired influent and effluent samples. Samples were collected with appropriate lags in each facility between influent and effluent sampling to approximate collection from the same mass of water before and after treatment. The first seven samples were analyzed for all of the indicator bacteria and fecal pathogens measured in this study, whereas the final three samples were only analyzed for *Cryptospordium* and *Giardia* as part of a spike recovery exercise to estimate the percent of total protozoa that were being counted during sample analysis. These spike recovery results were used to correct raw protozoa counts to more closely correspond to the actual concentrations of protozoa in the samples.

Average recoveries of spiked oocysts were much greater in effluent than in influent and differed substantially among wastewater discharges (identified only by number in Figure 23). There were significant differences between wastewater treatment facilities in the percent of spiked organisms that were recovered in effluent (Table 16). Based on these results, site-specific adjustments were applied to observed protozoa concentrations.

Seven out of 11 fecal pathogens analyzed were detected at all wastewater treatment facilities during the 2007–2008 sampling (Table 17). *Vibrio alginolyticus*, *Campylobacter* and Ecoli-O157 were not detected in any wastewater sample. Changes in measurement methods following the first one or two sampling periods affected results for two microbes. Firstly, it was found that greater dilutions were required to quantify *Vibrio parahaemolyticus* and, secondly, use of the US EPA method (USEPA 2006; Method 1682) was required in order to detect *Salmonella*. These two issues resulted in counts of *V. parahaemolyticus* that were too numerous to count (TNTC) and non-reported data for *Salmonella*.

Two one-way ANOVAs (JMP, SAS Campus DriveBuilding SCary, NC 27513) using transformed [log(x+1)] data were performed to determine whether there were any statistically significant differences in influent and effluent concentrations of indicator bacteria and fecal pathogens between sampling times or sites. All values below the detection limit were included at one half the detection limit (Arnone and Walling, 2006). One influent indicator bacterium and fecal pathogen exhibited significant temporal variation, but no spatial variation; influent *Enterococcus* concentrations in December 2008 were lower than in any other month and influent concentrations of *Vibrio parahaemolyticus* were greater in June 2007 than in any other month (Table 18). Effluent concentrations of *V. parahaemolyticus* were also greater in June 2007 than in any other month. Site 1 had significantly lower effluent concentrations of total coliform, fecal coliform and *Enterococcus*, while sites 1 and 2 had significantly lower concentrations of *Giardia* than did either Site 3 or Site 4.

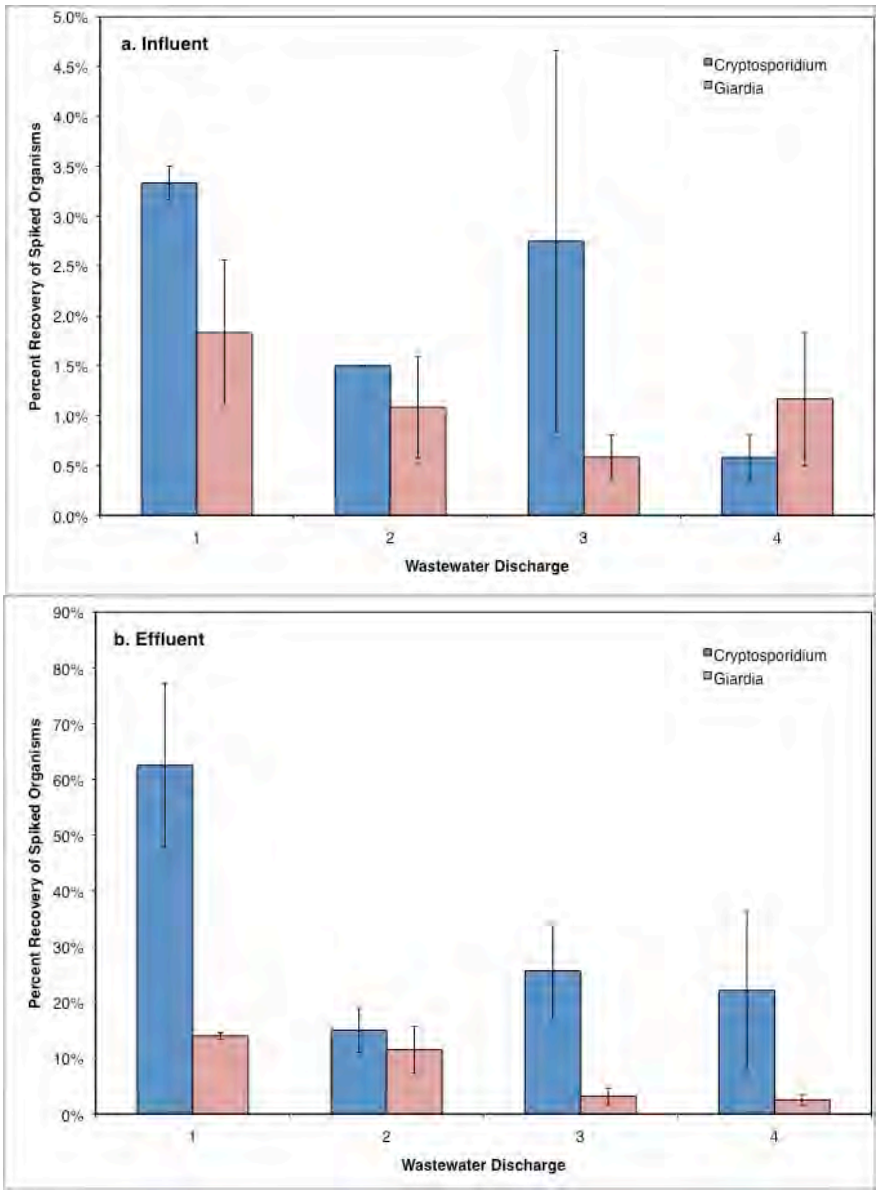


Figure 23 (a,b). Mean recoveries of spiked protozoal oocysts into influent and effluent samples (n = 3) from four wastewater treatment facilities. Bars indicate standard errors.

Table 16. Results from analysis of variance for differences among sites in the recovery of spiked protozoal oocysts. Arcsine-transformed data used.

Variable	R^2	*P*	*A posteriori*
Cryptosporidium in influent	0.4635	0.1536	–
Cryptosporidium in effluent	0.5591	0.0747	1=3, 1>4=2, 3=4=2
Giardia in influent	0.1974	0.6016	–
Giardia in effluent	0.6976	0.0179	1=2, 1>3=4, 2=3=4

Table 17. Results of fecal pathogen sampling in influent and effluent from four wastewater treatment facilities in the Monterey Bay area. Concentrations of *Cryptosporidium* and *Giardia* have been corrected recoveries of spiked organisms.

Date	Site	Source[1]	TotColi[2] 100ml	FecColi[3] 100ml	Enteroco[4] 100ml	Crypto[5] 10L	Giardia 10L	Vibpar[6] 100ml	Vibchol[7] 100ml	Vibalg[8] 100ml	Campy[9] 100ml	Salm[10] 100ml	Ecoli-O157 100ml
5/7/07	1	1	14400000	5000000	9300000	300	162000	TNTC[11]	<1000	<1000	<100	NR[12]	<1000
6/5/07	1	1	19800000	9000000	630000	1800	972000	10000	<1000	<1000	<100	NR	<1000
9/24/07	1	1	5000000	32000000	450000	<15	16364	<1000	55000	<1000	<100	35	<1000
12/17/07	1	1	42000000	15000000	1600000	<15	1623273	<1000	900	<1000	<100	2	<1000
3/31/08	1	1	3000000	7200000	500000	5400	693818	<1000	<1000	<1000	<100	43	<1000
6/2/08	1	1	8700000	8400000	2000000	<15	494182	<1000	<1000	<1000	<100	35	<1000
9/29/08	1	1	2000000	1100000	<100	27300	568909	<1000	<1000	<1000	<100	92	<1000
3/7/11	1	1	NA[12]	NA	NA	<15	515455	NA	NA	NA	NA	NA	NA
3/8/11	1	1	NA	NA	NA	<15	216273	NA	NA	NA	NA	NA	NA
3/9/11	1	1	NA	NA	NA	<15	2487273	NA	NA	NA	NA	NA	NA
5/7/07	2	1	99000000	36000000	900000	667	47538	TNTC	<1000	<1000	<100	NR	<1000
6/5/07	2	1	187000000	88000000	3500000	3333	889846	71000	<1000	<1000	<100	NR	<1000
9/24/07	2	1	52000000	38000000	900000	<33	1170462	<1000	<1000	<1000	<100	161	<1000
12/17/07	2	1	53000000	69000000	4500000	667	1357846	<1000	1000	<1000	<100	5	<1000
3/31/08	2	1	3400000	8400000	470000	8000	548308	<1000	<1000	<1000	<100	1	<1000
6/2/08	2	1	40000000	10000000	590000	<33	605538	<1000	<1000	<1000	<100	11	<1000
9/29/08	2	1	64000000	31000000	45000	<33	3744462	<1000	<1000	<1000	<100	8	<1000
3/1/11	2	1	NA	NA	NA	<33	25763077	NA	NA	NA	NA	NA	NA
3/2/11	2	1	NA	NA	NA	103333	8967692	NA	NA	NA	NA	NA	NA
3/3/11	2	1	NA	NA	NA	74667	12221538	NA	NA	NA	NA	NA	NA
5/7/07	3	1	9000000	1800000	600000	182	547714	TNTC	<1000	<1000	<100	NR	<1000
6/5/07	3	1	86000000	26000000	180000	627273	1218857	53000	<1000	<1000	<100	NR	<1000
9/24/07	3	1	4500000	3600000	300000	<18	10440000	100	<1000	<1000	<100	161	<1000
12/17/07	3	1	12000000	39000000	6300000	727	5629714	<1000	600	<1000	<100	16	<1000
3/31/08	3	1	4900000	4400000	110000	<18	51429	<1000	<1000	<1000	<100	5	<1000
6/2/08	3	1	34000000	4400000	200000	5818	13302857	<1000	<1000	<1000	<100	161	<1000
9/29/08	3	1	22000000	10000000	200000	1091	293143	<1000	<1000	<1000	<100	16	<1000
3/1/11	3	1	NA	NA	NA	<18	45162857	NA	NA	NA	NA	NA	NA
3/2/11	3	1	NA	NA	NA	<18	44777143	NA	NA	NA	NA	NA	NA
3/3/11	3	1	NA	NA	NA	24727	26084571	NA	NA	NA	NA	NA	NA

Date	Site	Source[1]	TotColi[2] 100ml	FecColi[3] 100ml	Enteroco[4] 100ml	Crypto[5] 10L	Giardia 10L	Vibpar[6] 100ml	Vibchol[7] 100ml	Vibalg[8] 100ml	Campy[9] 100ml	Salm[10] 100ml	Ecoli-O157 100ml
5/7/07	4	1	70000000	18000000	1500000	<86	154286	TNTC	<1000	<1000	<100	NR	<1000
6/5/07	4	1	29000000	20000000	2400000	<86	475714	180000	<1000	<1000	<100	NR	<1000
9/24/07	4	1	47000000	17000000	90000	22286	156000	<1000	<1000	<1000	<100	16	<1000
12/17/07	4	1	47000000	4500000	18000	13714	541714	15000	190	<1000	<100	10	<1000
3/31/08	4	1	4200000	6900000	230000	12000	6558000	<1000	<1000	<1000	<100	16	<1000
6/2/08	4	1	26000000	3400000	380000	<86	2514857	<1000	<1000	<1000	<100	161	<1000
9/29/08	4	1	750000000	6200000	<100	211543	64312886	<1000	1200	<1000	<100	6	<1000
3/1/11	4	1	NA	NA	NA	23143	1288286	NA	NA	NA	NA	NA	NA
3/2/11	4	1	NA	NA	NA	<86	2082000	NA	NA	NA	NA	NA	NA
3/3/11	4	1	NA	NA	NA	8571	895714	NA	NA	NA	NA	NA	NA
5/7/07	1	2	<100	<100	900	80	643	<1	<1	<1	<100	NR	<10
6/5/07	1	2	<100	<100	1800	8	643	196	<1	<1	<100	NR	<10
9/24/07	1	2	<100	<100	<100	<1	143	<1	<1	<1	<100	<1	<10
12/17/07	1	2	100	100	100	1	643	<1	<1	<1	<100	<1	<10
3/31/08	1	2	800	1200	100	8	<4	<1	<1	<1	<100	1	<10
6/2/08	1	2	1100	320	310	48	71	<1	<1	<1	<100	1	<10
9/29/08	1	2	2500	1200	350	1576	750	<1	2	<1	<100	1	<10
3/7/11	1	2	NA	NA	NA	176	2786	NA	NA	NA	NA	NA	NA
3/8/11	1	2	NA	NA	NA	144	1214	NA	NA	NA	NA	NA	NA
3/9/11	1	2	NA	NA	NA	112	1536	NA	NA	NA	NA	NA	NA
5/7/07	2	2	4200000	1700000	20000	33	130	126	<1	<1	<100	NR	<10
6/5/07	2	2	1400000	900000	47000	33	130	3000	<1	<1	<100	NR	<10
9/24/07	2	2	5700000	3400000	72000	<3	478	<1	<1	<1	<100	8	<10
12/17/07	2	2	9000000	2700000	6300	<3	1739	<1	<1	<1	<100	1	<10
3/31/08	2	2	27000	35000	7000	<3	87	<1	<1	<1	<100	1	<10
6/2/08	2	2	550000	110000	2300	<3	43	<1	<1	<1	<100	2	<10
9/29/08	2	2	2800000	460000	6200	<3	348	<1	<1	<1	<100	54	<10
3/1/11	2	2	NA	NA	NA	<3	1609	NA	NA	NA	NA	NA	NA
3/2/11	2	2	NA	NA	NA	233	1043	NA	NA	NA	NA	NA	NA
3/3/11	2	2	NA	NA	NA	<3	1391	NA	NA	NA	NA	NA	NA
5/7/07	3	2	4200000	1300000	1400000	<2	2842	TNTC	<1	<1	<100	NR	<10
6/5/07	3	2	180000	90000	8000	156	4263	24000	<1	<1	<100	NR	<10

Date	Site	Source[1]	TotColi[2] 100ml	FecColi[3] 100ml	Enteroco[4] 100ml	Crypto[5] 10L	Giardia 10L	Vibpar[6] 100ml	Vibchol[7] 100ml	Vibalg[8] 100ml	Campy[9] 100ml	Salm[10] 100ml	Ecoli-O157 100ml
9/24/07	3	2	540000	5000000	36000	39	2053	<1	<1	<1	<100	54	<10
12/17/07	3	2	47000000	84000000	1000	<2	7421	<1	<1	<1	<100	<1	<10
3/31/08	3	2	300	55000	2300	<2	3316	<1	<1	<1	<100	1	<10
6/2/08	3	2	270000	6500	1300	<2	1895	<1	<1	<1	<100	2	<10
9/29/08	3	2	680000	320000	<100	292	9474	<1	<1	<1	<100	92	<10
3/1/11	3	2	NA	NA	NA	58	15158	NA	NA	NA	NA	NA	NA
3/2/11	3	2	NA	NA	NA	117	8053	NA	NA	NA	NA	NA	NA
3/3/11	3	2	NA	NA	NA	2	12316	NA	NA	NA	NA	NA	NA
5/7/07	4	2	200000	90000	900	23	3600	<1	<1	<1	<100	NR	<10
6/5/07	4	2	1700000	180000	8100	23	<20	<1	<1	<1	<100	NR	<10
9/24/07	4	2	<100	<100	<100	<2	200	<1	<1	<1	<100	<1	<10
12/17/07	4	2	4700000	<100	<100	<2	4000	<1	<1	<1	<100	<1	<10
3/31/08	4	2	2000	2000	100	<2	17800	<1	<1	<1	<100	1	<10
6/2/08	4	2	<100	300000	100000	113	7000	<1	<1	<1	<100	1	<10
9/29/08	4	2	600	250	<100	23	1600	<1	<1	<1	<100	1	<10
3/1/11	4	2	NA	NA	NA	113	4400	NA	NA	NA	NA	NA	NA
3/2/11	4	2	NA	NA	NA	361	54400	NA	NA	NA	NA	NA	NA
3/3/11	4	2	NA	NA	NA	541	25200	NA	NA	NA	NA	NA	NA
	Influent Mean		59962069	18689286	1353329	29430	7238840	13713	2103	<1000	<100	48	<1000
	Influent Standard Error		21893768	3271347	341279	16462	2273706	6261	1640	-	-	10	-
	Effluent Mean		2969818	3594717	61520	109	5011	2047	<1	<1	<100	11	<10
	Effluent Standard Error		1409885	2498869	41649	42	1533	1125	-	-	-	4	-

[1] = 1 = influent, 2 = effluent; [2] = total coliform ; [3] = fecal coliform; [4] = *Enterococcus*; [5] = *Cryptosporidium*; [6] = *Vibrio parahaemolyticus*; [7] = *Vibrio cholerae*; [8] = *Vibrio alginolyticus*; [9] = *Campylobacter*; [10] = *Salmonella*; [11] = too numerous to count, dilutions changed for subsequent sampling events; [12] = not reported; [13] = not analyzed

Table 18. Significant results of analysis of variance using transformed data [log(x+1)] for differences among sites (spatial) and times (temporal) in the concentrations of indicator bacteria and fecal pathogens at four wastewater treatment facilities in the Monterey Bay area.

	Main Effect	R^2	P	*A posteriori*[1]
Influent				
Enterococcus	Time[1]	0.5339	0.0113	2=4=6=3=5>7
Vibrio parahaemolyticus	Time	0.8279	<0.0001	2>4=7=5=6=3
Effluent				
Total Coliform	Site	0.5332	0.0058	2=3, 3=4, 2>4=1
Fecal Coliform	Site	0.6454	0.0007	3=2>4=1
Enterococcus	Site	0.3383	0.0177	2=3=4, 3=4=1, 2>1
Giardia	Site	0.4097	0.0049	3=4> 2=1
Vibrio parahaemolyticus	Time	0.6110	0.0022	2=1, 1=3=4=5=6=7, 2>3=4=5=6=7

[1] = 1= 5/7/07, 2 = 6/5/07, 3 = 9/24/07, 4 = 12/17/07, 5 = 3/31/08, 6 = 6/2/08, 7 = 9/29/08; [2] = Site 1, Site 2, Site 3, Site 4; sites or times with the highest concentrations are on the left and those with the lowest concentrations are on the right.

Percent removal of indicator bacteria and fecal pathogens through the wastewater treatment process often exceeded 99% (Table 19). Moreover, all wastewater treatment facilities appeared to remove more than 99% of the actual fecal pathogens measured. Across all sites, *V. cholerae*, *Giardia* and *Cryptosporidium* were most easily removed, with average removals exceeding 99.6%. *Salmonella* had the lowest average removal at 76.94%, with no site achieving 99% removal, and fecal coliform had the second lowest average removal due to very high concentrations measured in the December 2007 effluent sample at Site 3 (see Table 14). Site 1 had the best overall removal, averaging 99.99% over all microbes measured and 99.86% for fecal pathogens. Site 3 had the lowest overall removal, averaging 65.24%, although it removed 99.94% of fecal pathogens.

4.3.2.3 Comparisons of Fecal Pathogen Loads

This project provided data that enabled comparisons of concentrations of indicator bacteria and fecal pathogens in surface waters (streams and rivers), storm runoff and wastewater. The average concentrations from each source were averaged among sites in each source type and were not flow averaged. The mean concentrations of fecal pathogens from wastewater, surface waters, and storm runoff exhibited very different profiles (Figure 24). Compared to the other sources, wastewater had higher concentrations of *Giardia* and *Vibrio parahaemolyticus*, whereas storm runoff had higher concentrations of *Cryptosporidium* and surface waters had higher concentrations of *Vibrio cholerae*.

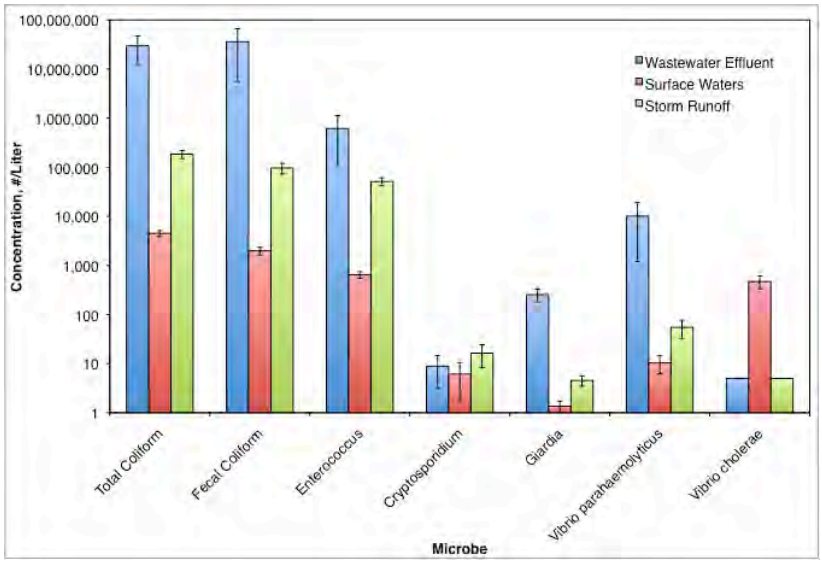


Figure 24. Mean concentrations of indicator bacteria and fecal pathogens from wastewater effluent, surface waters (streams and rivers) and storm runoff. Wastewater and streams and rivers were sampled in 2007 and 2008, whereas storm runoff was sampled in 2008 and 2010. Concentrations are not flow-weighted. Error bars indicate standard errors.

The concentration data for indicator bacteria and fecal pathogens were used with available flow data to calculate very rough estimates of loads from surface waters, storm runoff and wastewater. Load estimates for all three sources were subject to considerable uncertainty, due especially the absence of flow data for many sites. Of the 10 surface water sites that were sampled, only San Lorenzo River, Soquel Creek, Pajaro River, Salinas River, Carmel River and Big Sur River have readily available flow data (http://cdec.water.ca.gov/). Estimates of flow from storm runoff required assumptions about the proportion of runoff from the three sampled storm drains to total storm runoff around Monterey Bay. Estimated loads from wastewater did not consider periods when several of the dischargers have much reduced flows due to water reclamation efforts.

Table 19. Percent removal of indicator bacteria and fecal pathogens from wastewater during treatment in four wastewater treatment facilities in the Monterey Bay area. Red text indicates >99% removal efficiency.

	Percent Removal									
Site	TotColi[1] 100ml	FecColi[2] 100ml	Enteroco[3] 100ml	Crypto[4] 10L	Giardia 10L	Vibpar[5] 100ml	Vibchol[6] 100ml	Salm[7] 100ml	Fecal Pathogens	All
1	>99.99	>99.99	99.98	93.83	99.89	98.41	99.99	98.57	99.86	99.99
2	95.25	96.68	98.53	99.83	99.99	95.74	99.91	64.14	99.98	96.08
3	69.33	-1.76	81.64	99.90	99.95	56.44	99.90	58.57	99.94	65.24
4	99.32	99.25	97.63	99.59	99.85	>99.99	99.91	98.58	99.85	99.35
All	95.22	80.77	95.45	99.63	99.93	91.92	99.98	76.94	99.92	92.83

[1] = total coliform ; [2] = fecal coliform; [3] = *Enterococcus*; [4] = *Cryptosporidium*; [5] = *Vibrio parahaemolyticus*; [6] = *Vibrio cholerae*; [7] = *Salmonella*

The load estimates suggest that loads from surface waters and storm runoff, when combined, exceed those from wastewater for every fecal pathogen, except *Giardia* (Figure 25). Surface waters were the largest source of *Vibrio cholerae*. Nevertheless, estimated loads of fecal pathogens should not be the sole criterion for management actions to reduce fecal contamination in receiving waters. The location and mode of discharge from each source affect their respective probabilities of causing human and wildlife health concerns. For example, wastewater discharges are required to be located far from shore in deep water, such that high dilution of the discharge occurs before it reaches areas frequented by humans. Conversely, streams, rivers and storm drains discharge directly to nearshore regions resulting in very little dilution at the shoreline. Consequently, the similarity in loads of all fecal pathogens, except *Giardia* and *V. parahaemolyticus* suggest that efforts to make nearshore waters cleaner should focus on the two sources with direct discharges. Nevertheless, concerted efforts to generate more accurate load estimates could alter this conclusion.

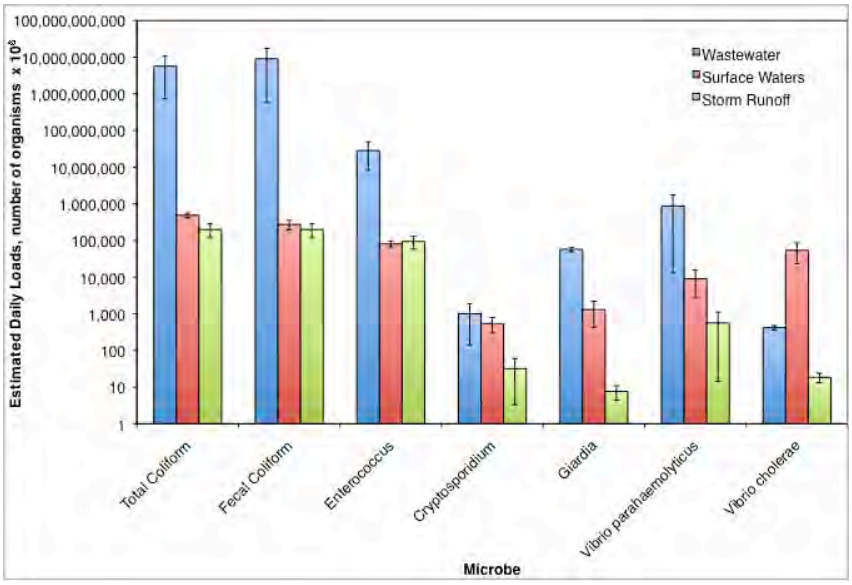


Figure 25. Mean daily loads of indicator bacteria and fecal pathogens from wastewater effluent, surface waters (streams and rivers) and storm runoff. Wastewater and streams and rivers were sampled in 2007 and 2008, whereas storm runoff was sampled in 2008 and 2010. Error bars indicate standard errors.

4.3.2.4 Recommendations

Assuming that fecal pathogens from different sources respond similarly to being discharged into ocean waters, the relative effects on human health and wildlife from each source should be proportional to the discharge locations and loads of fecal pathogens from each. Consequently, management actions to reduce fecal pathogen loads into the ocean should be based on improved load estimates, especially for surface waters and storm runoff, which will require concerted efforts to improve measurement of flow volumes from these two sources. Event-based sampling should be included to document flows and loads of fecal pathogens during high-flow events.

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Appendix A

Screening of Effects of Endocrine Disrupting Compounds with the 21-day Fathead Minnow Reproduction Assay: Evaluation of Wet and Dry Weather Samples

Screening of Effects of Endocrine Disrupting Compounds with the 21-day Fathead Minnow Reproduction Assay: Evaluation of Wet and Dry Weather Samples

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February 11, 2011

Final Report prepared for:
Central Coast Long-term Environmental Assessment Network (CCLEAN)

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INTRODUCTION

This report presents results from the screening of sewage treatment plant effluents in the Monterey Bay region for endocrine disrupting compounds (EDCs) using a short-term reproduction assay with fathead minnows (*Pimephales promelas*). This was a two-part screening, designed to capture wet and dry season effluent from the cities of Santa Cruz and Watsonville, the Monterey Regional Water Pollution Control Agency, and the Carmel Area Wastewater District wastewater treatment facilities. The wet season assay was conducted in the fall of 2009, and the dry season assay was conducted in the spring of 2010. This report was produced for the Central Coast Long-Term Environmental Assessment Network (CCLEAN) as an initial assessment of potential effects of EDCs on fish reproduction using effluents from CCLEAN participant facilities. The study, funded by the State Water Resources Control Board, will provide CCLEAN with data to be used in future management decisions.

Because of concerns over the potential effects of EDCs on reproduction and development of wildlife, the U.S. EPA has placed EDCs on the high priority research area list (USEPA, 1996). Studies in marine systems have documented endocrine disruption effects linked to sewage effluent in marine flatfish in California (Schlenk et al., 2005), Japan (Hashimoto et al., 2000) and the United Kingdom (Lye et al., 1997).

The UC Davis Marine Pollution Studies Laboratory (UCD-MPSL, Monterey, CA) conducted the assay following methods adapted from *A Short-term Test Method for Assessing the Reproductive Toxicity of Endocrine-Disrupting Chemicals Using the Fathead Minnow (Pimephales promelas) EPA/600/R-01/067* (USEPA, 2008). The tests measured the reproductive performance, morphology, and biochemical endpoints of groups of fathead minnows as indicators of potential endocrine disruption from the four treatment plant effluents. This report details the methods and results of the two five-week assays, including 14-day pre-exposure periods, 21-day effluent exposure periods, and 96-hour reference toxicant tests.

Test Description

The test begins with a 14-day pre-exposure period, designed to verify that all fish are in good reproductive health. This is followed by a 21-day exposure period, where effluents are introduced to the test aquaria, and a number of endpoints are monitored (Table 1). These include daily fecundity (measured as egg production), and fertilization success (monitored daily by quantifying cleavage in eggs), behavior (territorial posturing by females, loss of equilibrium, un-coordinated swimming, atypical quiescence, lack of feeding, hyperventilation), and appearance (color). Several other endpoints are enumerated at test termination (Day 21). These include development of secondary sex characteristics (presence of nuptial tubercles, measurement of dorsal fat pad, and coloration), weight of surviving fish, calculation of gonadosomatic index (GSI) and fat pad index (FPI), and enzyme-linked immunosorbent assay (ELISA) analysis of plasma vitellogenin.

The assay was modified for this project to omit two endpoints recommended in the EPA protocol: plasma sex steroid analysis and gonad histopathology. These analyses were deemed to

be optional in the scoping process for this project, and are outside of the expertise of UCD-MPSL. If significant differences were observed between test and control organisms in the assay, these analyses could be conducted to provide further lines of evidence. In the event that future analyses were to be performed to assess these endpoints, plasma and gonad samples were preserved at test termination. Plasma samples have since been discarded as storage beyond 30 days is not recommended. The gonad samples remain in storage at UCD-MPSL.

Table 1. Observations and Endpoints for the Assay

Pre-Exposure	Exposure	Termination
Survival	Survival	Survival
Fecundity	Fecundity	Weight
Fertility	Fertility	Plasma Vitellogenin
Hatch success	Hatch success	Gonadosomatic Index
	Behavior	Fat Pad Index
	Secondary Sex Characteristics	

METHODS

Exposure System

The EPA protocol was adapted to use a modified flow-through renewal system based on the system described in Martinovic et al. (Martinovic et al., 2007). Aquaria were set up in a randomized design as described in the EPA protocol, with four replicate aquaria for each treatment (effluent). Each aquarium (20 x 40 x 20cm) contained three 3-inch diameter PVC spawning substrates. During the pre-exposure period, heated UCD-MPSL well water was pumped continuously at a rate of 100 mL per minute, resulting in 14.4 renewals of each 10L exposure aquarium every 24 hours. Well water was warmed in a leached fiberglass holding tank, and was delivered using digital peristaltic pumps through leached silicone tubing.

Effluent samples were collected daily by each wastewater treatment facility during the 21-day exposure. Twenty-four-hour composites (after Martinovic et al., 2008) were collected in pre-cleaned amber glass containers. Chilled samples were transported each morning to UCD-MPSL for immediate dilution and introduction into the test system.

The effluent exposure was initiated by introducing diluted effluent into the aquaria so that final effluent concentrations would reflect ocean discharge dilution ratios (Santa Cruz 140:1, Watsonville 84:1, Monterey Regional 145:1, and Carmel 121:1). Effluents were initially diluted to the following concentrations in 20L glass carboys: Santa Cruz 36%, Watsonville 60%, Monterey Regional 34%, and Carmel 41%. Diluted effluents pumped at 2 mL/minute and well water pumped at 98 mL/minute were mixed in a T-fitting and delivered to each replicate aquarium to create the final discharge ratios. Flow rates were measured during pre-exposure and exposure periods to ensure maintenance of effluent dilutions (Appendix A).

Dissolved oxygen was measured daily, and pH, conductivity and ammonia were measured weekly. Water quality samples from each replicate were collected sub-surface and composited. Dissolved oxygen, pH, and conductivity were measured with a Fisher Scientific Accumet meter and appropriate electrodes (Fisher Scientific, Pittsburgh, PA). Un-ionized ammonia was measured using a Hach 2010 spectrophotometer (Hach, Loveland, CO). During the wet season test, water temperature was recorded with a continuous-recording HOBO-temp thermometer during the pre-exposure test and with a continuous recording Optical Stowaway during the exposure (both from Onset Computer Corporation, Pocasset, MA). During the dry season test, water temperature was recorded with a continuous-recording Optical Stowaway. Additional daily temperatures were measured using a glass spirit thermometer during both tests. The tests were conducted with a photoperiod of 16 h light and 8 h dark, per EPA protocol, with fluorescent lighting intensity of 10-20 $\mu E/cm^2$.

Each replicate aquarium was loaded with two males and four females. Eight extra aquaria were maintained during the pre-exposure for use as replacement aquaria in case of high mortality or low reproductive output in the experimental aquaria. Fish were fed wet flake food slurry twice daily *ad libitum*. Aquaria were cleaned daily with siphon tubes to remove waste and leftover food, as recommended in the EPA protocol.

Test Organisms and Distribution

Fathead minnows were obtained from commercial suppliers and held at UCD-MPSL for a minimum of two months before initiation of the pre-exposure test. All fish were separated by supplier source. Fish were maintained in communal tanks in flow-through UCD-MPSL well water at ambient well water temperature (~17 °C), and under fluorescent lighting (10-20 $\mu E/M^2/s$, 16 h light/8 h dark). Fish were initially fed dry flake food (Zeigler Bros. Inc., Gardiners, PA) and frozen brine shrimp twice daily, but the feeding regime was changed after observing some mortality in the culture tanks. The majority of the mortality was considered to be due to inability to swallow large food items and items that floated on the surface, resulting in food obstructing the passage to the air bladder and the fish to develop buoyancy regulation problems. Wet flake food slurry (*ad libitum*) was substituted and mortality was significantly reduced.

The origin of the fish used in the tests varied based on their availability from the various suppliers, and differed in the two tests. The wet season test used fish from three suppliers. Fish from Osage Catfisheries (Osage Beach, MO) and Aquatic Bio Systems (ABS, Fort Collins, CO) were used in the effluent test and fish from Aquatic Research Organisms (Hampton, NH) were used in the reference toxicant test. Osage fish arrived in two batches and were aged 10-months and 8-months, respectively, at initiation of the pre-exposure test. Fish from ABS arrived in one batch, and were aged 10 to11 months at the initiation of the pre-exposure test. ARO fish were 8.5 months old at the initiation of the reference toxicant test. Osage fish were distributed among the first two replicates of each effluent treatment, a mixture of Osage and ABS fish were used in the third replicate, and ABS fish were used in the fourth replicate (Table 2). Aquatic Bio Systems fish were used to supplement the limited number of Osage Catfisheries fish that exhibited clear external sexual characteristics at initiation of the pre-exposure test. At the end of the pre-exposure period (Day 15), two replicate aquaria which had exhibited low egg production

were replaced with two of the reserve aquaria that were maintained during the pre-test (Control Replicate 3, and Carmel Replicate 3).

Table 2. Distribution of fish among replicate wet season aquaria. Data below reflect replicates which were replaced by reserve aquaria at the end of the pre-exposure period, but do not identify mortalities that occurred during the pre-exposure period, as fish source could not be identified.

		Replicate			
Treatment	Sex	1	2	3	4
Control	♀	4 Osage	4 Osage	4 Osage	4 ABS
	♂	2 Osage	2 Osage	2 Osage	2 ABS
Santa Cruz	♀	4 Osage	4 Osage	4 ABS	4 ABS
	♂	2 Osage	2 Osage	2 Osage	2 ABS
Watsonville	♀	4 Osage	4 Osage	4 Osage	4 ABS
	♂	2 Osage	2 Osage	2 Osage	2 Osage
Monterey	♀	4 Osage	4 Osage	4 ABS	4 ABS
	♂	2 Osage	2 Osage	2 Osage	2 ABS
Carmel	♀	4 Osage	4 Osage	4 Osage	4 ABS
	♂	2 Osage	2 Osage	2 Osage	2 ABS

A mixture of fish from Osage and ARO were used in all replicates of the dry season test (Table 3). Osage fish were aged 8-months at initiation of the pre-exposure test and ARO fish were aged 11 months at the initiation of the pre-exposure test. Fish from both suppliers were used in the reference toxicant test. At the end of the pre-exposure period (Day 15), five replicate aquaria which had exhibited egg production below the 15 eggs/female/day criterion were replaced with five of the reserve aquaria that were maintained during the pre-exposure test (Control replicates 1 and 4, Watsonville replicates 2 and 4, and Monterey replicate 2).

Table 3. Distribution of fish among replicate dry season aquaria. Data below reflect replicates which were replaced by reserve aquaria at the end of the pre-exposure period, but do not identify mortalities that occurred during the pre-exposure period, as fish source could not be identified.

Treatment	Sex	Replicate 1	Replicate 2	Replicate 3	Replicate 4
Control	♀	2 Osage, 2 ARO	2 Osage, 2 ARO	2 Osage, 2 ARO	2 Osage, 2 ARO
	♂	1 Osage, 1 ARO	1 Osage, 1 ARO	1 Osage, 1 ARO	1 Osage, 1 ARO
Santa Cruz	♀	2 Osage, 2 ARO	2 Osage, 2 ARO	2 Osage, 2 ARO	2 Osage, 2 ARO
	♂	1 Osage, 1 ARO	1 Osage, 1 ARO	1 Osage, 1 ARO	2 Osage
Watsonville	♀	2 Osage, 2 ARO	1 Osage, 3 ARO	2 Osage, 2 ARO	2 Osage, 2 ARO
	♂	1 Osage, 1 ARO	1 Osage, 1 ARO	1 Osage, 1 ARO	1 Osage, 1 ARO
Monterey	♀	2 Osage, 2 ARO	2 Osage, 2 ARO	2 Osage, 2 ARO	2 Osage, 2 ARO
	♂	1 Osage, 1 ARO	1 Osage, 1 ARO	2 Osage	2 Osage
Carmel	♀	2 Osage, 2 ARO	2 Osage, 2 ARO	2 Osage, 2 ARO	2 Osage, 2 ARO
	♂	1 Osage, 1 ARO	1 Osage, 1 ARO	2 Osage	2 Osage

Reference Toxicant Test

Sodium chloride (NaCl) reference toxicant tests were conducted synoptically with both wet and dry season effluent exposures. These positive control tests were conducted under similar conditions as the effluent exposures, but were 96-hour static-renewal tests, with a single renewal of the test solution at 48 hours. The wet season test concentrations were 0, 1, 1.8, 3.2, 5.6, 10, and 18 g/L, and the dry season concentrations were 0, 3.2, 5.6, 10, 18, and 32 g/L. Reagent grade NaCl was diluted into MPSL-UCD well water to prepare the test solutions. Each concentration was tested in replicates of two aquaria, with 4 fish per replicate. Fish were fed minimally with wet flake food slurry to avoid fouling the aquaria.

Observations and Endpoints

Fecundity and Fertilization Success

Fecundity was determined on the basis of number of females per reproductive day for each test aquarium (4 females for 21 days = 84 reproductive days). Successful reproductive performance for controls during the pre-exposure and exposure periods required a minimum of 15 eggs/female/day. Eggs were removed from spawning substrates and counted daily. Percent fertilization was determined by examining a sub-sample of 100 eggs under the microscope and quantifying cleavage.

Occasionally the eggs were very soft and would rupture when removed from the substrates. We assumed that these eggs were recently spawned and had not yet hardened. During the wet season test these eggs were scraped into the bowl after counting, but the soft eggs would appear deflated and were not evaluated for fertilization. The fertilization counts would be made using any hard eggs that were present. For the dry season test the eggs were counted and the fertilization evaluation was omitted.

Behavior and Appearance

Observations of fish behavior and appearance were recorded daily during the pre-exposure and exposure. Territorial behavior, such as defense of spawning substrate was noted. Observations of appearance included alterations in secondary sex characteristics such as nuptial tubercles, dorsal fat pad, darkened coloration and vertical bands in males, and distended abdomen and swollen ovipositor in females. Nuptial tubercles and dorsal fat pads were measured as part of the final endpoint quantification. The other physical appearance endpoints were noted as part of the testing procedure while the fish were in the replicate aquaria. Prior to initiation of test termination, fish demonstrating deviations from normal appearance were noted.

Nuptial Tubercle Scores

Each fish was examined under magnification, and the number, size (value of 1-3), and location of nuptial tubercles were recorded and compiled into a total score, per EPA protocol. Abnormal appearance of any tubercles (i.e. sunken) was noted.

Weight

A representative subset of fish was weighed to the nearest 0.01g at the initiation of the pre-exposure period. Initial weights were determined using weighted averages per aquaria. Wet weights of all surviving fish were recorded to the nearest 0.01g at test termination after blood was sampled. Blood weight was not determined, as less than one milliliter of blood was collected from each fish. Although larger fish had larger blood volumes, the weight of the blood was considered minimal relative to the weight of the fish, and the EPA protocol does not require adjustments to the net weight based on blood loss.

Gonadosomatic Index (GSI)

The gonadosomatic index (weight of the gonads relative to the total body weight of the fish) can be used to assess the reproductive status of the fish. At test termination, after wet weight was measured, gonads were removed and weighed to the nearest 0.1 mg. GSI was calculated using the following formula: GSI = (gonad weight/body weight) x 100. Gonads were preserved with Davidson's fixative and stored for potential future histological analysis per recommendations by Jeff Wolf at Experimental Pathology Laboratories, Inc. Had the histopathology endpoint been pursued, this laboratory would likely have conducted the analysis.

Dorsal Fat Pad Index (FPI)

Each fish was examined for the presence of a dorsal nape fat pad according to EPA protocol. When present, the fat pad was scored on a size scale of 1 (no fat pad visible) to 5 (very prominent, overhanging body surface). The fat pad was then removed and weighed to the nearest 0.01g. The FPI was calculated using the following formula: FPI = (fat pad weight/body weight) x 100.

Embryo Hatch Success

Hatch success, an optional endpoint of the EPA protocol, was recorded for each replicate aquarium during the pre-exposure and exposure period. Twenty eggs were selected from a single spawn and were placed individually in vials filled with well water. Embryos were maintained under the same temperature and lighting conditions as the test, and percent hatch success was recorded for each replicate.

During the wet season test, egg collection for determining the pre-exposure hatch success did not begin until the second week of the pre-exposure. There was not enough time to ensure that enough eggs could be collected with time to hatch before beginning the exposure period. One replicate each from the control, Santa Cruz, and Watsonville had fewer than twenty eggs available for determining hatch success. When a total of twenty eggs were not available from a single spawn, fewer eggs were collected and percentages were calculated based on total number. Pre-exposure hatch success from one Santa Cruz replicate and one Watsonville replicate were not measured. During the exposure period, hatching was not measured in one Watsonville replicate because no eggs were available before the end of the exposure. During the dry season test, pre-exposure hatch success was not recorded for three replicates. Two replicates had

insufficient egg production, and were replaced for the exposure test. The third replicate had sufficient egg production, but embryos were not isolated.

Plasma Vitellogenin

At test termination fish were anaesthetized with MS-222, and the caudal peduncle was partially severed with micro-dissection scissors. Blood was collected from the caudal vein with a heparinized microhematocrit capillary tube and transferred to a centrifuge tube. Vitellogenin is an unstable molecule and requires immediate processing to accurately capture plasma concentrations. Plasma was immediately isolated by centrifugation for 3 min at 14,000 rpm and stored at -80ºC until analysis.

Plasma samples were assayed by ELISA using the commercially available quantitative fathead minnow vitellogenin assay kit per manufacturer's instructions (Biosense Laboratories, Inc., Bergen, Norway). The kit uses specific binding between antibodies and vitellogenin to quantify the vitellogenin in the plasma. Vitellogenin standards ranged from 0.05 ng/mL to 50 ng/mL, but not all standard absorbance values are used in each standard curve. Absorbance values at either end of the range can be dropped to improve the curve fit and confidence in the measurements (as per Biosense instructions). Absorbance values and concentrations are plotted on log scales with a power curve fit. The equation from the curve fit was used to calculate vitellogenin concentrations from the sample absorbance values. Plasma samples were serially diluted 50, 5000 and 500,000 times with buffer, and all dilutions were analyzed in duplicate. Sample dilutions are prepared serially by introducing 10 μL of plasma to 490 μL of buffer to create a 50:1 dilution. The 5,000:1 dilution is prepared by adding 10 μL of 50:1 to 990 μL of buffer, and the 500,000:1 dilution is prepared by adding 10 μL of 5,000:1 to 990 μL of buffer. Once the vitellogenin concentrations were calculated using the absorbance value and the calibration curve equation, the concentration was multiplied by the dilution factor to determine the final concentration.

The vitellogenin samples were analyzed in two batches per test. Mean absorbance values for the wet season analysis events were corrected for the background absorbance of the buffer used to prepare the standards and make dilutions (non-specific binding - NSB). A mean NSB absorbance value was used for the correction. The mean NSB absorbance values in the dry season analysis events were approximately twice as high as those of the wet season events for unknown reasons. All methods and procedures were exactly the same in all analyses. Low standard concentrations that were prepared with this buffer had absorbance values that were lower than background. When NSB-corrected values were used to create calibration curves, the range of usable standards was narrow, and the number of valid results would have been limited. For this reason, the calibration curves in the latter two events were calculated using the mean absorbance values (as per the recommendation of Biosense).

Sample dilutions with absorbance values outside of the calibration range were not used. Once the acceptable absorbance values are identified, the coefficients of variation (CVs) were examined to determine if there was excessive variability between the duplicate measurements. Although Biosense recommends using absorbance values with CVs less than 10%, four calibrators with CVs greater than 10% were used to create calibration curves. Among all of the

analysis events there were several instances where data were reported even though sample duplicates had CVs greater than 10%. The decision to report these data was based on whether or not there were additional acceptable dilutions with lower CVs, and best professional judgment of the magnitude of the CV and whether or not the absorbance values were within a factor of two from each other. Whenever possible, the lowest dilution with acceptable absorbance values was used to calculate the final vitellogenin concentration. Most of the vitellogenin measurements for females were out of the calibration range, as the assay is intended to capture lower than normal levels for females and higher than normal levels in males.

Statistical Analysis

Data were analyzed following the guidance provided in the EPA protocol. Because the samples were tested in single concentrations, analysis of variance was replaced with separate-variance t-tests conducted between individual treatments (effluents) and controls. The mean fecundity endpoint was calculated using data collected daily during the exposure period; all other data was collected at test termination. Fecundity was also compared using a separate-variance t-test conducted between the pre-exposure and exposure periods, to represent changes based on effluent exposure. Fecundity data from the pre-exposure period were calculated after an acclimation period of seven days, per guidance in the EPA protocol.

RESULTS and DISCUSSION

Quality Assurance

A number of criteria need to be met for tests to be considered acceptable. These include acceptable survival (>90%), active spawning (>15 eggs/female/day), high rate of fertility and hatching (>90%), consistent exposure concentrations, and water quality parameters that are within acceptable limits.

All of the quality assurance criteria were met. Percent survival in the wet season pre-exposure control was 100%, the spawning rate was 29 eggs/female/day, fertility was 96%, and hatching success was 96%. During the wet season effluent exposures, control survival was 100%, spawning rate was 19 eggs/female/day, fertility was 97%, and hatching success was 99%. Percent survival in the dry season pre-exposure control was 96%, the spawning rate was 42 eggs/female/day, fertility was 90%, and hatching success was 97%. During the dry season effluent exposures, control survival was 100%, spawning rate was 41 eggs/female/day, fertility was 96%, and hatching success was 98%.

There are no specific criteria for the consistency of the effluent flows, but throughout the five-week wet season test, the 98 mL/minute dilution water flow rates were within 7% of target and the 2 mL/minute effluent flow rates were within 5% of target. Throughout the five-week test dry season test, the 98 mL/minute dilution water flow rates were within 6% of target and the 2 mL/minute effluent flow rates were within 20% of target.

Fish used in each of the effluent exposures were composed of organisms from two different commercial suppliers, and fish used in the reference toxicant test for the wet season test were

from a third commercial supplier. The use of fish from different sources was necessitated by a lack of available fish during the period in which fish were ordered. The use of fish from different sources did not appear to have implications for the general health or reproductive potential of the fish culture as a whole. All fish ranged from 8 -11 months at test initiation, and were maintained in the same culture facility at UCD-MPSL for at least 2 months prior to testing. This ensured that all fish cultures had the same water, temperature, lighting, and feeding conditions prior to test initiation.

Table 4: Wet season and dry season test mean (standard deviation) and range of water quality parameters measured during the combined pre-exposure and exposure periods.

	DO (mg/L)		pH		Conductivity (uS/cm)		Unionized NH_3 (mg/L)		Hardness (mg/L)		Alkalinity (mg/L)	
Treatment	Mean (SD)	Range	Mean (SD)	Range	Mean (SD)	Range	Mean (SD)	Range	Mean (SD)	Range	Mean (SD)	Range
Wet Season												
Control	6.79 (0.80)	6.14-8.21	7.87 (0.20)	7.58-8.13	716 (47)	623-745	0.70 (0)	ND-0.4	166 (11)	152-181	131 (15)	114-154
Santa Cruz	6.65 (0.71)	5.81-7.67	8.03 (0.08)	7.95-8.13	711 (47)	617-740	0.27 (0.31)	ND-0.7	159 (5)	154-164	123 (10)	109-136
Watsonville	6.76 (0.69)	5.97-7.85	8.03 (0.08	7.95-8.18	710 (46)	618-743	0.60 (0.08)	ND-0.6	156 (5)	151-165	123 (14)	109-143
Monterey	6.97 (0.62)	6.12-7.91	8.12 (0.07)	8.01-8.22	711 (48)	614-742	0.53 (0.39)	ND-0.7	155 (7)	149-168	119 (10)	108-130
Carmel	6.90 (0.77)	5.96-8.07	8.11 (0.12)	7.92-8.23	726 (46)	642-768	0.40 (0)	ND-0.9	161 (5)	154-167	126 (13)	109-140
Dry Season												
Control	6.26 (0.83)	4.43-8.50	8.12 (0.18)	7.81-8.33	831 (39)	804-889	0.0 (0)	NA	184 (17)	168-209	130 (6)	126-139
Santa Cruz	6.31 (0.78)	4.43-8.50	8.11 (0.17)	7.89-8.33	817 (42)	782-883	0.20 (0.45)	ND-1.0	214 (57)	174-296	132 (7)	124-138
Watsonville	6.28 (0.76)	4.43-8.50	8.13 (0.14)	7.97-8.33	818 (41)	780-878	0.18 (0.25)	ND-0.5	188 (15)	178-209	136 (15)	124-158
Monterey	6.12 (0.80)	4.43-8.50	8.12 (0.13)	8.01-8.33	814 (43)	778-882	0.30 (0.42)	ND-0.9	183 (20)	162-209	130 (3)	126-134
Carmel	6.29 (0.76)	4.43-8.50	8.17 (0.11)	8.06 - 8.33	821 (64)	734-911	0.14 (0.31)	ND-0.7	195 (11)	183-209	126 (8)	119-136

Acceptable ranges: D.O. >4.9 mg/L; pH = 6.5-9.0; Alkalinity >20 mg/L; Unionized NH_3<4.7 mg/L

All WQ parameters were within range of the test criteria (Table 4). Water temperatures during the wet season pre-exposure period were lower than the recommended range, but were increased by the time the exposure was initiated. Mean water temperature was 22.8 (±1.6°C) during the pre-exposure period and 24.3 (±1.1°C) during the exposure period. The wet season test was conducted during a period of extremely low ambient temperatures and this affected the temperature of the well water used for the test. The capacity of the water heating system was increased in order to raise the temperature to meet the protocol specification of 25 (±1°C). Fecundity and fertilization levels were all above minimum test requirements during the period with the coolest temperatures, so the lower temperatures did not appear to have an adverse effect on spawning activity or success.

Reference Toxicant Tests

In the wet season reference toxicant test, complete mortality was observed in the highest concentration of sodium chloride (18 g/L). All other concentrations had complete survival with normal swimming behavior and appearance. The calculated median lethal concentration (LC50) was 14 g/L, and was slightly higher than the range of threshold concentrations reported in the literature. An additional concentration was added for the dry season test and complete mortality was observed in both the 18 and 32 g/L concentrations. All lower concentrations had partial or complete survival, and the calculated LC50 was 4.61 g/L.

The reference toxicant test stipulated in the EPA protocol is designed to test the overall health of the test organisms, in order to ensure that underlying poor health is not a factor in the organism response to exposure treatments. The use of NaCl as the toxicant in these reference tests is not meant to represent or indicate sensitivity to endocrine disrupting compounds. The calculated median lethal concentrations of 14.0 g/L and 4.61 g/L indicate the reference toxicant test organisms had comparable sensitivity to NaCl as other test populations.

Survival

Survival in the pre-exposure and exposure phases of both wet and dry season tests was greater than 92% (Table 5). There were no significant differences between the survival of the individual effluent treatments and those of the control. In the wet season test two reserve aquaria were substituted for treatment aquaria based on low fecundity and percent survival, and five reserve aquaria were substituted for treatment aquaria in the dry season test. All other reserve aquaria were discontinued at the end of the pre-exposures. Due to mortalities that occurred during the pre-exposure period¸ five of the twenty replicates in the dry season test contained five, rather than six fish, at initiation of the effluent exposure. These aquaria were distributed among the effluent treatments based on their pre-exposure fecundity data.

Fecundity and Fertilization

Mean fecundity (as measured by egg production/female/day) varied considerably during pre-exposure and effluent exposure periods of both tests. Although there was high among-replicate variability, the output of eggs in all treatments and the control exceeded the 15 eggs/female/day test criterion except for the Santa Cruz effluent in the dry season test.

In the wet season test, there were no significant differences between the effluent exposure fecundity of the individual treatments and that of the control (Table 6). There was a significant decrease in fecundity between the pre-exposure and effluent exposure periods in the controls, and although there were slight increases and decreases in the treatments between these periods, none of these differences were statistically significant.

In the dry season test, there was a significant difference between the Santa Cruz effluent exposure fecundity and that of the control ($p = 0.008$), but there was no significant decrease in fecundity between the pre-exposure and effluent exposure periods in the Santa Cruz effluent, or any other effluent (Table 6). Although the fecundity during the pre-exposure exceeded the test acceptability criterion, fish used for the Santa Cruz exposures had a lower mean fecundity than

the other treatments throughout the pre-exposure and effluent exposure periods. Based on the low fecundity in the Santa Cruz replicates during the pre-exposure period, the low fecundity observed during the effluent exposure may not represent endocrine disruption caused by exposure to effluent.

Table 5. Mean (standard deviation) percent survival of fathead minnows in wet and dry season pre-exposure and exposure periods.

	Pre-Exposure		Exposure		
Treatment	Mean	SD	Mean	SD	Mortalities during Effluent Exposure
Wet Season					
Control	100	0	100	0	None
Santa Cruz	100	0	96	9	1 Male, Rep 4, Day 4
Watsonville	100	0	92	10	1 Male, Rep 3, Day 11; 1 Female, Rep 2, Day 19
Monterey	100	0	100	0	None
Carmel	100	0	92	10	1 Male, Rep 1, Day 18; 1 Male, Rep 2, Day 20
Reserve	98	6			NA
Dry Season					
Control	96	9	100	0	None
Santa Cruz	96	9	100	0	None
Watsonville	90	13	100	0	None
Monterey	96	9	96	9	1 Female, Rep 4, Day 15
Carmel	96	9	100	0	None
Reserve	96	8			NA

All pre-exposure and exposure treatments had mean percent fertilization greater than 90% with the exception of the Monterey pre-exposure replicates from the dry season test (Table 6). One of the Monterey replicates was replaced for the effluent exposure and percent fertilization was increased to 93%. Fertilization did not vary between the pre-exposure and effluent exposures or between the two tests. Fertilization also did not vary between the effluent treatments and the control.

Table 6. Mean (standard deviation) number of eggs produced per female per day (fecundity) and mean (standard deviation) percentage fertilized in wet and dry season tests. * indicates significant differences between treatments.

	Fecundity (eggs/female/day)				Percent Fertilized				Fecundity T-Test Probability	
	Pre-Exposure		Effluent-Exposure		Pre-Exposure		Effluent - Exposure		Effluent vs.	Exposure vs.
Treatment	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Control	Pre-Exposure
Wet Season										
Control	33	10	19	8	97	3	97	2	NA	0.034*
Santa Cruz	21	4	22	11	97	2	92	10	0.311	0.397
Watsonville	22	8	17	7	97	2	93	13	0.362	0.186
Monterey	29	21	28	16	96	3	96	5	0.183	0.485
Carmel	37	14	33	15	94	5	95	5	0.079	0.361
Dry Season										
Control	35	18	33	8	90	17	96	8	NA	0.361
Santa Cruz	19	9	10	10	94	11	92	14	0.008*	0.120
Watsonville	35	19	35	31	92	15	95	14	0.401	0.498
Monterey	29	15	25	15	89	15	93	10	0.254	0.348
Carmel	27	13	21	14	97	4	92	19	0.134	0.274

Fish Appearance and Behavior, and Quantification of Nuptial Tubercles

While there is variability between individuals, mature male fathead minnows typically have darker dorsal areas with dark vertical bands. Female fish are usually uniformly lighter in color.

In the wet season test, there were no changes in male appearance (coloration and vertical banding) over the course of the test, although one male from Watsonville and several males from Monterey and Carmel maintained light coloration and lack of banding throughout the test (Table 7). One female fish from the Monterey treatment exhibited a slightly dark coloration and vertical banding during Days 6-21, which is atypical of females. Two females from the Santa Cruz treatment had light coloring typical of lighter male fish, but no banding or tubercles. No other females exhibited male coloration, banding, or tubercles. Control and treatment males had variable levels of dark coloration and banding, with no clear patterns emerging per treatment.

Territorial behavior appeared normal for all males during the pre-exposure and exposure periods, with males exhibiting aggressive defense of spawning substrates. Larger males were typically more aggressive than smaller males, and in aquaria where one male was noticeably larger than the other, the larger male appeared to dominate the aquarium. Pairings of larger males with smaller males likely led to the observed mortality of males in some of the replicate aquaria. These fish had signs of fighting such as loss of skin in the caudal peduncle, and chewed tail and pectoral fins. One female fish from the Monterey treatment, which exhibited darker coloration and vertical bands characteristic of males, was observed regularly defending spawning substrates

in a territorial manner during Days 7-21 of the exposure period. This female was observed in a replicate containing two males.

Individual tubercle scores were variable within treatments, and there were no significant differences between the control and the individual treatments. The mean tubercle score for the control (17.6 ± 10.4) was lower than literature values for unexposed males (31.2 ± 8.0, Watanabe et al., 2007). Two fish from the Monterey treatment exhibited low scores of 7 and 9, and some tubercles on these fish were sunken, indicating that they were smaller than the lowest rating level, but still visible.

Table 7. Summary of coloration, banding, and nuptial tubercle scores for wet season fish. D, SD and L indicate dark, slightly dark and light, respectively. Y and N indicate yes and no. * indicates that tubercles appeared sunken. ** indicates female fish with male coloration.

	Control			Santa Cruz			Watsonville			Monterey			Carmel		
Male	Color	Bands	Tuber.	Color	Bands	Tuber.	Color	Bands	Tuber.	Color	Bands	Tuber.	Color	Bands	Tuber.
1	D	Y	25	D	Y	48	D	Y	24	D	N	22	D	Y	25
2	L	N	21	D	Y	30	D	Y	28	SD	Y	22	L	N	31
3	D	Y	10	D	Y	20	D	Y	29	L	N	24	L	Y	35
4	D	Y	25	SD	N	21	SD	N	15	L	N	*7	L	N	11
5	SD	Y	6	SD	N	21	L	N	8	D	Y	17	L	N	15
6	SD	Y	35	SD	N	10	D	Y	26	L	Y	*9	L	N	10
7	SD	N	8	SD	N	24	SD	Y	12	D	Y	19			
8	SD	N	11							L	N	15			
Mean			17.6			24.9			20.3			16.9			21.2
Female															
1				L	N	0				SD**	Y**	0			
2				L	N	0									

All dry season control males exhibited dark coloration and clear vertical bands at test termination (Table 8), and all control females had light coloration with no vertical banding. Males with lighter coloration occurred in all effluent treatments except Santa Cruz. All effluent treatments had at least one male without clear vertical banding.

Two female fish from the Santa Cruz treatment exhibited darker coloration during the exposure period, and one of these females also had clear vertical banding at test termination. This female exhibited territorial behavior during the exposure period, aggressively defending the hatching substrates in a replicate that contained two males. This coloration and behavior is atypical of females, and could be an indicator of endocrine disruption. No females in any other treatment exhibited male coloration, banding, or territorial behavior. Territorial behavior appeared normal for males in all treatments during the pre-exposure and exposure periods. Nuptial tubercles were not observed on females in any of the treatments.

Individual tubercle scores were variable within treatments. The mean tubercle score for the control (23.5 ± 6.7) was comparable to literature values for unexposed males (31.2 ± 8.0, Watanabe et al., 2007). Male fish from all of the effluent treatments had lower mean tubercle scores than the control males. Watsonville, Monterey, and Carmel each had a male with no

tubercles present. One male from the Santa Cruz treatment exhibited a low score of 6. The absence or reduced number of tubercles could be an indication of endocrine disruption.

Table 8. Summary of coloration, banding, and nuptial tubercle scores for dry season fish. D, SD and L indicate dark, slightly dark and light, respectively. Y and N indicate yes and no. * indicates that tubercles appeared sunken. ** indicates female fish with male coloration.

	Control			Santa Cruz			Watsonville			Monterey			Carmel		
Male	Color	Bands	Tuber.	Color	Bands	Tuber.	Color	Bands	Tuber.	Color	Bands	Tuber.	Color	Bands	Tuber.
1	D	Y	27	SD	Y	6	L	N	15	L	N	16	L	N	0
2	D	Y	12	SD	Y	11	D	Y	21	D	Y	23	L	N	13
3	D	Y	24	SD	Y	23	ND	ND	15	D	Y	15	SD	N	16
4	D	Y	19	D	Y	20	L	N	16	SD	N	17	D	Y	41
5	D	Y	30	SD	N	19	D	Y	16	SD	Y	14	L	N	19
6	D	Y	24	D	Y	34	SD	N	0	L	N	0	L	Y	42
7	D	Y	33	SD	Y	38				L	N	26	L	N	29
8	D	Y	19							L	N	24			
Mean			23.5			21.6			13.8			16.9			22.9
Female															
1				D	N	NA									
2				SD	Y	NA									

Weights

Initial weights for the different treatments varied based on the origin of the fish (Table 9). In the wet season test the mean initial weights of the control fish were 8.20 (± 1.6g) and 2.98 (± 0.61g) for males and females, respectively. The mean initial weights of the control fish in the dry season test were 8.91 (± 1.63g) and 5.33 (± 1.01g) for males and females, respectively. Fish used in this study were approximately five months older than the minimum reproductive age required in the EPA protocol, and had a greater mass than previously reported studies. Watanabe et al. (2007) list the average weights for unexposed fathead as 4.20 (± 1.04g) and 1.61 (± 0.34g) for males and females, respectively.

In both tests, mean weights were lower at test termination and comparable between tests for females. Males lost more weight during the dry season test. Weight loss could be attributed to egg production, competition for food, and fish being weighed at test termination after blood was removed. There were no significant differences between the final weights of the individual treatments and that of the control in the wet season test, but the final dry season weights of the male fish from the Santa Cruz effluent were significantly higher than those of the control ($p = 0.031$). However, weights that are higher than the control are not considered an indication of endocrine disruption.

Table 9. Mean (standard deviation) initial and final weights wet season male and female fish.

Treatment	Sex	Initial Weight (g) Mean	Initial Weight (g) SD	Final Weight (g) Mean	Final Weight (g) SD	Growth T-Test Probability
Wet Season						
Control	Male	8.20	1.60	5.28	0.88	NA
	Female	2.98	0.61	2.36	0.17	NA
Santa Cruz	Male	8.20	1.60	6.24	0.33	0.186
	Female	2.69	0.70	2.11	0.28	0.257
Watsonville	Male	9.10	1.46	5.76	0.52	0.252
	Female	2.98	0.61	2.14	0.30	0.153
Monterey	Male	8.20	1.60	4.68	1.53	0.153
	Female	2.69	0.70	2.10	0.36	0.191
Carmel	Male	8.20	1.60	4.57	0.57	0.457
	Female	2.98	0.61	2.15	0.09	0.243
Dry Season						
Control	Male	8.91	1.63	6.16	0.63	NA
	Female	5.33	1.01	2.66	0.11	NA
Santa Cruz	Male	8.91	1.65	7.43	0.42	0.031 *
	Female	5.41	1.09	2.70	0.22	0.389
Watsonville	Male	9.06	1.74	6.54	0.70	0.074
	Female	5.33	1.01	2.92	0.11	0.096
Monterey	Male	8.91	1.65	7.13	0.88	0.141
	Female	5.50	1.17	2.91	0.07	0.120
Carmel	Male	8.91	1.65	7.11	1.06	0.487
	Female	5.50	1.17	2.57	0.18	0.260

Gonadosomatic Index (GSI)

Wet season GSI values ranged from 1.15 to 1.52% for males and 13.56 to 15.28% for females (Table 10). The dry season values were similar with range of 1.29 to 1.79% for males and 12.13 to 17.70% for females. Wet season males from the Monterey exposure had significantly larger gonads than the controls. Dry season males from the Santa Cruz and Carmel exposures, and females from the Watsonville exposures, also had significantly larger gonads than the controls.

A larger GSI value for the wet season Monterey males was not considered an indicator of endocrine disruption, particularly since fecundity for these fish was higher than the controls. Dry season fecundity for Santa Cruz and Carmel were lower than the controls, but endocrine disruption was not suspected because the gonads were significantly larger. Although the GSI values were significantly greater than the control, typical GSI values for the fathead minnow range from 1 to 2% for males and 8 to 13% for females (Smith, 1978; Jensen et al., 2001).

Fathead minnow ovaries can undergo rapid cyclical changes as successive batches of eggs are produced, and female GSI values can vary significantly between individuals based on their

spawning interval. However, standard deviation in female GSI values was low (1.78 - 4.17), indicating that this was not a factor for this test.

Table 10. Mean (standard deviation) gonadosomatic index (GSI) for male and female fish. * indicates significant difference between sample GSI and control GSI ($\alpha = 0.05$).

		Wet Season			Dry Season		
		Gonadosomatic Index (%)		GSI T-Test	Gonadosomatic Index (%)		GSI T-Test
Treatment	Sex	Mean	SD	Probability	Mean	SD	Probability
Control	Male	1.15	0.12	NA	1.32	0.17	NA
	Female	14.74	2.67	NA	12.13	3.60	NA
Santa Cruz	Male	1.29	0.33	0.224	1.61	0.23	0.048 *
	Female	13.56	2.20	0.260	14.84	4.17	0.182
Watsonville	Male	1.52	0.40	0.080	1.29	0.25	0.411
	Female	15.28	3.93	0.415	17.70	3.49	0.034 *
Monterey	Male	1.45	0.20	0.023*	1.40	0.21	0.286
	Female	13.77	2.66	0.313	14.69	1.78	0.133
Carmel	Male	1.17	0.33	0.438	1.75	0.22	0.013 *
	Female	15.19	2.23	0.404	12.95	1.96	0.353

Dorsal Fat Pad Index (FPI)

The mean wet season FPI for control males was 2.04%, which was lower than all mean treatment values (Table 11). Index values of unexposed males in Watanabe et al. (2007) were 3.99%. Monterey and Carmel FPIs were significantly higher than the control values, at 4.68 and 4.57, respectively. Two female fish from the Santa Cruz treatment developed dorsal fat pads during the exposure period, with a mean FPI of 0.82%. Fat pad development is atypical for female fish, and can be an indicator of endocrine disruption.

The mean dry season FPI for control males was 2.47%, and was also lower than all mean treatment values. There were no significant differences between control and treatment FPI values in the dry season test and no female fish from the control or treatments developed dorsal fat pads during the exposure period.

Table 11. Mean (standard deviation) fat pad index (FPI) for male and female fish.

		Wet Season			Dry Season		
		Fat Pad Index (%)		FPI T-Test	Fat Pad Index (%)		FPI T-Test
Treatment	Sex	Mean	SD	Probability	Mean	SD	Probability
Control	Male	2.04	0.91	NA	2.47	1.19	NA
	Female	NA	NA	NA	NA	NA	NA
Santa Cruz	Male	4.58	0.30	0.106	3.22	2.74	0.321
	Female	0.82	0.32	NA	NA	NA	NA
Watsonville	Male	2.83	0.24	0.239	3.02	2.39	0.349
	Female	NA	NA	NA	NA	NA	NA
Monterey	Male	4.68	1.53	0.028*	2.93	1.08	0.294
	Female	NA	NA	NA	NA	NA	NA
Carmel	Male	4.57	0.57	0.016*	3.93	1.70	0.107
	Female	NA	NA	NA	NA	NA	NA

Embryo Hatching Success

The mean percent hatching success for all treatments in both periods of both tests was greater than 96% (Table 12). There was no significant difference in hatch success between controls and individual treatments in either the wet or the dry season tests.

Table 12. Mean (standard deviation) percent hatching success of embryos during the pre-exposure and exposure periods

	Wet Season				Dry Season			
	Pre-Exposure		Effluent Exposure		Pre-Exposure		Effluent Exposure	
Treatment	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Control	96	8	99	3	97	6	98	3
Santa Cruz	97	6	100	0	100	0	100	0
Watsonville	98	5	98	3	100	0	100	0
Monterey	100	0	100	0	100	0	100	0
Carmel	100	0	100	0	100	0	100	0

Plasma Vitellogenin

Analysis Issues

Several difficulties were encountered when conducting the ELISAs for vitellogenin. Some of these issues might be related to the use of the Biosense kits, whereas others might be related to

interfering factors in the samples. Although all appropriate procedures were followed, the data that were produced were highly variable and render quantitative data interpretation impossible.

The first issue encountered was the high buffer solution absorbance values measured in the dry season test. The elevated absorbance values were only observed in the blanks, which consisted of straight buffer preparation. The buffer was used to create the calibrators and dilute the plasma samples, but lower absorbance values were measured in many of these preparations, when compared to the blanks. The high background absorbance values created the need to generate the calibration curves in an unorthodox manner without background correction. The calibrations were conducted under the advisement of Biosense technical staff, but they could not provide a reason for the elevated absorbance in the blanks. Although elevated absorbance values in the blanks slightly altered the final concentrations of vitellogenin, they did not explain the high variability observed throughout all of the analysis events.

The second issue encountered was a lack of proportional dilution among the three plasma dilutions prepared for each sample. The first dilution is prepared by adding a small amount of plasma to a large proportion of buffer, and the subsequent dilutions are prepared serially from the first dilution. A wide range of dilutions is prepared to span the potential vitellogenin concentrations that could be measured in the samples. Similarly, a wide range of calibrators are prepared to span potential measured concentrations. As per the Biosense methods, several upper and lower-end calibrators are discarded to improve the curve fit, and only one or two of the sample absorbance values should fit within the absorbance range of the calibrators. We would expect to see a hundred-fold difference in concentrations between these samples, but we observed much lower and much more variable differences. Biosense was not able to provide guidance on this issue, but EPA researchers were consulted regarding this and other issues and suggested that absolute linearity among the dilutions was not to be expected based on the large dilution factors. The calibrators were also prepared with a serial dilution, but the proportions were on the order of 2:1 instead of 100:1. All of the absorbance values from the calibrators were proportional, suggesting that the cause of the non-proportional dilutions in the samples originated with the plasma.

Vitellogenin induction in male fish provides evidence of exposure to EDCs. Test control fish that originated from uncontaminated environments, held in contaminant-free culture water, and exposed in the same control water would be expected to have very low concentrations of vitellogenin. Although reported values in the literature vary among studies, there can be orders of magnitude differences between unexposed males and females. Vitellogenin concentrations in unexposed males range from < 10 ng/ml (Thorpe et al., 2007) to 20,000 ng/ml (Watanabe et al., 2007). Concentrations in unexposed females can range from approximately 400,000 ng/ml (Thorpe et al., 2007) to 17,400,000 ng/ml (Jensen et al., 2001, 2007; Watanabe et al., 2007). At the suggestion of Dr. Gerald Ankley, Kathleen Jensen at the U.S. EPA Duluth laboratory was consulted regarding some of the issues regarding the ELISA measures of VTG in these assays. She suggested that she would expect VTG in control male fish to be near the detection limit of the Biosense ELISA kits. This was clearly not the case with some of the control male fish.

Based on the data from the other endpoints, such as fecundity, GSI, and behavior, little or no endocrine disruption and low vitellogenin concentrations were expected. Regardless, a number

of vitellogenin concentrations greater than 20,000 ng/mL were measured in male fish in both tests, but in the wet season test one concentration was greater than 100,000 ng/mL and two concentrations were greater than 1,000,000 ng/mL (Table 13). The dry season test had two concentrations greater than 100,000 ng/mL and eight concentrations greater than 1,000,000 ng/mL. Although no induction of vitellogenin would be expected in control males, one wet season male control and four dry season male controls had concentrations greater than 1,000,000 ng/mL.

Table 13. Individual male vitellogenin concentrations (ng/mL). Concentrations greater than 100,000 ng/mL are bolded and concentrations greater than 1,000,000 are italicized.

		Vitellogenin (ng/ml)			
	Male	Replicate 1	Replicate 2	Replicate 3	Replicate 4
Wet Season					
Control	1	36,365	23,653	***1,542,692***	477
	2	4,197	High Variability	13,532	441
Santa Cruz	1	6,456	246	10,899	8,084
	2	16,352	35,722	**125,135**	Dead
Watsonville	1	2,447	12,448	3,215	12,853
	2	1,988	16,051	Dead	High Variability
Monterey	1	9,294	19,301	971	58,501
	2	379	Below Range	High Variability	589
Carmel	1	163	343	***2,973,540***	178
	2	Dead	Dead	11,409	56
Dry Season					
Control	1	48,697	684	87,007	***1,386,717***
	2	***2,273,539***	***1,201,226***	9,549	***11,586,872***
Santa Cruz	1	61,389	18,905	11,033	344
	2	334	**110,227**	Dead	933
Watsonville	1	**697,772**	Over Range	318	***8,754,352***
	2	460	Dead	Dead	***9,290,116***
Monterey	1	2,857	***3,075,306***	6,951	10,407
	2	3,496	***1,080,978***	18,279	68
Carmel	1	4,814	42,378	8,342	3,953
	2	5,064	31,535	Dead	2,682

The upper range of vitellogenin detection in the wet season test was 12,500,000 ng/mL, and the upper range in the dry season test was 25,000,000 ng/mL. The normal range of vitellogenin in females is approximately 400,000 ng/ml (Thorpe et al., 2007) to 17,400,000 ng/ml (Jensen et al., 2001, 2007; Watanabe et al., 2007). The upper range of detection in the wet season test was approximately five million ng/mL lower than the upper range from normal unexposed females (17,400,000 ng/ml), but the upper range of the dry season test was well above the upper end of the normal range. All concentrations of vitellogenin in plasma from wet season female fish were over the range of test detection, with the exception of one female from the second control replicate with a concentration of 768,685 ng/ml. This measured concentration was within the

normal range for unexposed female fish, but because the upper range of detection in the wet season test was lower than the upper range for unexposed females, many concentrations could have been in the normal range. Eleven female fish in the dry season test had vitellogenin concentrations that ranged from 857,629 to 11,610,999 ng/mL. These concentrations were all within the normal range for unexposed female fish. One dry season female from the fourth replicate of the control had a concentration of 115,731 ng/ml, which would be considered lower than normal for an unexposed fish. It was interesting to note that most of the vitellogenin concentrations of the female fish from the dry season tests were outside of the range of calibrators. This suggests that the same issues affecting the measurement of vitellogenin in the males (including control males) were occurring with the female measurements.

High vitellogenin concentrations reported in the control male fish make it difficult to interpret results of other treatments, and render the vitellogenin results inconclusive. Fish in the control treatment were not exposed to any known sources of EDCs in the UCD-MPSL laboratory, and were held on-site for a minimum of two months prior to initiation of the pre-exposure test. Although fish were obtained from two different suppliers, no trends in vitellogenin concentrations were observed based on supplier source. The majority of replicates contained one male from each supplier, and results did not exhibit obvious patterns such as one high and one low value per replicate. If fish in the control treatment were exposed to EDCs at any point prior to or during testing, it would be expected that resultant high vitellogenin concentrations would appear throughout the four control aquaria, or at least in both male fish within a replicate. When this assay was recently conducted by the Los Angeles County Sanitation District (Carlita Barton, personal communication, LACSD, Los Angeles, CA), concentrations in control males averaged 300,000 ng/ml, which is higher than published normal values of 20,000 ng/ml or less. Even if the upper range of unexposed fish were increased, five control fish in the current study had apparent vitellogenin concentrations greater than 1,000,000 ng/mL. U.S. EPA researchers measure an average concentration of 4,000 ng/mL, and maximum concentrations of 20,000 ng/mL in unexposed males, and would expect concentrations in the millions of ng/mL to only occur in female fish and male fish that have been induced by endocrine disrupting compounds (Kathleen Jensen, personal communication U.S. EPA, Duluth, MN)

There were no obvious behavioral differences or significant reductions in fecundity, as would be expected if EDCs were affecting fish physiology. Thorpe et al. (2009) described an inhibitory effect on spawning events and egg production when vitellogenin levels in males were elevated to concentrations in the millions of ng/mL range. These levels resulted in feminization of male fish and inhibited breeding behavior. At these levels, the authors observed a reduction in egg production of 51-66% during the exposure, as compared to the pre-exposure period. This reduction was attributed to elevated vitellogenin induction in male fish. However, as feminization of male fish was not observed in the present study, and fecundity was not affected, it is unlikely that the apparent vitellogenin concentrations were accurate. There were also no clear correlations between individual replicate aquaria egg production and presence of male fish with elevated vitellogenin levels. There were a number of replicates where one or both male fish appeared to have concentrations of vitellogenin greater than 1,000,000 ng/mL, but there was no effect on the reproductive output of these replicates.

It is unclear why the vitellogenin concentrations were variable and randomly elevated, particularly in the control males. Discussions with the technical staff of Biosense, vitellogenin experts at U.S. EPA, and a number of researchers contacted at the national Society of Environmental Toxicology and Chemistry (SETAC) conference have suggested some possible causes. In an attempt to explain the unexpected results, we first confirmed the ELISA procedure step-by-step in consultation with Biosense. This confirmed that no mistakes were made in the preparation of standards and samples. The calibrators from all of the analysis events produced proportional absorbance values, so we determined that the preparations were accurate, and the plate reader was operating properly. It was assumed that the high concentrations, and therefore the variability, were the product of something in the plasma samples themselves. Biosense suggested interference could possibly occur from hemolysis, the rupturing of the blood cells during centrifugation, but U.S. EPA researchers have not experienced interference from hemolysis. Other possible interferences from the handling of the plasma samples could have been caused by not keeping the samples cold, or repeatedly thawing and re-freezing samples. Plasma samples in the current study were maintained on ice for no more than 5 minutes after centrifugation before they were cryo-frozen and placed in a -80 ºC freezer for storage. All other sample handling and analysis procedures followed standard or published methods obtained through the literature or personal communication.

The causes of (possible) interference and the resulting variability of the vitellogenin concentrations were not determined as part of this study, but advice for using the current method and suggestions for future analysis methods were gathered from fellow researchers consulted as part of this project. The use of the Biosense ELISA kits entail using microliter quantities of plasma in high dilutions (up to 1: 500,000), which could have a high potential for user error. Researchers at the Los Angeles County Sanitation District indicated similar variability in vitellogenin results when using Biosense ELISA kits. Daniel Schlenk (UC Riverside, CA) suggested that using such small quantities of plasma in the ELISA may lend itself to user error that would result in the high level of variability seen in this test. His recommendations included either homogenizing plasma samples from a minimum of five fish or to conduct the analysis on liver tissue. Vitellogenin is produced in the liver before entering the blood stream, and as such, is potentially a more direct way of analyzing vitellogenin levels in fish. This alternative procedure is not discussed in the EPA (2000) protocol but is apparently used by researchers conducting this type of analysis. It has also been suggested that a subsample of fish be measured for vitellogenin upon arrival in order to determine if there are elevated concentrations of prior to testing.

Future studies of vitellogenin concentrations in fathead minnows exposed to effluent should incorporate higher replication, validation of vitellogenin levels in representative male fish by ELISA prior to testing, analysis of liver rather than plasma, and could incorporate homogenization of samples from replicate fish. Additionally, analysis of plasma sex steroid levels, which was not conducted in the current project, could provide further indication of whether endocrine disruption has occurred. Future studies could also incorporate the use of polymerase chain reaction (PCR), which has been used to evaluate gene expression linked to vitellogenin induction related to EDC exposure (Kolok et al. 2007). For example, in the present study, use of gene expression techniques could have provided additional evidence (or lack

thereof) of endocrine disruption in the control male fish where elevated vitellogenin was measured using ELISA.

SUMMARY and CONCLUSIONS

The overall fecundity of the fathead minnow populations in both the wet season and dry season tests were quite variable among replicates and treatments, and between the tests themselves. Wet season fecundity ranged from 75 to 133 eggs/day, whereas the dry season fecundity ranged from 14 to 46 eggs/day. Standard deviations among replicates were sometimes equivalent to the mean values indicating extremely high variability and reduced statistical power. Low replication in the EPA protocol design (4 replicates per treatment) may not be sufficient to adequately capture and account for variability within treatments. Even if individual concentrations varied considerably between fish, it might be possible to determine trends per treatment if replication was higher.

The dry season effluent from Santa Cruz was the only treatment that had significantly lower fecundity than the control (Table 14). The average fecundity in the exposure was 10 eggs/female/day, which was lower than the control test acceptability criterion of 15. However, this result was not significantly different from the fecundity in the Santa Cruz pre-exposure aquaria. There were no statistically significant differences in egg production between the pre-exposure and exposure periods in any of the effluents. Mean fecundity in the Santa Cruz treatment was greatly influenced by the lack of egg production in the first replicate. The other three replicates had egg production that ranged from 7 to 23 eggs/female/day. One female in the third replicate of the dry season Santa Cruz treatment also exhibited territorial behavior, and although this could be a sign of endocrine disruption, this replicate had the second highest egg production. The reduced reproductive output observed in the dry season Santa Cruz effluent exposure was most likely due to low reproductive capacity of the fish. In the wet season test, fat pads were observed on two Santa Cruz females. This could also be a sign of endocrine disruption, but egg production in this replicate was also high. Although some masculinization was observed in replicates from both of the tests with Santa Cruz effluents, the preponderance of evidence does not suggest this effluent is causing endocrine disruption.

There were several other minor observations and significant differences in the other three effluents, but only one might be considered a result of endocrine disruption (Tables 15 to 17). The only other incidence of masculinization occurred in the wet season effluent from MRWPCA. A dark and banded territorial female was observed in a replicate that contained two males, but the presence of this female did not impact egg production in this aquarium. There were a number of males with light coloration and no banding observed in Watsonville, MRWPCA and Carmel treatments, but these fish did not appear to be feminized, and the egg production of these replicates was not impacted. There were also several observations of significantly larger gonads. These occurred in the wet season males from the Monterey treatment, the dry season males from Santa Cruz and Carmel, and the dry season females from Watsonville. Because the gonads from these effluents were significantly larger than the controls, it was assumed they were not impacted by the effluents.

The vitellogenin analysis produced abnormally high results in several male control fish, and in many other male fish in the effluents of both tests. Similar, though less dramatic results were

observed in the female fish analyzed for the dry season test. The high vitellogenin concentrations were variable throughout the tests and were not linked to any reproductive effects in the effluents. After many detailed discussions with the ELISA kit manufacturer and other researchers, we were still unable to determine the cause of the elevated and variable concentrations, although it is likely there was some interfering factor in the plasma samples themselves. Because of the difficulties encountered in the analysis of vitellogenin, these results were largely inconclusive and considered unreliable, and are not included in the summary tables below.

The results suggest that there was likely no impact on fathead minnow reproduction in any of the treatment plant effluents at the concentrations tested. While some parameters measured in the test are possible indicators of endocrine disruption due to effluent exposure, only the Santa Cruz treatment had significantly lower fecundity, the primary endpoint indicating reproductive impairment. Based on the fact that the pre-exposure egg production in the Santa Cruz aquaria did not significantly differ from the effluent exposure egg production, it was concluded that reduced egg production in the Santa Cruz effluent was not caused by endocrine disruption.

Table 14. Summary of wet season and dry season test endpoints for Santa Cruz.

	Wet Season			**Dry Season**		
Endpoint	Control	Effluent	Significance	Control	Effluent	Significance
Survival (%)	100 (±0)	96 (±9)	No	100 (±0)	100 (±0)	No
Fecundity (eggs/female/day)	19 (±8)	22 (±11)	NA	31 (±5)	10 (±10)	Fecundity was significantly lower than control. Could indicate endocrine disruption, but more likely due to fish with low reproductive capabilities based on low pre-test fecundity.
Fertility (%)	97 (±2)	92 (±10)	NA	96 (±8)	92 (±14)	NA
Reproductive Behavior	No abnormal observations	No abnormal observations	NA	No abnormal observations	No abnormal observations	NA
Coloration, Banding, and Nuptial Tubercles	No abnormal observations	No abnormal observations	NA	No abnormal observations	Two females with dark coloration (one with banding). One female with territorial behavior. One male with no banding.	Masculinization could be an indicator of endocrine disruption.
Gonadosomatic Index (%)	Male: 1.15 (±0.12) Female: 14.74 (±2.67)	Male: 1.29 (±0.33) Female: 13.56 (±2.20)	No	Male: 1.32 (±0.17) Female: 12.13 (±3.60)	Male: 1.61 (±0.23) Female: 14.84 (±4.17)	Male GSI significantly greater than control. Not an indicator of endocrine disruption.
Dorsal Fat Pad Index (%)	2.04 (±0.91)	4.58 (±0.30)	No, but two females developed dorsal fat pads, which could indicate endocrine disruption.	2.47 (±1.19)	3.22 (±2.74)	No
Embryo Hatch (%)	99 (±3)	100 (±0)	No	98 (±3)	100 (±0)	No

Table 15. Summary of wet season and dry season test endpoints for Watsonville.

	Wet Season			**Dry Season**		
Endpoint	Control	Effluent	Significance	Control	Effluent	Significance
Survival (%)	100 (±0)	92 (±9)	No	100 (±0)	100 (±0)	No
Fecundity (eggs/female/day)	19 (±8)	17 (±7)	No	31 (±5)	35 (±31)	NA
Fertility (%)	97 % (±2)	93 (±13)	NA	96 (±8)	95 (±14)	NA
Reproductive Behavior	No abnormal observations	No abnormal observations	NA	No abnormal observations	No abnormal observations	NA
Coloration, Banding, and Nuptial Tubercles	No abnormal observations	Some males with no banding.	NA	No abnormal observations	Some males with light coloration and no banding. One male with no tubercles.	NA
Gonadosomatic Index (%)	Male: 1.15 (±0.12) Female: 14.74 (±2.67)	Male: 1.52 (±0.40) Female: 15.28 (±3.93)	No	Male: 1.32 (±0.17) Female: 12.13 (±3.60)	Male: 1.29 (±0.25) Female: 17.70 (±3.49)	Female GSI significantly greater than control. Not an indicator of endocrine disruption.
Dorsal Fat Pad Index (%)	2.04 (± 0.91)	2.83 (±0.24)	No	2.47(±1.19)	3.02 (±2.39)	No
Embryo Hatch (%)	99 (±3)	98 (±3)	No	98 (±3)	100 (±0)	No

Table 16. Summary of wet season and dry season test endpoints for Monterey.

	Wet Season			**Dry Season**		
Endpoint	Control	Effluent	Significance	Control	Effluent	Significance
Survival (%)	100 (±0)	92 (±9)	No	100 (±0)	96 (±9)	No
Fecundity (eggs/female/day)	19 (±8)	28 (±16)	NA	31 (±5)	25 (±15)	NA
Fertility (%)	97 % (±2)	96 (±5)	NA	96 (±8)	100 (±0)	NA
Reproductive Behavior	No abnormal observations	Some light males with no banding. One female with territorial behavior.	NA	No abnormal observations	Some light males with no banding.	NA
Coloration, Banding, and Nuptial Tubercles	No abnormal observations	One female had dark coloration, vertical banding, and territorial behavior.	Could be an indicator of endocrine disruption.	No abnormal observations	Four males with light coloration and no banding. One male with no tubercles.	NA
Gonadosomatic Index (%)	Male: 1.15 (±0.12) Female: 14.74 (±2.67)	Male: 1.45 (±0.20) Female: 13.77 (±2.66)	Male GSI was significantly larger than controls, not an indication of endocrine disruption	Male: 1.32 (0.17) Female: 12.13 (3.60)	Male: 1.40 (0.21) Female: 14.69 (1.78)	No
Dorsal Fat Pad Index (%)	2.04 (± 0.91)	4.68 (±0.1.53)	Fat Pad Index was significantly larger than controls, not an indication of endocrine disruption.	2.47(±1.19)	2.93 (±1.08)	No
Embryo Hatch (%)	99 (±3)	100 (±0)	No	98 (±3)	100 (±0)	No

Table 17. Summary of wet season and dry season test endpoints for Carmel.

	Wet Season			**Dry Season**		
Endpoint	Control	Effluent	Significance	Control	Effluent	Significance
Survival (%)	100 (±0)	100 (±0)	No	100 (±0)	100 (±0)	No
Fecundity (eggs/female/day)	19 (± 8)	33 (± 15)	NA	31 (±5)	21 (±14)	NA
Fertility (%)	97 % (± 2)	96 (± 5)	NA	96 (±8)	92 (±19)	NA
Reproductive Behavior	No abnormal observations	No abnormal observations	NA	No abnormal observations	No abnormal observations	NA
Coloration, Banding, and Nuptial Tubercles	No abnormal observations	Some light males with no banding.	NA	No abnormal observations	Some light males with no banding. One male with no tubercles.	NA
Gonadosomatic Index (%)	Male: 1.15 (±0.12) Female: 14.74 (±2.67)	Male: 1.17 (±0.33) Female: 15.19 (± 2.23)	No	Male: 1.32 (±0.17) Female: 12.13 (±3.60)	Male: 1.75 (±0.22) Female: 12.95(±1.96)	Female GSI higher than control. Not an indicator of endocrine disruption
Dorsal Fat Pad Index (%)	2.04 (± 0.91)	4.57 (±0.57)	No	2.47(±1.19)	3.93 (±1.70)	No
Embryo Hatch (%)	99 (±3)	100 (±0)	No	98 (±3)	100 (±0)	No

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Appendix

Table A1. Wet season test control water and effluent flow rates measured during the pre-exposure and effluent exposures (ml/minute).

	Date	10/30/09	11/17/09	11/17/09	11/24/09	11/25/09	12/3/09	12/3/09
	Target Flow	100	98	2.00	2.00	98	2.00	98
Control	1	98	100	2.02	2.03	102	1.88	103
	2	97	98	2.09	2.05	98	1.88	99
	3	97	98	2.08	1.98	99	2.02	99
	4	99	102	2.02	1.98	105	1.90	103
Santa Cruz	1	99	97	2.05	2.00	99	2.02	99
	2	98	96	2.05	2.02	98	2.03	98
	3	98	100	2.10	2.05	101	2.03	100
	4	98	102	2.11	2.05	104	1.88	104
Watsonville	1	98	95	2.00	1.92	98	2.08	98
	2	99	95	2.02	1.94	97	2.03	98
	3	98	96	2.05	1.97	99	1.93	98
	4	97	102	2.04	1.96	103	1.91	104
Monterey	1	103	101	1.99	2.02	101	2.01	102
	2	103	100	2.00	1.98	101	2.02	103
	3	101	98	2.03	2.03	100	1.99	101
	4	102	98	2.02	2.01	102	2.06	102
Carmel	1	99	97	2.05	1.96	100	2.00	99
	2	99	97	2.01	1.99	98	2.06	97
	3	103	101	2.03	1.97	101	2.03	101
	4	102	97	1.96	1.98	102	1.98	101
	Mean	99.4	98.5	2.04	1.99	100.4	1.99	100.5
	SD	2.11	2.33	0.04	0.04	2.19	0.07	2.24
	Minimum	97	95	1.96	1.92	97	1.88	97
	Maximum	103	102	2.11	2.05	105	2.08	104

Table A2. Dry season test control water and effluent flow rates measured during the pre-exposure and effluent exposures (ml/minute).

	Date	5/5/10	5/26/10	5/26/10	5/27/10	5/27/10	6/8/10	6/8/10
	Target Flow	98	2.0	98	2.0	98	2.0	98
Control	1	98	2.5	103	2.0	104	2.0	103
	2	97	2.6	96	2.1	98	2.1	98
	3	101	2.5	103	2.1	104	2.0	104
	4	102	2.4	102	2.0	103	1.9	103
Santa Cruz	1	100	2.3	101	1.9	100	1.9	102
	2	99	2.2	98	1.8	99	1.8	99
	3	100	2.2	100	1.7	101	1.8	100
	4	98	2.3	99	1.9	100	1.9	100
Watsonville	1	102	2.4	101	1.9	101	1.8	103
	2	99	2.1	100	1.8	103	1.8	103
	3	101	2.2	101	1.8	104	1.9	104
	4	99	2.2	102	1.8	102	1.8	103
Monterey	1	93	1.8	95	1.9	96	1.9	95
	2	99	2.1	95	2.0	95	2.0	96
	3	101	2.0	98	2.0	97	1.9	99
	4	98	2.2	96	2.0	96	1.9	96
Carmel	1	98	2.0	92	2.1	92	2.0	92
	2	98	2.1	94	2.0	94	1.9	96
	3	94	2.1	99	2.0	100	2.0	98
	4	98	2.1	93	2.0	93	2.0	95
	Mean	98.8	2.22	98.4	1.94	99.1	1.92	99.5
	SD	2.3	0.2	3.4	0.1	3.8	0.1	3.6
	Minimum	93	1.8	92	1.7	92	1.8	92
	Maximum	102	2.6	103	2.1	104	2.1	104

Appendix B

PEER REVIEW OF LABORATORY BIOASSAY REPORT ON POTENTIAL EFFECTS OF ENDOCRINE-DISRUPTING CHEMICALS ON FATHEAD MINNOW REPRODUCTION

May 3, 2011

Project No. 11-1421-0012
E/11/0655

Mr. Dane Hardin, Director
Central Coast Long-term Environmental Assessment Network (CCLEAN)
P.O. Box 8346
Santa Cruz, CA
95061

PEER REVIEW OF LABORATORY BIOASSAY REPORT ON POTENTIAL EFFECTS OF ENDOCRINE-DISRUPTING CHEMICALS ON FATHEAD MINNOW REPRODUCTION

Dear Mr. Hardin:

Golder Associates Ltd. (Golder) is pleased to provide the Central Coast Long-term Environmental Assessment Network (CCLEAN) with this letter report documenting the findings of our peer review of the laboratory bioassay report for a study commissioned by CCLEAN.

1.0 BACKGROUND

CCLEAN is a long-term monitoring program designed to help municipal agencies and resource managers protect nearshore marine water quality in the Monterey Bay area of California. Program participants include the City of Santa Cruz, City of Watsonville, Moss Landing Power Plant, Monterey Regional Water Pollution Control Agency (MRWPCA), and Carmel Area Wastewater District (CAWD), under the auspices of the Central Coast Regional Water Quality Control Board.

CCLEAN commissioned the Marine Pollution Studies Laboratory (MPSL) at the University of California (Davis) to conduct a laboratory study to investigate the potential effects of endocrine-disrupting chemicals (EDCs) on fathead minnow (*Pimephales promelas*) reproduction. This involved testing dilute effluent samples from four member wastewater treatment plant (WWTP) discharges during wet season (fall 2009) and dry season (spring 2010) conditions. Test procedures were based on methods described in USEPA (2002a)[1].

[1] The MPSL proposal and laboratory report referred to the USEPA protocol for the 21-d fathead minnow reproduction test as having a 2008 publication date (*i.e.,* USEPA, 2008). However, the protocol document shows a June 2002 publication date, and therefore we have identified it accordingly.

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CCLEAN requested that Golder undertake an independent peer review of the MPSL study report and related documentation. To that end, Golder was provided with the following documents for review:

- MPSL proposal to conduct 21-day fathead minnow reproduction tests on four WWTP effluents from the Monterey Bay area;
- Final MPSL laboratory report for the fathead minnow reproduction study (Siegler *et al.,* 2011); and,
- Three Microsoft™ Excel files containing data and calculations pertaining to the final MPSL laboratory report.

The specific objectives of Golder's independent peer review were to:

- Analyses of the technical information, including the USEPA (2002a) testing protocol and related documents and the MPSL proposal to complete the study as requested by CCLEAN, and the correspondence between the study objective (*i.e.,* to assess the endocrine disrupting activity of each participant's wastewater at real-world concentrations using the fathead minnow) and study design, including whether study results could address other ancillary objectives;
- Review of the laboratory data, narratives and analyses from the study, for completeness and data quality assessment;
- Review of the study conclusions relative to study objectives in relation to standard endocrine effects, including but not limited to fathead minnow fecundity;
- Review and quality checks of the results and conclusions, including checking of the calculations used for statistical analyses; and,
- Recommendations for improving the linkage between study design, objectives and execution.

The scope of Golder's independent review did not include review of MPSL benchsheets or laboratory logbooks, nor did it include review of any analytical chemistry data associated with the WWTP effluent samples provided to MPSL for the toxicity tests. Therefore, it was not possible to evaluate linkages between the toxicity test results and the possible presence of EDCs in the effluent samples tested.

2.0 OVERVIEW OF THE TEST METHOD

A brief overview of the fathead minnow reproduction test method, as described in USEPA (2002a), is provided here for reference. This is a short-term test designed to assess the potential for EDCs to adversely affect fathead minnow reproduction. The test is conducted in a flow-through system using reproductively mature male and female fish that have not previously been held under active spawning conditions. The test containers are 16-L aquaria, with 10-L test solution volumes, and three spawning substrates (sections of PVC pipe split lengthwise and placed with the concave side down so that fish can lay their eggs on the underside). Each treatment has four replicates, each containing four female fish and two male fish.

The baseline reproductive performance of the test fish is established during a 14 to 21-d pre-exposure period, followed by a 21-d exposure period during which the fish are exposed to the test material. Experimental conditions during the pre-exposure period are the same as during the 21-d exposure period, except that the fish are only exposed to laboratory dilution water. During this pre-exposure period, the fish are monitored for evidence of reproductive behaviour, alterations in secondary sex characteristics (in males these are nuptial tubercles on the snout, a dorsal fat pad, and darkened coloration; in females these are a distended abdomen and swollen ovipositor), spawning activity, and embryo characteristics (fertilization success, hatching success and larval morphology). Spawning should occur every three to four days, and the following performance criteria need to be met: fecundity (≥15 eggs/female/day), fertilization success (≥90%), embryo hatching success (≥90%), and normal appearance and swimming behavior of fry.

The primary test endpoints measured during the 21-d exposure period are: adult survival, reproductive behavior, secondary sex characteristics, gonadosomatic index (GSI), gonadal histology, plasma vitellogenin and sex steroid concentrations, fecundity and fertility. Additional optional endpoints are: embryo hatching success, larval survival, and morphology. Test acceptability criteria for a valid test are that water quality conditions be within acceptable limits, and that the negative (clean) control fish demonstrate the following: ≥90% mean survival, active spawning, and ≥90% fertility.

USEPA (2002a) was written from the perspective of testing chemical substances rather than whole-effluent samples such as those used for this study. It therefore does not specify requirements for effluent sample collection, storage conditions or maximum holding times.

3.0 REVIEW OF THE MPSL LABORATORY STUDY PROPOSAL

Golder reviewed the MPSL study proposal that was provided by CCLEAN. This consisted of an undated four-page technical proposal plus a cost estimate (the latter was not reviewed). The objective of the MPSL study was to conduct fathead minnow reproduction tests on dilute effluent samples from four WWTPs (Santa Cruz, Watsonville, MRWPCA, and CAWD) during wet season and dry season conditions. The MPSL proposal identified the type of testing that would be performed, provided an overview of the experimental design and endpoints that would be measured, and identified two modifications to the USEPA (2002a) protocol that were incorporated into their study design. The MPSL proposal did not provide information on the laboratory's past experience performing this particular test method.

The MPSL proposal stated that the exposure system would use four male fish and two female fish per replicate. Presumably this was a typographical error as USEPA (2002a) specifies the use of four female and two male fish per replicate, and the MPSL laboratory report showed that the correct numbers as specified by USEPA (2002a) and gender of fish were used for all tests.

MPSL's proposal identified two modifications to USEPA (2002a) for this study, to exclude two test endpoints:

- Plasma sex steroid (β-estradiol, testosterone, and 11-ketotestosterone) analyses by radioimmunoassay (RIA) were excluded because MPSL was unable to identify a contract laboratory able to perform these analyses. Plasma samples were collected following each round of testing, but were subsequently discarded once the recommended holding time was exceeded.
- Gonad histopathology was excluded because MPSL lacked the expertise for these analyses. However, the MPSL proposal included collection and preservation of tissue samples for later analysis if necessary, and costs to have those analyses performed. Gonad tissue samples collected following each round of testing were being stored by MPSL as of February 2011.

In deciding to exclude the above two endpoints, the MPSL proposal referenced communications with Dr. Gerald Ankley (USEPA, Duluth, MN), who was involved in development of the fathead minnow reproduction test method (e.g., Ankley *et al*., 2001). The other test endpoints would provide enough information to determine whether reproduction effects had occurred, but if such effects did occur, then it would be a matter of being more difficult to identify their potential cause without information on plasma sex steroid concentrations or gonad histopathology.

USEPA (2002a) specified that the fathead minnow reproduction test be conducted under flow-through conditions and that there be at least six renewals of test solutions every 24 h. MPSL proposed the use of a modified flow-through renewal system for delivering test solutions, based on a system used by Martinovic *et al*. (2007). The flow-through system used by MPSL is described in Section 4.1 below; it exceeded the requirements for daily renewal of test solutions and was considered suitable for the MPSL study.

MPSL proposed that 24-h composites of WWTP effluent samples be collected and used daily during the 21-d exposure period, and that samples be collected in glass containers rather than in plastic. The 24-h composite samples were to be generated by collecting 0.08-L volumes each hour to yield a final sample volume of 2 L of each WWTP effluent each day. The collection of 24-h composite samples for short-term chronic toxicity tests is routinely used for effluent discharge permit testing (USEPA, 2002b). The MPSL proposal cited a study by Martinovic *et al.* (2008) that showed fluctuations in the estrogenic activity of WWTP effluent (from the City of Duluth, MN) depending on the time of day. However, Martinovic *et al.* (2008) reported that male fish were exposed to fluctuating or time-weighted constant concentrations of 17β-estradiol exhibited similar responses. Those results showed that the timing of sample collection could be important if grab samples were being collected, but that the 24-h composite sampling of effluent proposed by MPSL was appropriate for this study. The MPSL proposal also noted that there was no information available on the potential variability of estrogenic activity in the WWTP effluents that would be used for this study. This further supported the use of 24-h composite effluent samples.

Although clean plastic Cubitainers™ or milk jugs are routinely used to collect effluent samples for acute and short-term chronic toxicity tests (*e.g.,* USEPA, 2002b), MPSL was concerned about the potential for interactions between the plastic containers and the effluent samples, either through sorption of effluent constituents to the containers or leaching of EDCs from the containers. MPSL's decision to collect the effluent samples in amber glass containers was therefore appropriate.

4.0 REVIEW OF MPSL STUDY METHODS AND TEST CONDITIONS

4.1 Exposure System

The exposure system used by MPSL for this study was consistent with that described in USEPA (2002a). Replicate test containers were 16-L glass aquaria (20 x 40 x 20 cm) with 10-L test solution volumes and three spawning substrates. There were four replicates per treatment, each containing four female fish and two male fish. Extra replicates were prepared for the pre-exposure period, in case errors in gender identification or poor reproduction necessitated replacement of one or more replicates for the 21-d exposure period. The study was conducted at 25 ± 1°C under a 16:8 h light:dark photoperiod, and fish were fed twice daily. Heated MPSL well water was used as the laboratory dilution water; this water was used for the pre-exposure period, for preparation of effluent dilutions during the 21-d exposure period, and for the negative (clean) control. The target flow rate for delivering test solutions to each test container was 100 mL/min. This resulted in approximately 14.4 water renewals occurring every 24 h, which was more than double the minimum specified in USEPA (2002a) and therefore acceptable.

In each of the wet season and dry season testing programs, MPSL tested single concentrations of four WWTP effluents (Santa Cruz, Watsonville, MRWPCA, and CAWD), plus a negative (clean) control. Testing was performed once each season. Effluent samples were not tested at full-strength (*i.e.,* 100% concentration), but were each diluted to a concentration that reflected their dilution ratio upon discharge to Monterey Bay. These final effluent dilutions ranged from 84:1 to 145:1 depending on the WWTP, and the same dilutions were tested during both the wet and dry seasons. Each effluent underwent an initial dilution and then the flow delivery system was adjusted so that effluent delivered at 2 mL/min and well water delivered at 98 mL/min were mixed together to deliver the final effluent dilution to each replicate at 100 mL/min. The same system was also used to deliver well water to the negative control replicates during the 21-d exposure period. Calculations for preparation of the final effluent dilutions and flow rates were checked and confirmed to be correct.

The wet season pre-exposure period began November 3, 2009 and continued for 16 days until November 18, 2009; this was also Day 0 of the 21-d exposure period, which continued until December 9, 2009. The dry season pre-exposure period began May 8, 2010 and continued for 17 days until May 24, 2010; this was also Day 0 of the 21-d exposure period, which continued until June 14, 2010.

Results from the pre-exposure and exposure periods from both rounds of testing were reviewed and discussed in Section 5.0.

4.2 Test Organisms

USEPA (2002a) specifies that the fish used for this test need to be reproductively mature, which is typically 120 to 180 days old (*i.e.,* 4 to 6 months old). Using fish obtained from an in-house culture or purchased from a commercial supplier is acceptable, as long as purchased fish are held in the laboratory for at least one month before use. MPSL used purchased fish that were 8 to 11 months old at test initiation and held these fish in their laboratory for at least two months prior to testing (under flow-through conditions at approximately 17°C). No information was provided in the MPSL report about the procedure used to acclimate these fish to the test temperature of 25 ± 1°C, and therefore it was not known whether this could have been a stressor to the fish. However, fathead minnow are tolerant of a wide range of temperatures (USEPA, 2002a),

An important aspect of laboratory toxicity testing is elimination of as many sources of variability as possible, so that only the effect of the test material that is being evaluated. This includes conducting the test under controlled environmental conditions (*e.g.,* temperature, photoperiod), and using test organisms of consistent size/age. USEPA (2002a) does not specify using fish from a single source, whereas USEPA (2002b) states that test organisms should be from the same source but does not make this a “must” requirement. Nevertheless, using test organisms from multiple sources in a particular test is a practice that is to be avoided. Organisms from different sources can have different sensitivities to contaminants even when culture conditions are similar, which can introduce variability to the test system and make interpretation of the results more difficult.

For both rounds of testing, MPSL used fish from more than one source because of a lack of availability from a single supplier. MPSL used fish from Osage Catfisheries (Osage Beach, MO) and Aquatic Biosystems (ABS, Fort Collins, CO) for wet season testing, and fish from Osage Catfisheries and Aquatic Research Organisms (ARO; Hampton, NH) for dry season testing.

The strategy used by MPSL to distribute fish from the two sources to test containers differed between the two rounds of testing, with no explanation or rationale provided. For wet season testing, the first three replicates of each treatment were mostly Osage fish and the fourth replicate of each treatment used mostly ABS fish.

In contrast, for dry season testing, fish were more evenly distributed so that most replicates had equal numbers of male and female fish from the two suppliers. We assume that the intent of the strategy used for the wet season testing was to allow differences in reproductive performance (if they did occur) to be more readily identifiable, and that based on the results this was not necessary for the dry season. The fish distribution used for wet season testing may also have been to avoid grouping larger Osage male fish with smaller ABS male fish in the same test containers, although this mixing of sizes was not entirely eliminated based on observations noted in the MPSL report. Fish used in each round of testing were of different ages, and also different initial wet weights (see Section 5.2). We reviewed the daily spawning data provided by MPSL for both pre-exposure periods, and although fecundity (number of eggs/female) varied between replicates and among treatments it was not possible to relate this to the source of fish used for either round of testing. Regardless, future testing should be performed using fish from a single source even if fewer samples can be tested concurrently. USEPA (2007) noted that fecundity can be quite variable and that fish should be of similar size and known age to improve consistency associated with this endpoint.

4.3 Feeding

USEPA (2002a) recommends that fish be fed frozen adult brine shrimp twice daily *ad libitum* during the experimental period. Prior to testing, MPSL initially fed the fish a dry flake fish food and frozen brine shrimp twice daily, but switched to feeding a wet flake food slurry after mortalities were observed. This wet slurry was also fed twice daily *ad libitum* during the reproduction tests. The dry flake food may not have been suitable (large particle size and flakes floating on the water surface), but it would have been useful to know the outcome of feeding thawed frozen brine shrimp alone since it has been used successfully by other researchers.

4.4 Reference Toxicant Testing

MPSL conducted a reference toxicant test concurrently with each round of testing. Reference toxicant testing is an important component of a laboratory's quality assurance program, providing information about the relative health of each batch of test organisms. MPSL conducted acute 96-h LC50 tests with sodium chloride (NaCl); USEPA (2002a) does not require that the reference toxicant be tested using the 21-d reproduction test, and use of an acute test is acceptable. The concentration range was increased for the dry season test after mortality only occurred in the highest NaCl concentration in the wet season, which was appropriate.

The 96-h LC50s for the wet season and dry season reference toxicant tests were 14.0 and 4.6 g/L NaCl, respectively. The result for the wet season reference toxicant test was not relevant to that round of testing because it was conducted with fish from a third supplier (ARO) and provided no information about the relative health of the fish (from Osage and ABS) that were used for wet season testing. The dry season reference toxicant test was relevant to the current study; although not an ideal approach, it was conducted with a mixture of fish from the same two suppliers (Osage and ARO) used for the dry season reproduction test.

When laboratories report reference toxicant results, they are typically compared to the historical performance for that test organism-toxicant-endpoint combination and presented in the form of a control chart showing the mean and warning limits (± 2 standard deviations [SD]) calculated from the most recent 20 acceptable test results. Although MPSL reported that their reference toxicant test results for this study were comparable to other test populations, they did not provide a mean ± 2SD for comparison to past performance and it was not clear whether the reference to other test populations was about testing done in their laboratory or published literature values.

4.5 Test Endpoints

Procedures used by MPSL for assessing each test endpoint (apart from plasma sex steroid analyses and gonad histopathology, which were excluded) were described in the MPSL report, and were consistent with USEPA (2002a) procedures. Results obtained for each endpoint are discussed in Section 5.0.

Although the MPSL report was clear that fecundity was assessed daily, it was not clear how often fertilization success was assessed. Based on review of the daily spawning data, percent fertilization was assessed each time that spawning occurred during the pre-exposure and exposure periods, which was appropriate.

Embryo hatching success was assessed in each replicate by monitoring the hatching of 20 embryos sampled from a single spawning event. This was assessed once for each replicate in the pre-exposure and exposure periods during each round of testing. Dates were not reported for wet season testing, but hatching success was measured near the beginning of the pre-exposure and exposure periods for dry season testing. Although this is an optional endpoint, it would have been useful to repeat the assessment near the end of the exposure period to see if there were any changes in hatching success.

4.6 Environmental Conditions During Testing

MPSL measured water quality in each test solution as follows: temperature and dissolved oxygen (DO) daily; pH, conductivity and ammonia weekly; and hardness and alkalinity four times over the testing period. With the exception of temperature, these water quality measurements were summarized in Table 4 of the MPSL report as both the mean ± SD and range of measurements reported for each treatment during each round of testing. Water quality ranges could only be checked against raw data (provided in spreadsheet format) for dry season testing as raw data were not provided for wet season testing. The following discrepancies were identified during our review of the water quality data:

- Temperature was monitored continuously and also measured daily, but no raw data for temperature were provided for either round of testing and no summarized temperature data were provided for dry season testing; this is a data gap that should be corrected. The only temperature data reported were the mean ± SD for the wet season pre-exposure and exposure periods. Reporting the range of temperatures to which test organisms were exposed is as important, if not more so, than reporting the mean ± SD. MPSL noted that the well water temperature was affected by low ambient temperatures during wet season testing in fall 2009, and that although the heating system capacity was increased the mean pre-exposure period temperature (22.8 ± 1.6°C) was below the target of 25 ± 1°C. Mean temperature during the wet season exposure period of 24.3 ± 1.1°C. Given the small SDs associated with these mean temperatures, it is likely that the actual temperature range was small even though the protocol specification was not met. Fathead minnow are able to tolerate and spawn successfully in a wide range of temperatures and therefore it is unlikely that the low temperatures associated with wet season testing adversely affected the test results. No conclusions can be made about dry season temperatures as these data were not provided.
- DO concentrations were above the test acceptability criterion of ≥60% saturation (4.9 mg/L at 25°C) in all treatments during wet season and dry season testing with one exception. The DO concentration decreased to 4.43 mg/L in all treatments on one day of the dry season pre-exposure period, but this was not expected to adversely affect the test results.
- Based on our review of the raw data, the range of pH values reported for the dry season negative control should be 7.84 to 8.33, instead of 7.81 to 8.33 as reported by MPSL in Table 4.

- MPSL reported that un-ionized ammonia concentrations were measured using a Hach 2010 spectrophotometer, and reported concentrations as "ammonia" in the dry season raw data spreadsheet and as "un-ionized ammonia" throughout the report. The form of ammonia measured by MPSL needs to be confirmed. The procedures manual for the ach 2010 spectrophotometer (Hach, 2000) shows that it measures concentrations of ammonia-nitrogen, which is a measure of the total ammonia concentration in a sample and not just un-ionized ammonia. The proportion of ammonia-nitrogen present as un-ionized ammonia is dependent on the temperature and pH of the sample, and therefore un-ionized ammonia concentrations are calculated using that information rather than being determined by direct measurement. USEPA (2002a) recommended that the un-ionized ammonia concentration in laboratory dilution water should be <35 µg/L. In contrast, MPSL reported that the acceptable range for un-ionized ammonia concentrations was <4.7 mg/L (footnote to Table 4), which appears to be incorrect.

Flow rates were measured periodically during each round of testing; raw data were provided in spreadsheet format and presented in Appendix A of the MPSL report. For the wet season program, flow rates were measured just prior to the pre-exposure period, once during the pre-exposure period, and three times at approximately weekly intervals during the 21-d exposure period. For the dry season program, flow rates were measured just prior to the pre-exposure period and three times during the 21-d exposure period, but not during the pre-exposure period. MPSL reported that flow rates were within 5 to 7% of their targets (100 mL/min for pre-exposure period dilution water, 98 mL/min for exposure period dilution water, and 2 mL/min for exposure period effluent or dilution water) except that dry season 2 mL/min flows were within 20% of target and ranged from 1.7 to 2.6 mL/min based on three measurement events. The use of digital peristaltic pumps to deliver test solutions and the similarity of the flow measurements that were reported suggest that flow rates were consistent during both rounds of testing. However, it would be preferable to have flow data collected more frequently (*e.g.,* twice weekly) for confirmation purposes.

5.0 REVIEW OF MPSL STUDY RESULTS

MPSL presented the results for wet season and dry season testing together, to facilitate comparisons between the two rounds of testing. The results for each test endpoint were presented in tables (comparing pre-exposure and exposure conditions where appropriate), and then all the results were compiled into separate tables for each WWTP effluent. MPSL used two-sample t-tests for unequal variance to test for statistically significant different differences in mean responses between each effluent sample and the negative control, and in some cases to test between pre-exposure and exposure period responses. Tests were one-tailed, and although not specified in the MPSL report, it appears that differences were considered to be statistically significant at p <0.05, which was appropriate.

To facilitate our review, results from the exposure periods were compiled into the attached Table 1 to present all of the study results arranged by testing season and test endpoint, with the results for the four WWTP effluents grouped together for each endpoint. Results and statistical analyses presented in the MPSL report were checked against the raw data and calculations provided in the two spreadsheets of wet season and dry season raw data.

5.1 Pre-Exposure Period Results

USEPA (2002a) specifies that the pre-exposure period last between 14 to 21 days. During that time, spawning should occur in each test container every three to four days, fecundity should be ≥15 eggs/female/day, and adult survival, egg fertilization, and embryo hatching success should be ≥90%. These conditions need to be met in all four replicate chambers for each treatment, otherwise the unacceptable replicates need to be replaced or the entire system needs to be assessed and a new pre-exposure period started with a fresh batch of fish.

Survival

MPSL reported that mean survival during pre-exposure was 100% for all wet season treatments and ranged from 90 to 96% for the dry season treatments, which was acceptable. Raw data on pre-exposure survival were not provided so these results could not be confirmed, and we have also assumed that these results already incorporated the two replacement replicates used for wet season testing and five replacement replicates used for dry season testing. The result of 90% mean survival for the Watsonville dry season treatment appeared to be an error; with a total of 24 fish in this treatment, percent survival should be 88 or 92%. Two of the original Watsonville replicates were replaced following the dry season pre-exposure period; if an additional replicate had only 88% survival then it should also have been replaced. MPSL also reported that the dry season exposure period started with five replicates having only five fish instead of six as a result of mortalities during the pre-exposure period; that count of five pre-exposure mortalities does not match Table 5 of the MPSL report, which indicated that there were at least six mortalities during that period.

Fecundity

There are concerns with how MPSL calculated fecundity during the pre-exposure periods for both rounds of testing, and whether acceptability criteria were met for this endpoint. For both rounds of testing, MPSL considered the first week of the pre-exposure period to be for acclimation and did not include data from the first week in calculation of the fecundity and fertilization endpoints. Therefore, although the wet and dry season pre-exposure periods were 15 and 17 days long, MPSL only used data from the last 9 and 10 days, respectively. USEPA (2002a) described the experimental period has having three phases: the first seven days were for acclimation (data from this period can be excluded to reduce variability); the remaining time until the test material was added was the pre-exposure period; and the period of exposure to the test material was the exposure period. USEPA (2002a) also specified that the pre-exposure period should last from 14 to 21 days, and it is therefore our opinion that MPSL should have allowed additional time for acclimation so that the pre-exposure period would not be shorter than 14 days.

- The wet season pre-exposure period was 16 days long, although the MPSL report referred to it as lasting 15 days. This may have been because fecundity data from the first day of the pre-exposure period were excluded; total egg production on that day was more than five times higher than the average for the remainder of the pre-exposure period and excluding those data so as not to bias the results was reasonable. Based on the 9-d pre-exposure period reported by MPSL, overall mean fecundity ranged from 21 to 37 eggs/female/day for each treatment. However, there was considerable variability in mean fecundity among replicates. For the negative control, Watsonville and CAWD treatments, there was almost a two-fold difference in mean fecundity among replicates for those treatments, and for the MRWPCA treatment it was almost five-fold. Two of the MRWPCA replicates had mean fecundity of only 11 eggs/female/day, which did not meet the acceptance criterion of ≥15 eggs/female/day. Those two replicates should have been replaced before starting the exposure period, or the MRWPCA treatment should have been excluded from testing. We re-calculated fecundity results for the 15-d pre-exposure period, and overall mean fecundity ranged from 27 to 37 eggs/female/day for each treatment. Variability among replicates was still high, with a two-fold difference in the Watsonville and CAWD treatments and a four-fold difference in the MRWPCA treatment. Two MRWPCA replicates still had unacceptably low mean fecundities of 11 and 13 eggs/female/day.

- The dry season pre-exposure period was 17 days long, although the MPSL reported also referred to it lasting 15 days. Based on the 10-d pre-exposure period reported by MPSL, overall mean fecundity ranged from 19 to 35 eggs/female/day for each treatment. However, there was considerable variability in mean fecundity among replicates, with three- or four-fold differences among replicates for all treatments. One replicate each from the negative control, Santa Cruz, MRWPCA and CAWD treatments had mean fecundities ranging from 7 to 14 eggs/female/day, which did not meet the acceptance criterion of ≥15 eggs/female/day. Those four replicates should have been replaced before starting the exposure period (except that five of the eight replacement replicates were already used and others may not have been suitable), one treatment should have been excluded from testing and the remaining replicates re-allocated, or testing should not have proceeded. We re-calculated fecundity results for the 17-d pre-exposure period, and overall mean fecundity ranged from 30 to 47 eggs/female/day for each treatment. Variability among replicates was still high, but mean fecundity in all replicates was at least 15 eggs/female/day, and therefore acceptable for testing.

Fertilization

MPSL assessed egg fertilization success on a portion of eggs from every pre-exposure period spawning event, except for a few instances when small batches of eggs were not assessed. As with the fecundity endpoint, MPSL did not use data from the whole pre-exposure period to calculate the fertilization endpoint. For the wet season 9-d pre-exposure period, MPSL reported that mean fertilization ranged from 94 to 97% among treatments. For the dry season 10-d pre-exposure period, MPSL reported that mean fertilization ranged from 89 to 97% among treatments, and that when one of the MRWPCA replicates was replaced mean fertilization for that treatment increased from 89 to 93%. There was very little change to mean fertilization when these endpoints were recalculated using data from the full duration of the pre-exposure periods. Mean fertilization was considered acceptable.

Hatching Success

Hatching success was assessed once for each replicate in each round of testing, and ranged from 97 to 100% among treatments, which was acceptable.

5.2 Exposure Period Results

We reviewed the results reported by MPSL for each test endpoint assessed during the 21-d exposure period, and checked calculations using the raw data provided in spreadsheet format.

Survival

Mean survival was 100% in the negative controls for both rounds of testing. Mean survival in the four dilute effluent samples ranged from 92 to 100% during wet season testing, and from 96 to 100% during dry season testing. There were no statistically significant (p <0.05) differences in mean survival between the dilute effluent samples and their applicable negative controls. At the effluent dilutions tested, there were no adverse effects on adult fathead minnow survival.

Fecundity

Mean fecundity was 19 eggs/female/day for the wet season negative control, and ranged from 17 eggs/female/day in dilute Watsonville effluent to 33 eggs/female/day in dilute CAWD effluent. There were no statistically significant (p <0.05) differences in mean fecundity between the dilute effluent samples and the negative control in wet season testing. Mean fecundity in the negative control was significantly lower in the exposure period than in the pre-exposure period.

Mean fecundity was 33 ± 8 eggs/female/day for the dry season negative control; this was reported correctly by MPSL in Table 6 of their report but reported incorrectly as 31 ± 5 eggs/female/day in Tables 14 to 17. Mean fecundity ranged from 10 eggs/female/day in dilute Santa Cruz effluent to 35 eggs/female/day in dilute Watsonville effluent. Mean fecundity in the dilute Santa Cruz sample was significantly lower (p <0.05) than the negative control in dry season testing. MPSL considered that this could be an indication of endocrine disruption, but alternatively that it was more likely related to poor reproductive performance based on comparison to the pre-exposure fecundity for that treatment. Review of the pre-exposure data for the Santa Cruz treatment suggested that fecundity was decreasing over the course of the 17-d pre-exposure period, and as noted previously one replicate from this treatment should have been replaced prior to starting the 21-d exposure period because of poor fecundity.

Although replicate variability was high in most treatments, there were differences in fecundity between wet season and dry season testing. The Watsonville sample was the most consistent, decreasing by 11% in the wet season and increasing by 6% in the dry season. Mean fecundity increased in the Santa Cruz, MRWPCA and CAWD samples in the wet season (16 to 74%), but decreased in those three samples in the dry season (24 to 70%).

At the effluent dilutions tested, there were no adverse effects on fathead minnow fecundity, with the possible exception of the dilute Santa Cruz sample tested in the dry season round.

Fertility

Mean fertility was 97% for the wet season negative control, and ranged from 92% in dilute Santa Cruz effluent to 96% in dilute MRWPCA effluent. MPSL correctly reported mean fertility in the dilute CAWD sample as 95 ± 5% in Table 6 of their report, but incorrectly reported it as 96 ± 5% in Table 17.

Mean fertility was 96% for the dry season negative control, and ranged from 92% for the dilute Santa Cruz and CAWD samples to 95% for the dilute Watsonville sample. MPSL correctly reported mean fertility in the dilute MRWPCA sample as 93 ± 10% in Table 6 of their report, but incorrectly reported it as 100 ± 0% in Table 16.

At the effluent dilutions tested, there were no adverse effects on fathead minnow egg fertilization.

Reproductive Behavior

The MPSL report did not note unusual behavior in any treatments during either exposure period, in terms of altered swimming behavior, hyperventilation, loss or equilibrium, or changes in feeding.

Mature male fathead minnows normally exhibit territorial behaviour, and will defend the spawning substrates; loss of this behaviour is an indicator of endocrine disrupting effects (USEPA, 2002a). There were no observations of unusual reproductive behaviour in either round of testing, except that MPSL reported one female in the wet season MRWPCA treatment and one female in the dry season Santa Cruz treatment that exhibited aggressive territorial behaviour, in addition to dark coloration and vertical banding. MPSL suggested that this was an indicator of endocrine disruption, but this coloring and dominant behavior can occur infrequently in unexposed female fish (USEPA, 2002a; Ankley *et al*., 2001) when there are multiple females in a test container.

Of some concern were the observations noted by MPSL during wet season testing of greater aggression and fighting in replicates where large males were paired with smaller males, with evidence of damage to the skin and fins and also mortality among smaller males. While aggressive behaviour by male fathead minnows is normal and expected with this experimental design, if there is an inequality in fish size (among males or females) then this may adversely affect reproductive behaviour and it would be preferable to use fish of similar sizes in future testing.

Coloration, Banding and Nuptial Tubercles

USEPA (2002a) provides descriptions of the differences that occur in the appearance of mature male and female fathead minnows. Male fish become dark except for two light-colored vertical bars on their sides, and they develop nuptial tubercles on their snout and a dorsal fat pad (see below) in front of their dorsal fin. The number, size and location of nuptial tubercles were scored for each male fish. Female fish do not typically undergo changes in coloring but do develop a fleshy ovipositor on their abdomen.

The majority of fish did not exhibit unusual color changes during the 21-d exposure periods in either round of testing. In the wet season testing, a number of male fish from several treatments remained light in color and/or did not develop vertical banding (including in the negative control), and there was one female fish in the MRWPCA treatment that developed dark coloration and vertical banding. In the dry season testing, negative control fish exhibited their expected coloring; three of the four effluent treatments had at least one light colored male and all four effluent treatments had at least one male without vertical banding. Two females from the dry season Santa Cruz treatment exhibited dark coloring, and one also exhibited vertical banding and territorial behaviour. As noted above, although MPSL suggested this could be an indication of endocrine disruption, these characteristics have been observed in unexposed fish. The occurrence of light coloring and/or lack of vertical banding in males was more common in the MRWPCA and CAWD treatments in both rounds of testing.

MPSL summarized nuptial tubercle scores for male fish in Tables 7 and 8; these results are also summarized in Table 1 of this review along with the results of statistical comparisons not reported by MPSL. Mean nuptial tubercle scores for the negative controls were 17.6 and 23.5, respectively, for wet season and dry season testing. Mean scores for the dilute effluent samples ranged from 16.9 to 24.9 for wet season testing, and from 13.8 to 22.9 for dry season testing. The dry season Watsonville mean score was the only treatment that was significantly lower (p <0.05) than the corresponding negative control. During dry season testing, one male fish from each of the Watsonville, MRWPCA and CAWD treatments had no tubercles; these fish were also light or slightly dark in color and had no vertical banding. No female fish developed nuptial tubercles, in either round of testing.

Fish Wet Weights

MPSL reported initial and final mean wet weights for fish from both rounds of testing. Initial fish weights were estimated by weighing five male fish and five female fish from each of the two suppliers, and then calculating weighted average wet weights for each treatment based on the allocation of fish from each supplier. Final wet weights were determined for all surviving fish, following collection of blood samples (USEPA, 2002a).

For wet season testing, the Osage male and female fish had larger initial weights than the ABS fish. For dry season testing, Osage males had lower initial weights than ARO males, but females from both suppliers were of similar weight. The male and female fish used for both studies were approximately twice the wet weight of typical fathead minnow used for these studies (Watanabe *et al*., 2007), but they were also several months older.

In Table 9 of the MPSL report, the initial weight for male fish used for the dry season control was reported as 8.91 ± 1.63 g, when it should be 8.91 ± 1.65 g.

Based on comparisons of mean wet weights, male and female fish lost weight in all treatments in both rounds of testing. Some of that weight loss can be attributed to uncertainty associated with the initial weight estimates, as well as loss associated with blood collection although MPSL reported that the amount of blood collected from each fish was ≤1 mL (approximately ≤1 g). In the wet season testing, average weight decreases were approximately 20% for female fish and up to 45% for male fish. In the dry season testing, average weight decreases were approximately 50% for female fish and 20 to 30% for male fish. MPSL suggested that these weight losses could also be due to egg production and competition for food. However, the initial weights were determined prior to the pre-exposure period and therefore before spawning activity had begun, and if weight loss was due to a food shortage then that raises a concern about whether the wet slurry used for feeding was providing an adequate food supply and adequate nutrition (even though it was provided twice daily *ad libitum*).

Gonadosomatic Index (GSI)

The gonadosomatic index (GSI) is the fish gonad weight expressed as a percentage of the total body weight, and is an indicator of the reproductive condition of the individual fish. GSI values for female fish can vary considerably, depending on whether the fish has recently spawned (low GSI) or is about to spawn (high GSI).

For male fish, MPSL reported that the wet season mean GSI was 1.15% for the negative control, and ranged from 1.17 to 1.52% for the dilute effluent samples. The dry season mean GSI for male fish was 1.32% for the negative control, and ranged from 1.29 to 1.75% for the dilute effluent samples. Male GSIs were significantly higher (p <0.05) than the negative control in the wet season MRWPCA treatment, and the dry season Santa Cruz and CAWD treatments. However, this was not considered an indication of endocrine disruption. Although the MRWPCA and CAWD treatments had a higher incidence of male fish with light coloration and/or lacking vertical banding in both rounds of testing, their mean GSIs were similar to or higher than the negative controls.

For female fish, MPSL reported that the wet season mean GSI was 14.74% for the negative control, and ranged from 13.56 to 15.28% for the dilute effluent samples. The dry season mean GSI for female fish was 12.13% for the negative control, and ranged from 12.95 to 17.70% for the dilute effluent samples. Female GSIs were only significantly higher (p <0.05) than the negative control in the dry season Watsonville treatment; however, this was not considered an indication of endocrine disruption.

Typical ranges for GSIs for unexposed fathead minnow have been reported as 1 to 2% for males, and 8 to 13% for females (Smith, 178; Jensen *et al.,* 2001; Watanabe *et al.,* 2007). Male fish exposed to the dilute effluent samples in this study had GSIs that were within this range. Although GSIs for female fish were in some cases above the range reported above, these values were generally within the range reported by Watanabe *et al*. (2007) for individual fish and may have been related to the fact that fish used for this study were several months older and also larger.

Dorsal Fat Pad Index (FPI)

The dorsal fat pad is a secondary sex characteristic associated with reproductively mature male fathead minnow. It is an elongated fleshy pad that extends on the dorsal side of the fish from the nape to the front of the dorsal fin (USEPA, 2002a).

MPSL examined each fish and assigned a fat pad score that ranged from 1 (none visible) to 5 (very prominent, overhanging the body surface). However, the MPSL report did not define the criteria used for the intermediate values for this subjective assessment, nor were they provided in USEPA (2002a). Raw data for these fat pad scores were provided in the spreadsheets but the results were not presented in the MPSL report. As part of this review, we calculated mean fat pad scores for each round of testing. For wet season testing, mean fat pad scores were 3.25 for the negative control, and ranged from 3.33 to 4.14 for the dilute effluent treatments; one

male fish in the MRWPCA treatment was scored “1” as having no visible fat pad. For dry season testing, mean fat pad scores were 2.63 for the negative control, and ranged from 2.67 to 3.57 in the dilute effluent samples.

MPSL also calculated a fat pad index (FPI), in which the weight of the fat pad was expressed as a percentage of the total body weight of the fish. Based on review of the raw data, the wet season FPIs and statistical comparisons were reported incorrectly in Table 11, and Tables 14 to 17, of the MPSL report. The corrected results are provided in Table 1 of this review. For wet season testing, the mean FPI was 2.04% for the negative control, and ranged from 2.61 to 4.58% for the dilute effluent samples. For dry season testing, the mean FPI was 2.47% for the negative control, and ranged from 2.93 to 3.93% for the dilute effluent samples. There were no statistically significant differences between effluent treatments and negative controls in either round of testing. The mean FPIs reported by MPSL for this study were consistent with the value of 3.99 ±1.64% (mean ± SD) reported by Watanabe *et al.* (2007) for unexposed male fish.

MPSL reported that two female fish from the wet season Santa Cruz treatment developed fat pads, which were scored as “2” and “3”, and suggested that this was an indication of endocrine disruption. However, there were no other indications of masculinisation of female fish from this treatment, such as changes in coloration, presence of vertical banding, or territorial behaviour, to support this.

Embryo Hatching Success

Embryo hatching success was assessed once for each replicate during the exposure period, as was also done during the pre-exposure period. Mean embryo hatching success ranged from 98 to 100% among treatments in both rounds of testing.

Vitellogenin Analyses

Vitellogenin is a precursor to egg yolk protein that is synthesized by reproductively mature fathead minnow; its production is controlled by estrogens, particularly β-estradiol (USEPA, 2002a). Vitellogenin concentrations in unexposed female fathead minnow can range from 400,000 to 17,400,000 ng/mL (Jensen *et al*., 2001; Thorpe *et al*., 2007). Vitellogenin can also be produced by male fathead minnow if they are stimulated by estrogen receptor agonists, and therefore vitellogenin production in male fathead minnow is used as a biomarker for exposure to estogenic substances (USEPA, 2002a). Thorpe *et al.* (2007) reported vitellogenin concentrations in unexposed male fathead minnow ranging from <10 to 160 ng/mL, and Jensen *et al*. (2001) and Watanabe *et al*. (2007) reported concentrations of approximately 4,000 ng/mL.

At the end of the 21-d exposure period, MPSL anaesthetized the surviving fish, collected blood samples and immediately isolated the plasma by centrifugation, and then froze the plasma samples at -80°C. Plasma samples were analysed using enzyme-linked immunoassay (ELISA) kits obtained from Biosense Laboratories Inc. (Bergen Norway). This was consistent with procedures used by other researchers.

MPSL encountered difficulties in conducting the vitellogenin assays, which were documented in their report. MPSL consulted with Biosense to confirm that the assays were being performed correctly (they were), and with researchers at USEPA and elsewhere experienced in measuring fathead minnow vitellogenin by ELISA. The MPSL report made no reference to their previous experience with this assay, and whether it had been performed successfully or if problems had been encountered.

MPSL reported vitellogenin concentrations for male fathead minnow ranging from 163 to 1,500,000 ng/mL for wet season testing and from 68 to 11,600,000 ng/mL for dry season testing, with the highest concentration occurring in a control fish. In both rounds of testing, variability among replicates was very high, as was variability between fish from the same replicate. The higher vitellogenin concentrations reported for male fish were within

ranges expected for female fish, or for male fish exposed to EDCs. However, if male fish had been exposed to EDCs either during acclimation, the pre-exposure period, or the exposure period, then all replicates should have had elevated vitellogenin concentrations and there should have been evidence of other reproductive effects; neither was the case.

MPSL reported that vitellogenin concentrations for female fathead minnows were "over the range of test detection" except for one control female at 770,000 ng/mL for wet season testing, but did not indicate what that concentration was other than that it was lower than the upper range for unexposed females (presumably the 17,400,000 ng/mL concentration reported above). MPSL reported that concentrations measured for dry season testing ranged from 860,000 to 11,600,000 ng/mL for 11 fish, but that one control fish had a relatively low concentration of 116,000 ng/mL. No explanation was given for why only 12 female fish were analysed for the dry season. MPSL noted that most of the measurements for female fish were outside the calibration range, but did not express the same degree of concern about the results for female fish as for the male fish. The difficulties encountered with this assay were more obviously reflected in the results obtained for male fish (because male control fish did not have the expected low concentrations of vitellogenin), but that does not mean that the same difficulties did not also occur with analyses of the female fish. It may have simply been fortuitous that most results for female fish were within the range of expected values.

We have not commented on the specific details of the ELISA technique used by MPSL as we do not have direct experience with this assay. The efforts made by MPSL to consult with the manufacturer and other experts for assistance were more than reasonable, and MPSL should continue to investigate solutions to the difficulties that they encountered before offering this assay for clients. The vitellogenin results reported by MPSL for both male and female fish must be considered suspect and should not be included in interpretation of the study findings.

6.0 REVIEW OF MPSL STUDY CONCLUSIONS

MPSL emphasized the variability associated with the fecundity endpoint (which was correct), but reported ranges for this endpoint as "eggs/day" rather than "eggs/female/day" in the first paragraph of their "Summary and Conclusions" section that we were unable to verify from review of the raw data. MPSL suggested that the experimental design of four replicates per treatment might be insufficient to address the fecundity variability, which may be the case; however, we would recommend that the performance of the laboratory be evaluated, for example through testing that used fish from a single source that were of similar size, and where every replicate met the requirement of ≥15 eggs/female/day during a pre-exposure period lasting for at least 14 days, as other factors, as noted above could also have contributed to the results of the study.

MPSL noted that the dry season Santa Cruz treatment was the only one to have significantly lower (p <0.05) mean fecundity than its negative control (10 eggs/female/day versus 33 eggs/female/day). MPSL attributed this to generally poor reproduction by the fish assigned to this treatment and noted that there was no significant difference in fecundity between the pre-exposure and exposure periods; although this was correct, MPSL did not note that there was an almost 50% decrease in mean fecundity between those two periods. Two replicates in this treatment had poor reproduction during the exposure period, one of which had <15 eggs/female/day during the pre-exposure period and should have been replaced. MPSL also noted that two female fish from the wet season Santa Cruz treatment developed dorsal fat pads, and that two females from the dry season treatment developed darker coloring and vertical bands and that one exhibited territorial behavior. However, these latter characteristics can occur infrequently in unexposed female fish.

The overall conclusion of the MPSL report was that there were likely no adverse impacts on fathead minnow reproduction for any of the four WWTP effluents at the concentrations tested.

7.0 CONCLUSIONS AND RECOMMENDATIONS FROM THIS REVIEW

Based on our review of the information provided, we observed the following:

1) We identified a number of discrepancies regarding how MPSL conducted this study relative to the published protocols for the test and other applicable guidance, some of which we believe should have invalidated the study (specifically the use of multiple sources of fish in the same test, using a pre-exposure period shorter than 14 days, and not meeting the mean fecundity requirement for the pre-exposure period in every replicate). There were also several errors in the MPSL report. It was unclear whether MPSL had prior experience conducting this test, given some of the difficulties encountered as well as the general lack of reference to previous test performance or experience when discussing any unusual occurrences.

2) Notwithstanding our concerns about the quality of the study as it was executed, we agree with the MPSL conclusion that fathead minnow reproduction was not adversely affected by exposure to the four WWTP effluents, at concentrations representing ocean discharge dilutions in Monterey Bay. This study provided no information about potential reproductive effects associated with higher effluent concentrations, nor did it provide information about potential reproductive effects on marine species resident to Monterey Bay.

Based on our review of the information provided, we provide the following recommendations:

1) Consider repeating all or part of this fathead minnow reproduction study to provide confirmation that the same findings can be obtained, absent of test validity or data quality concerns. Alternatively, repeat the study using a marine fish species to provide information more relevant to conditions in Monterey Bay.

2) If future testing of this nature will be undertaken, confirm the laboratory's previous experience with the test method and develop a detailed work plan that defines testing conditions and is agreed to by CCLEAN and the testing laboratory. If testing multiple samples concurrently, be prepared to reduce the number of samples in the event that problems arise such as a shortage of suitable fish.

3) Conduct fathead minnow reproduction tests on the highest possible concentrations of WWTP effluent (*i.e.*, non-lethal concentrations), to more clearly characterize the potential for reproductive effects under more conservative exposure conditions.

8.0 CLOSURE

We trust that this letter report provides sufficient information for your present needs. If you have any questions, please do not hesitate to contact the undersigned at 604-296-4200.

Yours very truly,

GOLDER ASSOCIATES LTD.

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Attachments: Table 1

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Table 1: Summary of Results for Testing of Dilute Santa Cruz, Watsonville, MRWPCA and CAWD Wastewater Treatment Plant (WWTP) Effluent During Wet Season and Dry Season Conditions

Endpoint	WWTP	Wet Season (Mean ± Standard Deviation [SD])				Dry Season (Mean ± Standard Deviation [SD])			
		Control	Effluent	% Difference From Control	Statistically Significant?	Control	Effluent	% Difference From Control	Statistically Significant?
Survival (%)	Santa Cruz	100 ± 0	96 ± 9	4% ↓	No	100 ± 0	100 ± 0	0%	No
	Watsonville	100 ± 0	92 ± 9	8% ↓	No	100 ± 0	100 ± 0	0%	No
	MRWPCA	100 ± 0	92 ± 9	8% ↓	No	100 ± 0	96 ± 9	4% ↓	No
	CAWD	100 ± 0	100 ± 0	0	No	100 ± 0	100 ± 0	0%	No
Fecundity (eggs/female/day)	Santa Cruz	19 ± 8	22 ± 11	16% ↑	No	33 ± 8	10 ± 10	70% ↓	Yes. Could indicate endocrine disruption, but more likely due to fish with low reproductive capabilities based on low pre-exposure fecundity.
	Watsonville	19 ± 8	17 ± 7	11% ↓	No	33 ± 8	35 ± 31	6% ↑	No
	MRWPCA	19 ± 8	28 ± 16	47% ↑	No	33 ± 8	25 ± 15	24% ↓	No
	CAWD	19 ± 8	33 ± 15	74% ↑	No	33 ± 8	21 ± 14	36% ↓	No
Fertility (%)	Santa Cruz	97 ± 2	92 ± 10	5% ↓	Not calculated	96 ± 8	92 ± 14	4% ↓	Not calculated
	Watsonville	97 ± 2	93 ± 13	4% ↓	Not calculated	96 ± 8	95 ± 14	1% ↓	Not calculated
	MRWPCA	97 ± 2	96 ± 5	1% ↓	Not calculated	96 ± 8	93 ± 10	3% ↓	Not calculated
	CAWD	97 ± 2	95 ± 5	2% ↓	Not calculated	96 ± 8	92 ± 19	4% ↓	Not calculated
Reproductive Behavior, Coloration and Banding	Santa Cruz	No abnormal observations	No abnormal observations		Not calculated	No abnormal observations	Two females with dark coloration; one with banding exhibiting territorial behavior. One male with no banding.		Masculinization could be an indicator of endocrine disruption.
	Watsonville	No abnormal observations	No abnormal observations		Not calculated	No abnormal observations	Some males with light coloration and no banding.		Not calculated
	MRWPCA	No abnormal observations	Some light males with no banding. One female with dark coloration, vertical banding, and territorial behavior.		Masculinization could be an indicator of endocrine disruption	No abnormal observations	Four males with light coloration and no banding.		Not calculated
	CAWD	No abnormal observations	Some light males with no banding.		Not calculated	No abnormal observations	Some light males with no banding.		Not calculated

Endpoint	WWTP	Wet Season (Mean ± Standard Deviation [SD])				Dry Season (Mean ± Standard Deviation [SD])			
		Control	Effluent	% Difference From Control	Statistically Significant?	Control	Effluent	% Difference From Control	Statistically Significant?
Nuptial Tubercle Scores	Santa Cruz	17.6 ± 10.4	24.9 ± 11.8	41% ↑	No	23.5 ± 6.7	21.6 ± 11.5	8% ↓	No
	Watsonville	17.6 ± 10.4	20.3 ± 8.5	15%↑	No	23.5 ± 6.7	13.8 ± 7.1 One male with no tubercles.	41% ↓	Yes
	MRWPCA	17.6 ± 10.4	16.9 ± 6.2	4% ↓	No	23.5 ± 6.7	16.9 ± 8.2 One male with no tubercles.	28% ↓	No
	CAWD	17.6 ± 10.4	21.2 ± 10.7	20% ↓	No	23.5 ± 6.7	22.9 ± 15.4 One male with no tubercles.	2% ↓	No
Gonadosomatic Index (GSI; %) - Male	Santa Cruz	1.15 ± 0.12	1.29 ± 0.33	12% ↑	No	1.32 ± 0.17	1.61 ± 0.23	22% ↑	Yes, but not an indicator of endocrine disruption.
	Watsonville	1.15 ± 0.12	1.52 ± 0.40	32% ↑	No	1.32 ± 0.17	1.29 ± 0.25	2% ↓	No
	MRWPCA	1.15 ± 0.12	1.45 ± 0.20	26% ↑	Yes, but not an indication of endocrine disruption	1.32 ± 0.17	1.40 ± 0.21	6% ↓	No
	CAWD	1.15 ± 0.12	1.17 ± 0.33	2% ↑	No	1.32 ± 0.17	1.75 ± 0.22	32% ↑	Yes, but not an indicator of endocrine disruption.
Gonadosomatic Index (GSI; %) - Female	Santa Cruz	14.74 ± 2.67	13.56 ± 2.20	8% ↓	No	12.13 ± 3.60	14.84 ± 4.17	19% ↑	No
	Watsonville	14.74 ± 2.67	15.28 ± 3.93	4% ↑	No	12.13 ± 3.60	17.70 ± 3.49	46% ↑	Yes, but not an indicator of endocrine disruption.
	MRWPCA	14.74 ± 2.67	13.77 ± 2.66	7% ↓	No	12.13 ± 3.60	14.69 ± 1.78	21% ↑	No
	CAWD	14.74 ± 2.67	15.19 ± 2.23	3% ↑	No	12.13 ± 3.60	12.95 ± 1.96	7% ↑	No
Dorsal Fat Pad Index (FPI; %)	Santa Cruz	2.04 ± 1.47	4.58 ± 3.14	125% ↑	No, but two females developed dorsal fat pads.	2.47 ± 1.19	3.22 ± 2.74	23% ↑	No
	Watsonville	2.04 ± 1.47	2.83 ± 1.53	39% ↑	No	2.47 ± 1.19	3.02 ± 2.39	22% ↑	No
	MRWPCA	2.04 ± 1.47	3.63 ± 1.69	78% ↑	No	2.47 ± 1.19	2.93 ± 1.08	19% ↑	No
	CAWD	2.04 ± 1.47	2.61 ± 1.19	28% ↑	No	2.47 ± 1.19	3.93 ± 1.70	59% ↑	No
Embryo Hatch (%)	Santa Cruz	99 ± 3	100 ± 0	1% ↑	Not calculated	98 ± 3	100 ± 0	2% ↑	Not calculated
	Watsonville	99 ± 3	98 ± 3	1% ↓	Not calculated	98 ± 3	100 ± 0	2% ↑	Not calculated
	MRWPCA	99 ± 3	100 ± 0	1% ↑	Not calculated	98 ± 3	100 ± 0	2% ↑	Not calculated
	CAWD	99 ± 3	100 ± 0	1% ↑	Not calculated	98 ± 3	100 ± 0	2% ↑	Not calculated

CAWD = Carmel Area Wastewater District; MRWPCA = Monterey Regional Water Pollution Control Agency; SD = Standard Deviation