Environmental factors influencing survival of threespine stickleback (*Gasterosteus aculeatus*) in a multipurpose constructed treatment wetland in southern California

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ABSTRACT: Survival of the threespine stickleback, *Gasterosteus aculeatus*, differed among marshes in a demonstration 9.9-ha multipurpose constructed treatment wetland designed to improve the quality of secondary-treated municipal wastewater in southern California. At a mean loading rate of 3.3 kg NH_4-N ha^{-1} d^{-1} (6 kg total N ha^{-1} d^{-1}), the suitability of the wetland to support a population of sticklebacks was estimated to be low. The development of potentially toxic levels of un-ionized ammonia, particularly during periods when pH increased concomitantly with oxygen generation by phytoplankton biomass > 300 mg chlorophyll *a* liter^{-1}, and disinfection by-products were associated with lowered survivorship of sentinel fish. Moreover, the high oxygen demand from nitrification of NH_4-N created daily periods of low dissolved oxygen concentration (6-16 h at < 2 mg liter^{-1}) in the open water areas of the shallow marshes. Low dissolved oxygen concentration in open water zones of the seven marshes during a part of each day and persistent anaerobic conditions in the emergent vegetation rendered the majority of the wetland's substrate surface unavailable for successful reproduction by sticklebacks. The potential sites for *Gasterosteus* to replace mosquitofish, *Gambusia affinis* and *G. holbrooki*, as a biological control agent against mosquitoes are probably limited to comparatively cool-water habitats with high water quality, such as riverine wetlands. ***Journal of Vector Ecology*** **32 (1): 90-105. 2007.**

Keyword Index: Constructed treatment wetlands, *Gasterosteus*, mosquito abatement, un-ionized ammonia, disinfection by-products.

INTRODUCTION

The use of constructed treatment wetlands as an alternative to conventional wastewater treatment is increasing worldwide (Kadlec and Knight 1996, Cole 1998). In addition to tertiary treatment of municipal wastewater, man-made wetlands are used to improve the quality of agricultural wastewater (Karpiscak et al. 1999), mine runoff (Kadlec and Knight 1996), storm water (Metzger et al. 2002) and other water sources (Pries 2002). Constructed treatment wetlands can provide additional benefits to water resources management programs such as wildlife conservation, wetland habitat enhancement, public education, and recreational sites. When wetland systems treat water carrying comparatively high levels of nutrients and pollutants, it can be a considerable challenge to provide high quality habitat for native fishes, amphibians, waterfowl, and waterbirds (Nelson et al. 2000).

A drawback of using wetlands for treatment of wastewater is the potential for production of pestiferous and pathogen-vectoring mosquitoes (Mortensen 1983, Carlson et al. 1986, Walton et al. 1998, Russell 1999). This concern is particularly acute in regions where human development encroaches on historically isolated wetlands and where conditions, such as dense emergent vegetation, large expanses of shallow water, and high nutrient loads, are especially favorable for mosquito production (Walton 2002). Surveillance of mosquito abundance and pathogen activity in the vector and host/reservoir species (Rose 2001) is important and intervention to control mosquitoes may be necessary.

Larvivorous fish can be an important component of a mosquito abatement strategy for wetlands (Meisch 1985, Kramer et al. 1988, Walton and Mulla 1991, Walton 2007). Integrated pest management approaches combining mosquito-specific larvicides (e.g., *Bacillus thuringiensis* subsp. *israelensis* de Barjac and *B. sphaericus* Neide) and planktivorous fish reduce the need for chemical pesticides, diminish the potential for evolution of resistance in mosquitoes to mosquito control agents, potentially lower the cost of mosquito abatement, minimize negative effects of pesticide application on nontarget species, and lessen the likelihood of pathogen transmission to humans, wildlife, and domesticated animals.

Mosquitofish (*Gambusia affinis* (Baird & Girard) and *G. holbrooki* Girard) have been introduced worldwide as biological control agents for mosquitoes since the early 1900s (Meisch 1985). Their effectiveness as mosquito control agents and for reducing the incidence of disease caused by mosquito-borne pathogens (Gratz et al. 1996, Rupp 1996), as well as their potential negative impact on native fishes and other fauna (Courtenay and Meffe 1989, Gamradt and

Kats 1996, but see Lawler et al. 1999), are still debated. In localities where mosquitofish are not native, introductions are discouraged or prohibited (Swanson et al. 1996) and alternative biological control agents for mosquitoes to *Gambusia* spp. are needed.

The threespine stickleback (*Gasterosteus aculeatus* L.) may be an alternative biological control agent for mosquitoes to *Gambusia* in many regions along both coasts of North America. The stickleback is predaceous, feeding on small animals throughout the water column (Coykendall 1980, Schooley and Page 1984, Hangelin and Vuorinen 1988, Sanchez-Gonzales et al. 2001) and preying readily on immature mosquitoes (Bay 1985). The food preferences, foraging behavior (Wootton 1976, Whoriskey et al. 1985, FitzGerald and Wootton 1993), short generation time (Wootton et al. 2005), adaptability (McKinnon and Rundle 2002), and wide geographic distribution (Page and Burr 1991, Swift et al. 1993) suggest that the threespine stickleback may be an effective predator of mosquitoes in a wide variety of habitats (Hubbs 1919; but see Offill and Walton 1999).

Here we report on the survival and growth of the threespine stickleback in a multipurpose constructed treatment wetland receiving secondary-treated municipal effluent in southern California. Survival of fish and the potential for the wetland to support a population of native sticklebacks for mosquito control are related to physical and chemical factors measured in the wetland.

MATERIALS AND METHODS

This study was carried out in a 9.9-ha multipurpose demonstration constructed treatment wetland at Eastern Municipal Water District's (EMWD) Hemet-San Jacinto Regional Wastewater Reclamation Facility (HSJRWRF) in San Jacinto, CA (33°48'N, 117°1'W; 454 m ASL). The wetland received daily 3800-7600 m^3 of secondary-treated municipal effluent from a conventional wastewater treatment facility. Secondary-treated municipal effluent was blended with approximately 50% of the wetland effluent, divided among five inlet marshes, flowed to a central pond, and then was divided between two outlet (polishing) marshes (Figure 1) before being incorporated into a reclaimed water supply. The nominal operating depth of the inlet and outlet marshes was 0.55 m; the central pond was 1.9 m. Daily total inflow volume varied according to the operational needs of the HSJRWRF; hydraulic residence time was 9-14 days (Sartoris et al. 2000). The wetland did not contain fish because of concerns that fish might clog components of the reclaimed water conveyance system.

The shallow marshes comprised 80% of the wetland's surface area and contained two bulrush species. California bulrush [*Schoenoplectus* (=*Scirpus*) *californicus* (C.A.Meyer)] and hardstem bulrush [*S. acutus* G.H.E. Muhlenberg ex J. Bigelow)] were found in about 75% and 25% of the shallow marshes, respectively (Sartoris et al. 2000). At sites where fish survival was monitored, California bulrush was prevalent in Inlet Marsh 3 and Outlet Marsh A, and hardstem bulrush was prevalent near the inflow of Inlet Marsh 1 (Figure 1). The vegetation had been planted on 1.2- or 2.4-m centers in autumn 1994 (Sartoris et al. 2000). Between two and four 12.2-m wide open water zones were present initially in each of the seven shallow marshes (Figure 1).

Survival and growth of sentinel fish

Gasterosteus aculeatus L. sticklebacks were collected by dip net from the San Jacinto River near Cranston Station in the San Bernardino National Forest (Riverside County, CA). Collection permit restrictions limited the number of fish (n = 55) used in this study. Fish were transported in water from the collection site under aeration in a cooler and acclimated for 30 min in one-half water from the collection site and from the wetland. Standard length of each fish was measured to the nearest millimeter before placement into cages in the wetland.

Survivorship of sticklebacks was monitored by placing eight to eleven sentinel fish in duplicate cages at three locations in the wetland: Inlet Marsh 1, Inlet Marsh 3, and Outlet Marsh A (Figure 1). To reduce the likelihood that fish were inadvertently introduced into the wetland, each internal cage was surrounded by an external cage (length × width × height: 1.30 m × 1.20 m × 0.94 m) that was pushed into the substrate at the vegetation-open water interface. External cages consisted of a wooden frame covered on five sides with fiberglass window screen. Internal cages were 1.25 m^3 (1.22 m × 1.13 m × 0.91 m) and were constructed of fiberglass window screen affixed on five sides to a polyvinyl chloride (PVC) frame. To limit the handling of fish during censuses, the inner cage was lifted out of the water and the number of surviving fish in each cage was counted. Each cage was covered with chicken wire or plastic netting (BirdBlock™: Easy Gardner, Waco, TX; mesh opening: 0.016 m) to prevent predation of the sentinel fishes by piscivorous birds.

Survivorship of sticklebacks was monitored approximately biweekly from June 14 through October 3, 1996. Dead fish which were not significantly decomposed were collected at each census and preserved in 10% formalin. Standard length of each dead fish was measured to the nearest millimeter.

Environmental factors

Water quality parameters were measured continuously for four- to five-day intervals using two recording electronic water quality sensor arrays (ICM Water Analyzers, Perstop Analytical, Wilsonville, OR) positioned in the open water and in the vegetation near the sentinel cages. Dissolved oxygen concentration, pH, conductivity, and water temperature were recorded at 30 min intervals. One probe was placed in the open water near one of the sentinel fish cages and the second probe was placed at 2 or 10 m into the vegetation on the outlet side of the cages. The sensor array was situated in the middle of the water column at approximately 0.25 m below the water surface. The sensors were moved among the three marshes containing the sentinel fish during the study; physicochemical variables were measured continuously for two time periods in each marsh. After four to five days in the wetland, the sensors were cleaned and checked against standards. The sensors were also deployed at two sites along the west and southwest sides of the central pond (Figure 1) for 7 days in September. Additional measurements were taken at 5 m into the vegetation and in other marshes of the wetland for a one-year period that encompassed the study discussed here.

Nutrient concentrations, physicochemical parameters, and phytoplankton biomass were measured approximately bi-weekly across the wetland during the summer. Grab samples (2.2 liters) were taken with a Van Dorn bottle (Beta Plus Bottle: Wildlife Supply Co., Saginaw, MI) from the middle of the water column at 15 stations in the wetland during July and at 17 stations from August through October (Figure 1). Additional samples were taken in December 1996, and February, April, and June 1997. Samples were stored in acid-washed dark polyethylene bottles, placed immediately on ice after collection, and analyzed within 2-6 h of collection.

Ammonia-nitrogen, nitrate-nitrogen, and nitrite-nitrogen were measured using ion-specific electrodes (Orion Research Inc., Beverly, MA: #9512, 9307, 9346, respectively). The nitrogen samples were measured within 2 h of collection. Un-ionized ammonia concentration was calculated as

$$A = C\ /\ [1 + 10^{(0.09018\ +\ (2729.92/T)\text{-}pH)}]$$

where A is the aqueous NH_3 concentration (mg liter^{-1}), C is the ammoniacal nitrogen concentration in water (mg liter^{-1}), pH is pH of the water, and T is temperature (°K) (Denmead et al. 1982). Un-ionized ammonia concentration was calculated at 30 min intervals during the period that the sensor arrays were deployed.

Total phosphorus, total dissolved phosphorus, and particulate phosphorus were measured using the ascorbic acid method (APHA 1995). Total phosphorus samples were oxidized using persulfate prior to colorimetric analyses. Particulate phosphorus concentration was calculated as the difference between total phosphorus concentration in unfiltered water and total dissolved phosphorus concentration in water filtered through a glass fiber filter (Whatman GF/F; Whatman International Ltd., Maidstone, England, U.K.).

Total inorganic carbon in the water column was estimated using the known relationship between total alkalinity, pH, and water temperature (Wetzel and Likens 1991). Total alkalinity was measured by Gran titration (Wetzel and Likens 1991). Physico-chemical parameters were measured at each sampling station using one of the electronic sensors described previously.

Phytoplankton biomass was measured spectrophotometrically as chlorophyll *a* (Wetzel and Likens 1991) in duplicate samples at each sampling site. Phytoplankton was collected on 0.45 µm pore membrane filters (Supor 450: Gelman Sciences Inc., Ann Arbor, MI) and chlorophyllous pigments were extracted in 90% alkaline acetone. Total phytoplankton biomass includes phaeopigments.

Water quality variables were analyzed weekly in inflow and outflow water samples by EMWD staff following APHA (1995) methods. Sartoris et al. (2000) and Thullen et al. (2002) describe the variables and analytical procedures. In addition to nutrient concentrations, five-day biochemical oxygen demand (BOD_5), total suspended solids, residual chlorine, and coliform bacteria abundance were measured. Residual chlorine in influent water to the wetland was measured using the idiometric method (APHA 1995) on eight dates during the period that sentinel fish were present in the wetland. Coliform bacteria density in the influent water was measured using the fermentation tube test and calculated as the Most Probable Number (MPN) using the number of positive reactions in the dilution series (APHA 1995).

Statistical analyses

Survivor functions for sentinel sticklebacks were calculated using Kaplan-Meier estimation, a nonparametric product limit estimator (SURVIVAL module: SYSTAT 1999b). Survival quantiles were calculated for each survivor function. Location (marsh) was a stratification variable in the analysis and the statistical significance of differences of stickleback survival among the three marshes were compared by a log-rank (Mantel-Haenszel) test.

Backward stepwise regression was used to assess the importance of selected physico-chemical variables on the conditional probabilities of stickleback mortality between sampling dates for the environmental factors. Conditional probabilities of stickleback mortality within each interval between the measurement of environmental variables were estimated from the survival function for sentinel fish in each marsh. If only one fish remained in a cage and the fish died in the interval being considered, then the conditional probability of mortality (p=1.0) for that interval was not included in the regression. The full regression model considered the concentrations of ammonium-nitrogen, nitrate-nitrogen, nitrite-nitrogen, un-ionized ammonia, residual chlorine in the influent water, and log-transformed chlorophyll *a* biomass as independent variables. The higher-order interactions of un-ionized ammonia, residual chlorine, and log-transformed phytoplankton biomass were also included in the analysis. Un-ionized ammonia concentration was estimated as the product of ammonium-nitrogen concentration in the open water zone adjacent to the cages holding sentinel fish and the mean across dates for the maximum proportion of NH_4-N converted to un-ionized ammonia during the two periods that electronic water quality sensors were present in each marsh (Inlet Marsh 1=0.090, Inlet Marsh 3=0.083, Outlet Marsh A=0.009). Weekly residual chlorine samples were averaged across dates within each of the five time intervals. Because water residence time was 9-14 days, water entering the outlet marsh was >7 days old; therefore, residual chlorine was assumed to be below detection limits (<0.2 mg liter^{-1}) in the outlet marsh.

Growth of sentinel fish was compared by ANOVA

(SYSTAT 1999a) using the mean standard length of fish collected on three to four dates in each cage during the study. The slopes for the relationship between standard length versus time differed significantly among the cages in the three marshes so ANCOVA was inappropriate. Least squares regression was used to estimate growth rate.

The duration of potentially stressful dissolved oxygen concentration (<2 mg liter^{-1}) was determined daily for the two periods in each marsh when sensor arrays were deployed and compared among the open water zones of the three marshes using ANOVA. Warm-adapted *G. aculeatus* exhibit normal swimming behavior at dissolved oxygen concentration >2 mg liter^{-1} (Feldmeth and Baskin 1976).

RESULTS

Survival and growth of sentinel fish

Survival of sentinel sticklebacks differed among the marshes in the wetland. The relative survival of *Gasterosteus* in Outlet Marsh A was > Inlet Marsh 3 > Inlet Marsh 1 (Figure 2). The fish in both cages in Inlet Marsh 1 succumbed comparatively quickly and all individuals expired by day 58 after stocking. Sentinel fish in the two cages in Inlet Marsh 3 exhibited excellent survival through days 42 and 58; survival declined markedly thereafter. Approximately 78% of the sentinel fish in one cage in Outlet Marsh A survived for the duration of the study (126 days); however, all fish in the second cage survived for nearly two months before dying abruptly by day 64 after stocking.

One cage containing *Gasterosteus* in each marsh exhibited catastrophic mortality associated with blooms of the filamentous chlorophyte, *Hydrodictyon*. The algae rapidly filled a cage during the period between censuses. Dead sticklebacks were found throughout the water column, were entangled in the net-like *Hydrodictyon,* and nearly all of the individuals in a particular cage were dead: Inlet Marsh 1 cage 1, 26 June; Inlet Marsh 3 cage 1, 11 August; Outlet Marsh A cage 2, 17 August. Following the sudden appearance of *Hydrodictyon*, the percent mortality of fish extant in a cage at the previous census was 100%, 87%, and 100%, respectively.

Mean survival times for sentinel fish in cages not affected by *Hydrodictyon* differed significantly among the marshes in the San Jacinto demonstration constructed treatment wetland (log-rank test: χ^2=24.74, df=2, p <0.0005). Mean survival in Inlet Marsh 1 (29.7 days; 75% quantile=12 days, 25% quantile = 37 days) was < Inlet Marsh 3 (93.6 days; 75% quantile=64 days, 25% quantile=104 days). Mean survival in Outlet Marsh A was estimated to be 121 days and only the 78% quantile, 27 days, could be estimated because a large proportion of the fish survived.

The growth rate of sentinel fish (Figure 3) in Inlet Marsh 3 was 58% of that for fish caged in Outlet Marsh A (mean ± SE: Inlet Marsh 3: 0.07 ± 0.02 mm SL d^{-1}; Outlet Marsh A: 0.12 ± 0.01 mm SL d^{-1}). The length of fish in Inlet Marsh 1 did not change significantly during the comparatively short period that fish persisted.

Environmental factors

Nitrogen loading averaged 6 kg ha^{-1} d^{-1} and was dominated by ammonium nitrogen (Table 1). The mean total phosphorus concentration was ~2 mg liter^{-1} and did not decline significantly as water moved through the treatment wetland. Most of the phosphorus was in the dissolved phase and particulate phosphorus concentration in the middle of the water column did not change appreciably across the wetland. Municipal wastewater with a BOD_5 ≥10 mg liter^{-1} was the primary source of nutrients; water input from precipitation and groundwater input is effectively zero during the summer months (Smith et al. 2000). Alkalinity and conductivity (Table 1) were indicative of the hard water found in arid southern California.

The maximum daily dissolved oxygen concentration (D.O.) in the open water zones (Figure 4) differed significantly among the marshes (ANOVA, df=2, 20, p< 0.0005). Daily maximum D.O. concentrations in open water zones of Inlet Marshes 1 and 3 were significantly greater than in Outlet Marsh A (Bonferroni test, p<0.001). The mean daily maximum D.O. concentration in Inlet Marsh 1 was 18.7 ± 1.9 mg liter^{-1} (n=8), in Inlet Marsh 3 was 15.6 ± 4.5 mg liter^{-1} (n=9), and in Outlet Marsh A was 6.3 ± 2.7 mg liter^{-1} (n=9). Daily maximum D.O. concentration in the open water zone did not differ between periods within each marsh (ANOVA, df=3, 20, p>0.262).

The duration of potentially stressful D.O. concentration (<2 mg liter^{-1}) for sticklebacks in the open water zone differed significantly among the three marshes (ANOVA: df=2, 20, p <0.0005; Bonferroni test: p < 0.008). The duration of low D.O. concentration did not differ significantly between the two periods of study within each marsh (ANOVA: df=2, 18, p >0.170). The period of low D.O. concentration in the open water zone was shortest in Inlet Marsh 1 (mean ± SD: 6.2 ± 2.0 h; n=8), intermediate in Inlet Marsh 3 (10.4 ± 2.9 h; n=8), and longest in Outlet Marsh A (16.0 ± 2.3 h; n=7).

The mean daily pH in the Inlet Marshes was higher and more variable than in the Outlet Marsh (Figures 4a-f). The pH often exceeded 8.3 in the open water of the Inlet Marshes and increased concomitantly with the increase of daily dissolved oxygen levels. The pH in Outlet Marsh A was never greater than 7.4.

The pH in the open water of Inlet Marsh 1 increased during the day and the mean daily pH was higher than in water in the vegetation; however, the greater diel variation for pH in the open water zone overlapped the mean pH in the water column of the vegetated zone (Table 2). This trend was opposite to that observed in the two zones of Outlet Marsh A where pH of water in the emergent vegetation exceeded that in the open water zone (Table 2). The mean pH of water in the open and vegetated zones of Inlet Marsh 3 were similar.

Mean water temperature of the Inlet and Outlet Marshes was similar and ranged between 23.4° C and 26.8° C (Table 2). Water temperature at 0.25 m below the water surface never exceeded 29° C. Variation (SD) around the mean water temperature was 1-2° C.

Ammonium nitrogen (NH_4-N) dominated the total

Table 1. Mean nitrogen loading rates, nutrient concentrations and physicochemical factors in the HSJRWRF wetland from July through September 1996.

Variable	Mean ± SE	*n*
Nitrogen loading rates (kg ha^{-1} d^{-1})[a]		
organic nitrogen	1.08	
total ammonia nitrogen	3.30	
nitrite nitrogen	0.99	
nitrate nitrogen	0.61	
Total phosphorus (mg $liter^{-1}$)		
Inlet marshes	1.98 ± 0.36[b]	5
Outlet marshes	1.78 ± 0.18	5
Dissolved phosphorus (mg $liter^{-1}$)		
Inlet marshes	1.55 ± 0.21	5
Outlet marshes	1.43 ± 0.17	5
Particulate phosphorus (mg $liter^{-1}$)		
Inlet marshes	0.43 ± 0.19	5
Outlet marshes	0.35 ± 0.06	5
Alkalinity (μeq $liter^{-1}$)		
Inlet marshes	3316 ± 118	4
Outlet marshes	3209 ± 108	5
Conductivity (mS m^{-1})		
Inlet marshes	926.6 ± 9.9	5
Outlet marshes	908.0 ± 15.2	5
Dissolved inorganic carbon (mg $liter^{-1}$)		
Inlet marshes	0.090 ± 0.004	4
Outlet marshes	0.087 ± 0.004	2
Biological oxygen demand (BOD5: mg $liter^{-1}$)		
Inflow	~10-30[c]	
Outflow	4.82 ± 0.33	11
Total suspended solids (mg $liter^{-1}$)		
Inflow	11.27 ± 5.66	11
Outflow	7.27 ± 2.69	11

[a] Data from Sartoris et al. (2000): 30 May – 19 Sept. 1996.
[b] SE was calculated across five sampling dates for the mean concentration in the five inlet marshes or the two outlet marshes on each sampling date.
[c] EMWD (unpublished data) and Barber et al. (2001).

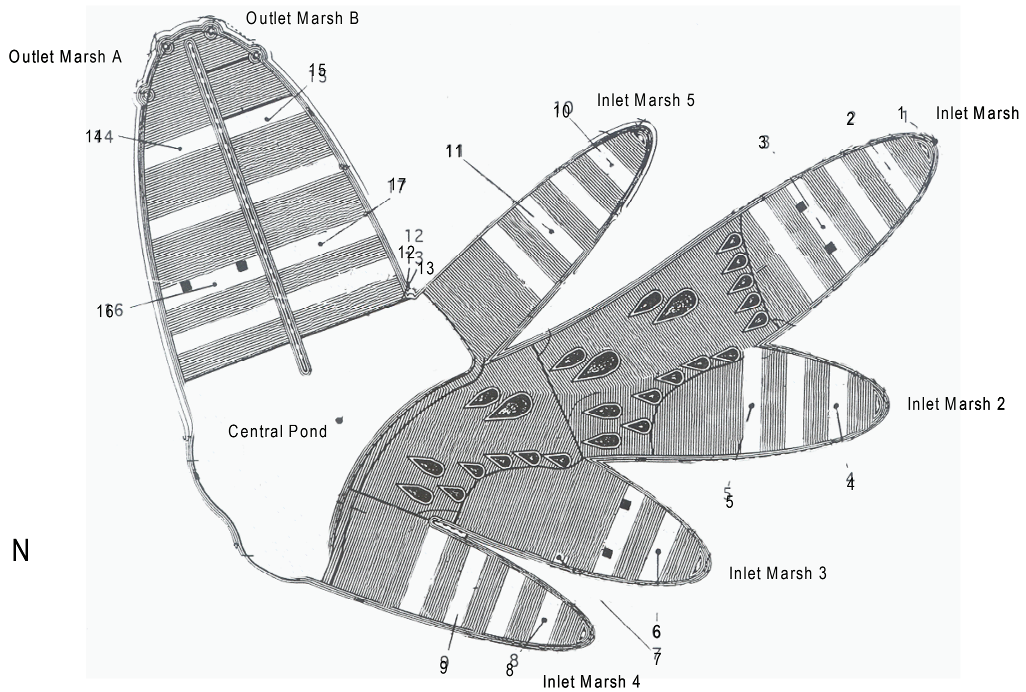

Figure 1. Schematic illustration of the 9.9-ha HSJRWRF wetland in San Jacinto, CA. Emergent vegetation (*Schoenoplectus* spp.) is illustrated by cross-hatching. Sentinel fish cages are illustrated by filled squares in Inlet Marsh 1, Inlet Marsh 3 and Outlet Marsh A. Water chemistry sampling stations are indicated by numbers 1 through 17. The teardrop-shaped objects are nesting islands for birds.

inorganic nitrogen (TIN) in the water column (Figure 5). Mean TIN in the open water zone closest to the inflow to Inlet Marsh 1 was 19.1 mg N liter^{-1} (n=6, SD= ± 3.7) and to the inflow to Inlet Marsh 3 was 19.9 mg N liter^{-1} (n=6, SD= ± 4.3). Mean TIN in the open water zone closest to the cages holding sentinel fish was 23-26% lower than in the open water zones near the inflow. Mean TIN near the cages in Inlet Marsh 1 was 11.2 mg N liter^{-1} (n=6, SD= ± 2.9) and in Inlet Marsh 3 was 13.5 mg N liter^{-1} (n=6, SD= ± 4.3). Ammonium nitrogen was 78% to 89% of TIN.

TIN and the percentage of TIN as NH_4-N in Outlet Marsh A decreased from the inlet marshes (Figure 5). Mean TIN in the two zones of the outlet marsh did not differ (station 14: 5.1 ± 3.3 mg N liter^{-1}; station 16: 5.8 ± 3.8 mg N liter^{-1}). Ammonium-nitrogen was 66-67% of TIN in Outlet Marsh A. The percentage of TIN as nitrate-nitrogen (NO_3-N) in the outlet marsh (31-32%) increased from that observed in the inlet marshes (7-10%). Nitrite-nitrogen was a comparatively small component (2-3%) of TIN.

Chlorophyll *a* concentration fluctuated more than two orders of magnitude across time in the three marshes in the wetland (Figure 6a). Phytoplankton biomass was lowest (<35 mg chlorophyll *a* liter^{-1}) in Outlet Marsh A on all dates when sentinel fish were present except the last date. Phytoplankton biomass was >300 mg chlorophyll *a* liter^{-1} in Inlet Marsh 1 in July and in Inlet Marsh 3 in late August when appreciable mortality of sentinel fish was observed.

Water column transparency was inversely related to the trends in phytoplankton biomass. Secchi depth in Inlet Marsh 1 was very shallow in July (Figure 6b) and indicated the uppermost limit of a dense layer of suspended matter (i.e., phytoplankton and bacteria). During late July and early August, the Secchi disk was visible on the substrate of Inlet Marsh 1 and Outlet Marsh A. Water transparency in Inlet Marsh 3 declined during August and Secchi depth was comparatively shallow in all of the marshes in early September.

Un-ionized ammonia concentration was >0.5 mg liter^{-1} for a part of every day in Inlet Marsh 1 and for 67% of the days in Inlet Marsh 3 (Figure 7a). Un-ionized ammonia concentration exceeded 1.25 mg liter^{-1} on four days in the inlet marshes during the 17 days that the sensors recorded physicochemical data, and fluctuated daily as pH in the water column changed. Un-ionized ammonia concentration was much lower in Outlet Marsh A, fluctuating between 0.02 and 0.05 mg liter^{-1} during late July and between 0.07 and 0.14 mg liter^{-1} during mid-August (Figure 7a).

Residual chlorine in the influent water was above detection limits (0.2 mg liter^{-1}) during three time periods while sentinel fish were present in the wetland: 11-31 July, 20 August, and 17 September (Figure 7b). Residual chlorine concentration was >8 mg liter^{-1} in July, >3 mg liter^{-1} in August and near the limit of detection in September. Coliform bacteria abundance was related inversely to residual chlorine concentration in the wetland influent and declined to ≤100 MPN (100 ml^{-1}) during periods that residual chlorine was present at >0.5 mg liter^{-1} (Figure 7b).

The conditional probability of mortality of sentinel *G. aculeatus* increased directly with residual chlorine concentration in the influent water and the estimated un-

Table 2. The mean (± SD) pH and temperature of water in open water adjacent to cages holding sentinel fish and in vegetated zones of three marshes in the HSJRWRF wetland during summer 1996. Data were recorded every 0.5 h and at 0.25 m depth.

Marsh	Dates	Open water			Vegetation			
		pH	°C	*n*	pH	°C	*n*	Distance of sensor in vegetation (m)
Inlet Marsh 1	July 16-19	7.38 ± 0.35	25.2 ± 1.6	186	7.06 ± 0.19	25.2 ± 1.5	141	2
	August 5-9	7.28 ± 0.27	24.5 ± 1.8	207	7.14 ± 0.03	23.4 ± 1.7	181	10
Inlet Marsh 3	July 22-27	7.42 ± 0.34	25.0 ± 1.0	191	7.42 ± 0.27	24.8 ± 0.9	191	2
	August 12-15	7.33 ± 0.26	26.7 ± 0.9	197	7.32 ± 0.07	26.0 ± 0.9	197	10
Outlet Marsh A	July 29-Aug. 2	7.12 ± 0.04	26.8 ± 0.9	196	7.23 ± 0.03	26.6 ± 1.0	146	2
	August 16-20	7.18 ± 0.04	24.7 ± 1.2	194	7.37 ± 0.10	24.7 ± 0.9	165	2

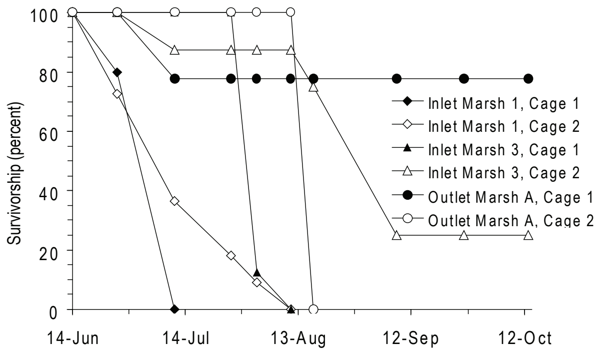

Figure 2. Survivorship of sticklebacks (*Gasterosteus aculeatus*) in cages placed in three marshes of the HSJRWRF wetland, San Jacinto, CA, during 1996.

ionized ammonia concentration in the open water zone adjacent to the cages holding fish. Stepwise regression identified the two main effects as significant predictors of conditional mortality of the sticklebacks ([NH_3]: (coefficient ± SE) 0.280 ± 0.102; t=2.733, df=10, p <0.02; [residual chlorine]: 0.087 ± 0.031; t=3.385, df=10, p < 0.006; ANOVA: F = 18.062, df=2, 11; p <0.001; R^2=0.77); however, the high conditional mortality in Inlet Marsh 1 when one of the two remaining fish in the cage died was identified as an outlier with significant influence on the model results. After deleting this point, the interaction between the two factors was identified as the best predictor of stickleback mortality within the sampling intervals of this study ([NH_3*residual

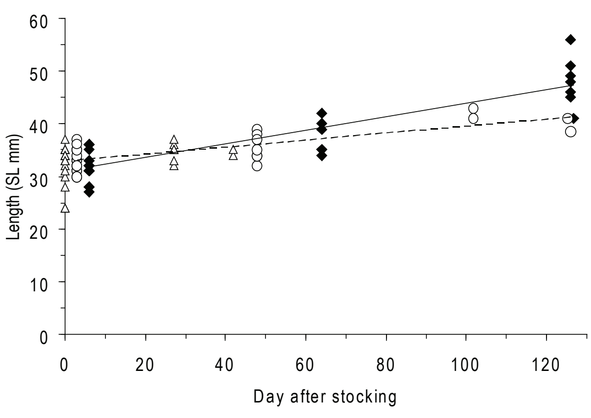

Figure 3. Growth (standard length) of threespine sticklebacks (*Gasterosteus aculeatus*) in cages within three marshes of the HSJRWRF wetland in San Jacinto, CA, during 1996. Symbols are displaced at t = 0 to facilitate illustration. Inlet Marsh 1: △; Inlet Marsh 3: ○ and dashed line [SL (mm) = (0.072 ± 0.015)*(days) + (32.4 ± 0.8); R^2 = 0.45]; Outlet Marsh A: ◆ and solid line [SL (mm) = (0.121 ± 0.011)*(days) + (31.8 ± 0.7); R^2 = 0.78].

chlorine]: 0.147 ± 0.024; t=6.22, df=9, p <0.001; ANOVA: F =36.64, df =1, 10; p <0.0005; R^2=0.79; Figure 7c). Whereas the predicted un-ionized ammonia concentration in open water zones of the three marshes was related significantly to stickleback mortality and was strongly correlated with NH_4-N (r=0.88), NH_4-N was not retained in the regression model.

Suitable sites for stickleback reproduction

The periphery of the center pond maintained dissolved oxygen concentration above 2 mg liter^{-1} for four days during September 1996 (Figure 8a). Dissolved oxygen concentration however was <2 mg liter^{-1} for a portion of three days at the end of the monitoring period. The fluctuation of pH of water adjacent to vegetation surrounding the center pond was lower than that observed in the open water zones of the two inlet marshes but was greater than that observed in Outlet Marsh A. Persistent anaerobic conditions were observed at 5 m into emergent vegetation.

Ammoniacal nitrogen concentration increased in the wetland during the autumn and winter 1996 and remained high (>15 mg liter^{-1}) during the first half of 1997 (Figure 8b). Unlike summer 1996 when ammonium levels in the outlet marsh were appreciably lower than in the inlet marshes, ammonium levels in the inlet and outlet marshes were similar during late 1996 and early 1997.

The shallow shelf along the periphery of the center pond may have provided the only region in the wetland capable of supporting stickleback nesting activities during 1996 (Figure 9). Anaerobic conditions persisted in the shallow marshes containing thick emergent vegetation. Daily periods (6-16 h) of low D.O. occurred in open water zones and approximately 2 m into the emergent vegetation of the shallow marshes of the wetland. In addition to low D.O. levels, sticklebacks residing in the inlet marshes would be subject to periods of ammonia stress as well as to potential toxicity of residual chlorine and halogenated compounds.

DISCUSSION

Survival of *G. aculeatus* differed among marshes in a multipurpose constructed treatment wetland in southern California and the suitability of the wetland to support a population of sticklebacks as part of an integrated pest management program for mosquitoes was estimated to be low. Of the probable factors contributing to differences of stickleback survivorship among marshes in the wetland, potentially toxic levels of disinfection by-products and un-ionized ammonia, particularly during periods when pH increased concomitantly with oxygen generation by large phytoplankton populations, were associated with lowered survivorship of sentinel fish. Moreover, the high oxygen demand from nitrification of NH_4-N created daily periods of low dissolved oxygen concentration (<2 mg liter^{-1}: 6-16 h) in the open water areas of the shallow marshes. Low dissolved oxygen concentration in open water zones of the marshes during a part of each day and persistent or periodic anaerobic conditions in the emergent vegetation rendered

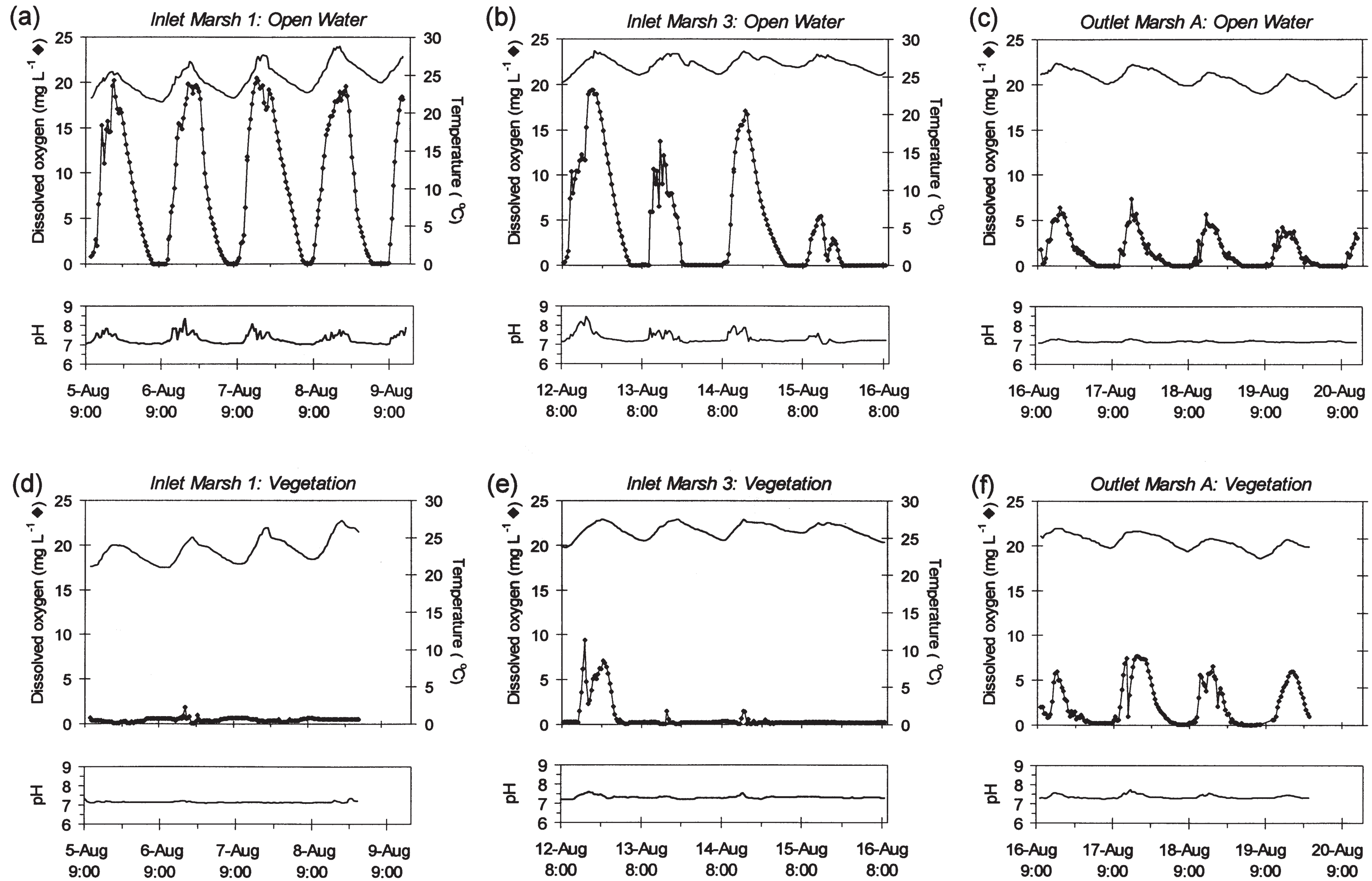

Figure 4. Dissolved oxygen concentration, water temperature and pH at sites in open water and within the vegetation near cages containing sentinel threespine stickleback in three marshes within the HSJRWRF wetland in San Jacinto, CA, during 1996. a: Inlet Marsh 1, open water; b: Inlet Marsh 3, open water; c: Outlet Marsh A, open water; d: Inlet Marsh 1, vegetation; e: Inlet Marsh 3, vegetation; f : Outlet Marsh A, vegetation.

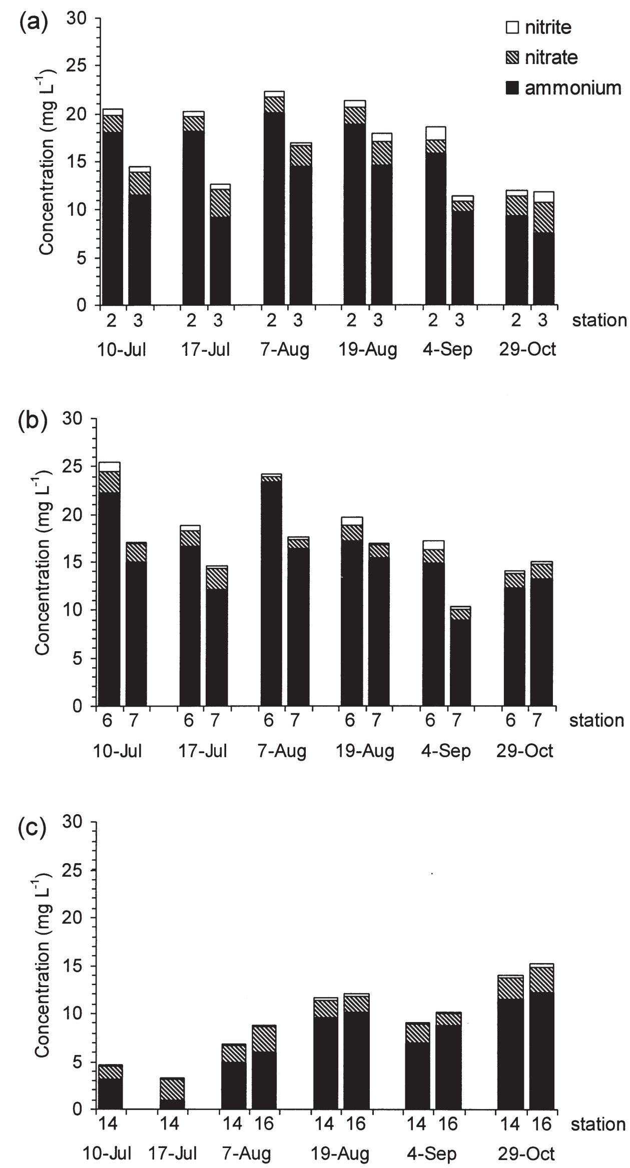

Figure 5. Concentration of inorganic nitrogen compounds in three marshes of the HSJRWRF wetland, San Jacinto CA, during 1996. a: Inlet Marsh 1, b: Inlet Marsh 3, c: Outlet Marsh A. Station refers to the sampling sites illustrated in Figure 1.

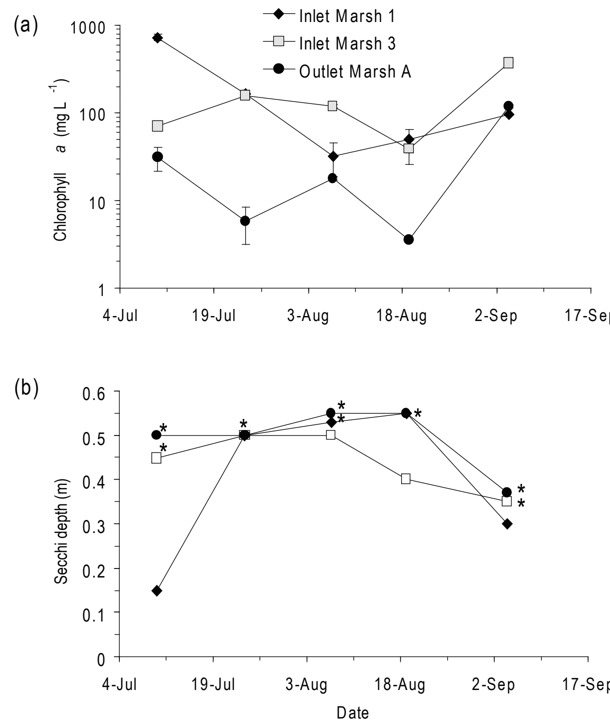

Figure 6. Phytoplankton biomass (a: total chlorophyll *a*) and water column transparency (b: Secchi depth) in the open water zone adjacent to cages holding sentinel fish in the HSJRWRF wetland during 1996. * indicates that the Secchi disk was visible on the bottom of the marsh.

the majority of the wetland's substrate surface unavailable for successful nest-building activities by sticklebacks.

Survival of sentinel *G. aculeatus* in the inlet marshes was lower than in the polishing marsh, Outlet Marsh A, and was related to changes in water quality from the inflow to the outflow of the treatment wetland. The mean NH_4-N concentration (<6 mg liter^{-1}) and suspended matter (measured by chlorophyll *a* biomass and water transparency) were low in the open water zone of the outlet marsh during the summer; stickleback survival was high throughout most of the study. Stickleback survival in Inlet Marsh 1 was the lowest among the three marshes where sentinel fish cages were positioned and survivorship declined rapidly at the beginning of the study. Stickleback survival declined markedly in Inlet Marsh 3 after two months. The declines in fish survival in the inlet marshes were associated with comparatively high concentrations of NH_4-N (mean: >16 mg liter^{-1}), suspended matter (shallow Secchi depth and algal biomass > 300 mg chlorophyll *a* liter^{-1}), and residual chlorine.

The high dissolved oxygen concentration and concomitant increase of pH associated with sustained primary production in the inlet marshes shifted the chemical equilibrium from ammonium to un-ionized ammonia creating potentially toxic levels of ammonia (>0.5 mg NH_3-N liter^{-1}) for the sticklebacks. The rates of oxygen production and demand in the water column did not differ significantly among the three marshes studied (unpublished data), but autotroph biomass in the water column varied over 100-fold in each marsh. The daily maximum dissolved oxygen concentration in open water zones (<13 mg O_2 liter^{-1} vs. ~19.5-20.5 mg O_2 liter^{-1} for outlet vs. inlet marshes) was directly related to algal biomass during the period that sentinel fish were monitored.

Factors known to affect the toxicity of ammonia to fish include dissolved oxygen concentration, temperature, pH, previous acclimation to ammonia, fluctuating or intermittent exposures to ammonia, carbon dioxide concentration, salinity, and concentrations of other toxicants (e.g., metals such as Al, Cu, Ni and Zn) (Hazel et al. 1971, Thurston et al. 1981, Nimmo et al. 1989). The 96-h LC_{50} for guppy (*Poecilia reticulata* Peters) juveniles was 1.24 mg NH_3 liter^{-1} (Ruffier et al. 1981). The 96-h LC_{50} for fathead minnow, *Pimephales promelas* Rafinesque, in well water (1.12 mg NH_3 liter^{-1}) was similar to *P. reticulata* but was reduced by 50% in municipal wastewater (0.56 mg NH_3 liter^{-1}: Nimmo et al. 1989). The chronic value for this species was 0.13 mg NH_3 liter^{-1}. Swanson et al. (1996) recommended that un-ionized ammonia nitrogen above 0.1 mg liter^{-1} should be avoided in culture systems of *G. affinis*. The 96-h TL_m (median tolerance limit) for *G. aculeatus* juveniles and adults in freshwater at 15° and 23° C were 2.1 and 1.8 mg undissociated NH_4OH liter^{-1} (Hazel et al. 1971); using the formula of Denmead et al. (1982) and substituting the values in Table 6 of Hazel et al., these levels are equivalent to LC_{50}s of 0.58 and 0.38 mg NH_3 liter^{-1}, respectively.

Residual chlorine in the influent water and the interaction of disinfection by-products with ammoniacal nitrogen and wetland-derived organic matter can create toxic conditions for fish and other aquatic organisms in constructed treatment wetlands where NH_4-N levels are elevated. Other compounds commonly found in wastewater, such as endocrine disruptor chemicals, can be detrimental to fish (Bell 2001, 2004, Pottinger et al. 2002, Wibe et al. 2002). The results of regression studies carried out in our study show that the interaction between residual chlorine and un-ionized ammonia was strongly related to stickleback mortality within sampling periods. If dechlorination of the influent water to a treatment wetland is not fully effective or the influent water supply is contaminated by chlorinated water, then chlorine and halogenated compounds (e.g., chloramines, trihalomethane, or other disinfection by-products; Nimmo et al. 1989, MESC, NBS, and CSU 2000[1], Rostad et al. 2000) can enter a constructed treatment wetland. Even though the concentrations of potentially toxic disinfection by-products released from water treatment plants decline as water moves through constructed treatment wetlands, enhanced concentrations of wetland-derived organic carbon (Sartoris et al. 2000, Barber et al. 2001) are thought to increase dramatically (~30-fold) the potential for toxic effects from disinfection by-products (Rostad et al. 2000).

Differences in the natural cycles of bulrush dieback may have contributed to the differences in survival of sticklebacks in the two inlet marshes. Despite being situated at a greater distance from the influent weir of Inlet Marsh 1 where the effects of chlorine contamination might be lessened as compared to being positioned closer to the influent weir of Inlet Marsh 3, *G. aculeatus* in Inlet Marsh 1 exhibited mortality higher than did the fish in Inlet Marsh 3. High mortality of sentinel sticklebacks might have been related to the interaction of disinfection by-products with higher levels of wetland-derived organic matter being released from a natural mid-winter (1995-1996) dieback of *S. acutus* that did not occur in *S. californicus*-dominated Inlet Marsh 3.

Reduced survival in inlet marsh water also was observed in other fish species. During toxicity studies at the HSJRWRF wetland in mid-May 1996, 100% of *P. promelas* juveniles succumbed in water collected near the weirs of the five inlet marshes and all fish survived in water collected from the outlet marshes (MESC, NBS and CSU 2000[1]). Organic or oxidizable compounds such as chloramines were thought to have contributed to the toxicity of inlet water (MESC, NBS and CSU 2000[1]). Subsequent bioassays in March 1999, when residual chlorine was not detected in the influent water, failed to show toxicity of inlet water to fathead minnows at NH_4-N concentrations between 9 and 14 mg liter^{-1} (Castle 1999[2]). Survival of sentinel mosquitofish also was lowest in Inlet Marsh 1; however, *Gambusia* survivorship in Inlet Marsh 3 and Outlet Marsh A was 100% (Walton et al. 1997). Stickleback survival was lower than that of mosquitofish in each of the three marshes of the HSJRWRF wetland.

Water temperatures in the HSJRWRF wetland were between 21° C and 29° C during the period that sentinel fish were monitored and did not exceed the upper thermal tolerance of the sticklebacks. The thermal tolerance of *G. aculeatus* varies among populations and is related to the ambient temperatures found in the native habitat and to the acclimation temperature (Jordan and Garside 1972, Blahm and Snyder 1975). The upper lethal temperature for warm-adapted sticklebacks collected from the Santa Clara River in southern California was 34.6° C (Feldmeth and Baskin 1976). Offill and Walton (1999) found that mortality of *G. aculeatus* collected from the Mojave River (San Bernardino County, CA) increased when the maximum water temperature of earthen ponds exceeded 33° C. The sticklebacks used in the present study were collected from the San Jacinto River (Riverside County, CA) and were expected to have an upper thermal tolerance (~34° C) that was similar to those observed in the other stickleback populations collected from shallow lotic environments of southern California.

The catastrophic mortality of sticklebacks associated with blooms of the net-like green algae, *Hydrodictyon* sp., is attributed to an unforeseen effect of the cages rather than a natural phenomenon in the open water zones of the constructed treatment wetland. Blooms of water net occurred in one replicate cage in each marsh and rapidly filled the water column of each cage. The natural mid-water activities of the stickleback (Schooley and Page 1984) were severely restricted by dense blooms of *Hydrodictyon*.

[1]MESC, NBS, and CSU (Midcontinent Ecological Science Center, National Biological Service and Colorado State University). 2000. Hemet/San Jacinto Regional Water Reclamation Facility: Demo Wetlands Toxicity Characterization. Phase 1 Report, May 1996. U. S. Geological Survey, U. S. Dept. Interior, Denver, CO. 11 pp.

[2]Castle, C. J. 1999. Hemet/San Jacinto Regional Water Reclamation Facility: Demo Wetlands Toxicity Assessment. Acute Baseline Conditions of Constructed Wetlands - Phase 1 Report, March 1999. U. S. Geological Survey, U. S. Dept. Interior, Denver, CO. 7 pp.

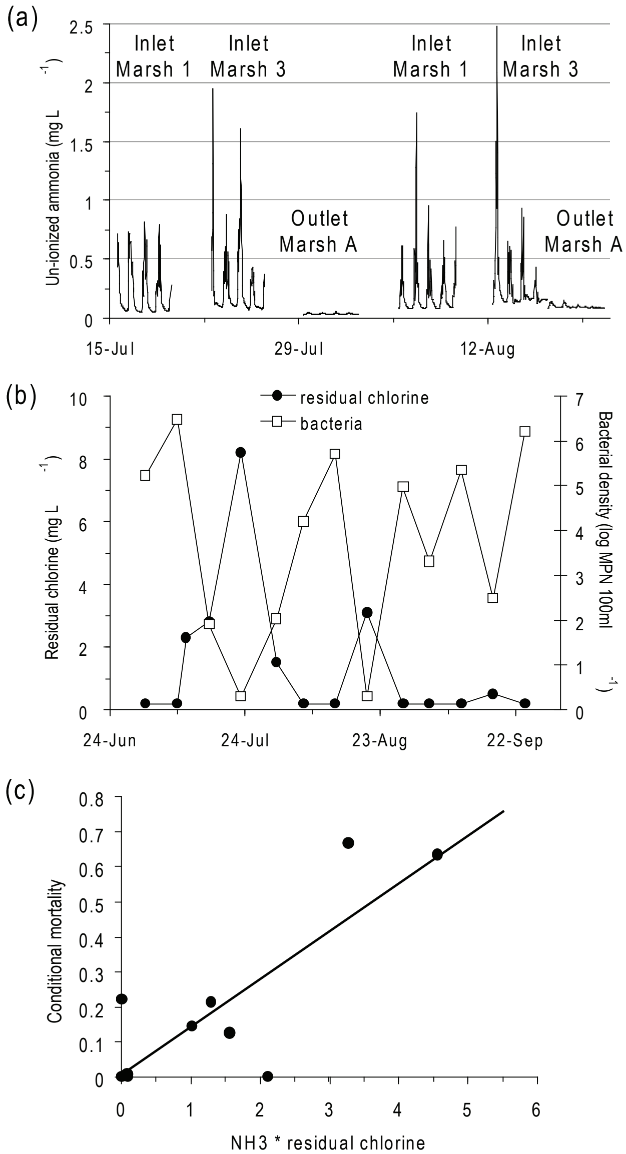

Figure 7. Factors associated with mortality of sentinel sticklebacks in the HSJRWRF wetland during the summer, 1996. a: estimated un-ionized ammonia concentration in the open water regions of marshes, b: residual chlorine concentration and coliform bacteria abundance in the influent water, c: conditional mortality of sentinel sticklebacks vs. the interaction of un-ionized ammonia and residual chlorine concentration.

Normal swimming activities of warm-adapted sticklebacks occurred at dissolved oxygen concentrations >2 mg L^{-1} (Feldmeth and Baskin 1976). Under hypoxic conditions, *G. aculeatus* gulp air/water at the air-water interface while either swimming parallel to the water surface or bobbing with the body maintained at a comparatively steep angle with respect to the water surface (Whoriskey et al. 1985, Walton et al. 1997); both behaviors would have been restricted by the dense blooms of water net. The moribund fish were entangled in the net-like algae throughout the water column and appeared to be very robust suggesting that the onset of mortality was rapid asphyxia. Large blooms of *Hydrodictyon* were never observed outside of the cages. Water movement, shading by dense planktonic algal populations in the inlet marshes, or herbivory by waterfowl may limit *Hydrodictyon* from proliferating in the open water outside the cages.

Because low dissolved oxygen concentration was observed in each of the three marshes in the HSJRWRF wetland, differences of fish survival in cages where *Hydrodictyon* was not present were unlikely to have been caused by hypoxia. Dissolved oxygen concentration in the water column of the three marshes was severely depressed at night and in the early morning. Dissolved oxygen levels were extremely low (~0 mg O_2 liter^{-1}) at all times at >5 m into the bands of vegetation and as close as 2 m from the open water-vegetation interface in the inlet marshes. Rose and Crumpton (1996) also found persistent oxygen depression in stands of emergent vegetation in a prairie pothole wetland. Unlike an Iowa wetland where open water areas adjacent to emergent vegetation were usually well-mixed (Rose and Crumpton 1996), the high oxygen demand from nitrification in the HSJRWRF wetland created stressful conditions for sticklebacks in open water zones adjacent to vegetation, particularly in the inlet marshes. Jones (1964) reported that sticklebacks can survive at dissolved oxygen concentrations as low as 0.25-0.5 mg liter^{-1} but when fish were exposed to water with different dissolved oxygen levels at 13° C they moved from water with oxygen concentration <3 mg liter^{-1} to water with higher dissolved oxygen concentration. Whoriskey et al. (1985) found that sticklebacks were able to survive periods of hypoxia when water temperature was <29° C; a similar result was found in the current study. The sticklebacks were able to withstand periods of oxygen deprivation in the treatment wetland provided there was access to the water surface.

Even though individuals are capable of surviving periods of hypoxia in the HSJRWRF wetland, it is projected that the wetland would provide very few habitats for stickleback reproduction. The periphery of the central pond in the HSJRWRF wetland may be the only site that provides sufficient dissolved oxygen concentration for stickleback males to build nests and tend developing eggs. Low dissolved oxygen concentrations found throughout most of the vegetated zones and found periodically in the open water areas between rows of vegetation in the marshes of the constructed treatment wetland would force a male stickleback to abandon the nest in order to meet his metabolic needs and probably kill the developing embryos in the eggs. Dissolved oxygen concentration in the central pond is enhanced by wind-driven mixing and can be >2 mg liter^{-1} (Sartoris et al. 2000, Smith et al. 2000) for periods sufficient to complete incubation of eggs (Wootton 1984).

In addition to few favorable nesting sites and the toxicity of constituents in municipal wastewater, other mortality factors limit the suitability of this constructed treatment wetland as a habitat for *G. aculeatus*. Sticklebacks inhabiting cool, well-oxygenated lakes or streams can reside well below the water surface; however, sticklebacks living in warm, hypoxic waters of constructed treatment wetlands

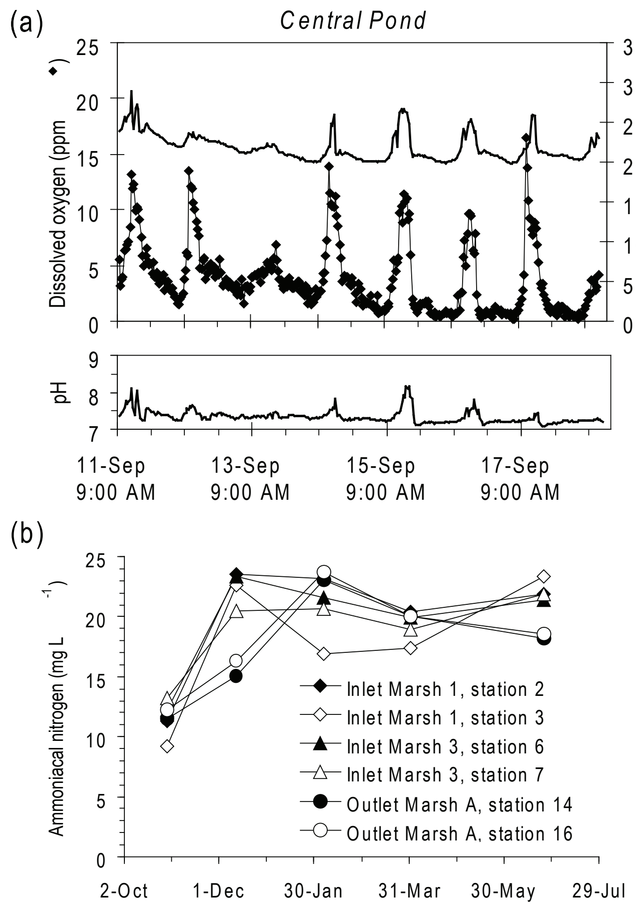

Figure 8. Physico-chemical factors along the periphery of the central pond during September 1996 and in three marshes between October 1996 and June 1997 in the HSJRWRF wetland, San Jacinto, CA. a: dissolved oxygen concentration, water temperature and pH; b: ammoniacal nitrogen concentration.

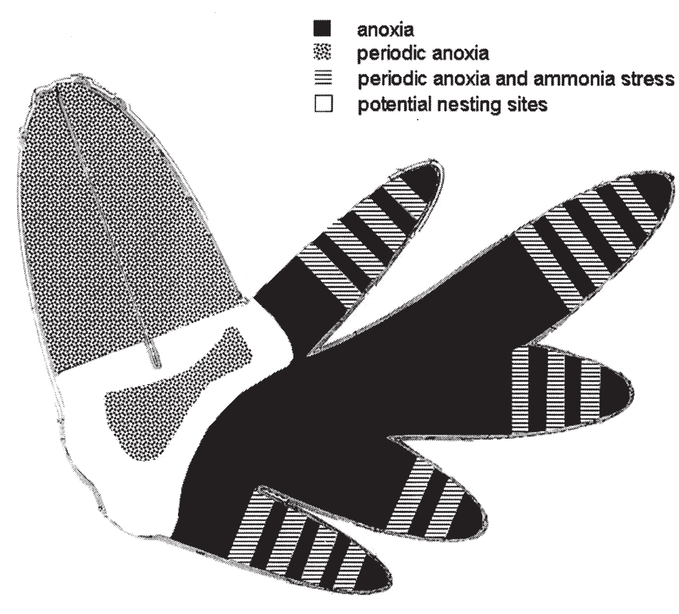

Figure 9. Schematic illustration of conditions affecting sites for stickleback reproduction in the HSJRWRF wetland during summer 1996.

in the arid southwestern United States must reside near the water surface and are at a greater risk of bird predation. Bird predation is a significant mortality factor for stickleback populations residing in natural wetlands and mortality increases appreciably where water depths are < 0.2 m (FitzGerald and Wootton 1993). The HSJRWRF wetland supports large populations of piscivorous birds (e.g., herons and egrets: Anderson et al. 2003). In wetlands containing piscivorous fish, hypoxia can limit the distribution of young fish to regions with little protective cover and reduce the survival of juvenile fish (Suthers and Gee 1986).

Seasonal and successional changes in the wetland can alter the nutrient removal efficiency of constructed treatment wetlands, exposing fish to potentially toxic levels of municipal wastewater constituents that fluctuate seasonally and increase temporally. Lower nitrogen removal efficiency associated with internal nitrogen loading by dense emergent macrophyte populations increased NH_4-N concentration in the outlet marshes (Sartoris et al. 2000) and phytoplankton biomass in open water zones (Smith et al. 2000). Ammoniacal nitrogen levels increased during autumn (Figure 8b) and remained comparatively high until spring. Warm-water aquatic communities were one and one-half to six times more sensitive to the toxic effects of ammonia at cold versus warm temperatures (Nimmo et al. 1989). The high ammoniacal nitrogen levels observed during the autumn and winter 1996 and in early 1997 would presumably be detrimental to overwintering sticklebacks.

Environmental factors associated with ammonia-rich municipal effluent entering a constructed treatment wetland in southern California are predicted to limit the suitability of the wetland as a habitat for the stickleback, an alternative to the mosquitofish as a biological control agent of mosquitoes. Both external and internal loading of potential toxins influenced the survival of sentinel *G. aculeatus*. If larvivorous/planktivorous fish species are to be incorporated into integrated pest management programs for mosquitoes inhabiting constructed treatment wetlands, then species with broad environmental tolerances will be needed in wetlands that treat ammonia-dominated municipal effluent. The functional and numerical responses of the fish populations are also important; mosquitoes should be an important component of the fish's diet and the habitat should be conducive to rapid increases of fish abundance following stocking and natural reductions in population size. The stickleback is commonly found in streams and rivers of southern California (Swift et al. 1993) and may be an effective larvivore of mosquitoes inhabiting the comparatively cool and high quality water found in riverine wetlands. For constructed treatment wetlands treating ammonia-rich municipal effluent that are effectively isolated from natural aquatic ecosystems, the mosquitofish is likely to be more effective than the stickleback as a biological control agent for mosquitoes.

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