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Eutrophication and Bacterial Pathogens as Risk Factors for Avian Botulism Outbreaks in Wetlands Receiving Effluents from Urban Wastewater Treatment Plants

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Due to the scarcity of water resources in the "Mancha Húmeda" Biosphere Reserve, the use of treated wastewater has been proposed as a solution for the conservation of natural threatened floodplain wetlands. In addition, wastewater treatment plants of many villages pour their effluent into nearby natural lakes. We hypothesized that certain avian pathogens present in wastewater may cause avian mortalities which would trigger avian botulism outbreaks. With the aim of testing our hypothesis, 24 locations distributed in three wetlands, two that receive wastewater effluents and one serving as a control, were monitored during a year. Sediment, water, water bird feces, and invertebrates were collected for the detection of putative avian pathogenic *Escherichia coli* (APEC), *Salmonella* spp., *Clostridium perfringens* type A, and *Clostridium botulinum* type C/D. Also, water and sediment physicochemical properties were determined. Overall, APEC, *C. perfringens*, and *C. botulinum* were significantly more prevalent in samples belonging to the wetlands which receive wastewater. The occurrence of a botulism outbreak in one of the studied wetlands coincided with high water temperatures and sediment 5-day biochemical oxygen demand (BOD_5), a decrease in water redox potential, chlorophyll *a*, and sulfate levels, and an increase in water inorganic carbon levels. The presence of *C. botulinum* in bird feces before the onset of the outbreak indicates that carrier birds exist and highlights the risk of botulinum toxin production in their carcasses if they die by other causes such as bacterial diseases, which are more probable in wastewater wetlands.

Water management is an essential aspect for the sustainable development of semiarid Mediterranean regions such as the "Mancha Húmeda" Biosphere Reserve in the Central Spanish Plateau, where recent drought periods have stressed the balance between crop irrigation and the conservation of wetlands (1) such as Las Tablas de Daimiel National Park, the most representative Spanish floodplain ecosystem (2, 3). In this situation of growing water scarcity, the use of treated wastewater has been proposed for the maintenance of Tablas de Daimiel National Park and to conserve its biodiversity (4). Within this region, many wastewater treatment plants already spill their effluents into natural lakes due to the absence of rivers near villages and also to maintain water bird populations. On the one hand, these wastewater lakes provide permanent resting and breeding areas for many species of water birds, including endangered species as the white-headed duck (*Oxyura leucocephala*), but on the other hand, pouring wastewaters has modified the ecology of the lakes in many ways, decreasing salinity, increasing eutrophication, and attenuating their natural hydrological cycles of drought-flooding periods (5). Moreover, this practice constitutes a health risk for birds because improperly treated effluents are an important source of pollutants and microorganisms, including avian bacterial pathogens (6, 7). A recent study revealed the role of invertebrates living on sewage filter beds as vectors of environmental pollutants that can affect immune function, neural development, and behavior in male starlings (*Sturnus vulgaris*) (8). Previous research linked the deaths of brown pelicans (*Pelecanus occidentalis*) with the presence of pathogenic clostridia on raw sewage discharges (9). Later on, enterocolitic bacteria, mainly *Salmonella*, were isolated from up to 58% of sick or dead water birds collected during the summer in a Mediterranean wetland (10). Strikingly, avian botulism outbreaks in the Mancha Húmeda frequently occur in lakes supplied with effluents from wastewater treatment plants (11).

Avian botulism is an intoxication produced by botulinum neurotoxins (BoNTs) which results in a flaccid paralysis of the muscles and the death of the affected birds. BoNTs are exotoxins produced by *Clostridium botulinum*, a strictly anaerobe spore-forming bacteria which is present in the sediments of wetlands and in the digestive tracts of water birds and fishes (12, 13). There are seven confirmed types of BoNTs (types A to G); the most frequent in Europe is mosaic type C/D (14, 15). High water temperatures and increases in the invertebrate biomass, which frequently occur in wastewater ponds, may attract water birds for feeding and provide optimal conditions for the occurrence of botulism outbreaks, although there is still not enough evidence to support this hypothesis (16). Other factors such as pH between 7.5 and 9, low redox potential (Eh), decreasing turbidity, and low salinity also contribute to botulism risk in wetlands (11, 16, 17). Avian mortalities due

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to any cause have the potential to be a major initiating factor of botulism outbreaks because they provide carcasses for the initial multiplication and toxinogenesis of *C. botulinum* (18). In this framework, urban wastewater that enters the wetlands has to be considered a potential source of avian pathogenic bacteria that may cause mortalities among a few water birds, with the risk of initiating a botulism outbreak affecting larger numbers of individuals.

With this study, we aimed (i) to evaluate the risk for water birds derived from the presence of pathogenic bacteria in wetlands that receive wastewater and (ii) to study possible changes in the ecological characteristics of the wetlands produced by wastewater and how these changes may favor the presence of *C. botulinum* type C/D and the outbreaks.

MATERIALS AND METHODS

Study area. The study area included wetlands located in the Mancha Húmeda Biosphere Reserve, a region situated in south-central Spain that covers a total surface of 25,000 ha (see Fig. S1 in the supplemental material). The climate in this area (~600 m above sea level) is cold-temperate continental, with a pronounced dry season and average annual rainfall between 400 to 500 mm. Within this region, we monitored three wetlands affected to different degrees by the effluent from urban wastewater treatment plants as follows (in the order of least to most affected): Tablas de Daimiel National Park (TDNP), Veguilla lake, and Navaseca lake. TDNP is a floodplain located at the junction of Cigüela and Guadiana rivers with a maximum flooded surface of 1.675 ha (19). This wetland occasionally receives the input of poorly treated wastewater from towns located upstream. Navaseca lake (24.3 ha) is located in the vicinity of TDNP (at a distance of about 6.5 km). This lake was seasonal in the past, but now it is permanently flooded and highly eutrophic because it receives the effluents of the wastewater treatment plant of Daimiel town. Veguilla lake (128 ha) is a semiartificial saline and seasonal wetland included in the complex of Alcázar de San Juan Natural Reserve (695 ha). It receives discontinuous inputs from the wastewater treatment plant of Alcázar de San Juan town with the purpose of maintaining water bird breeding populations. The effluent is alternatively discharged into a floodplain at the junction of Záncara and Cigüela rivers, especially in summer, when the lake is naturally desiccated to avoid avian botulism outbreaks. There is a landfill near Veguilla where some bird species that inhabit the lagoon (i.e., gulls [*Larus* sp.] and white storks [*Ciconia ciconia*]) usually feed. This is a region of endemicity for botulism, and in the last 20 years, outbreaks have occurred in the three studied wetlands, with the outbreaks being more frequent in Veguilla and Navaseca lakes (11).

Field sampling and data collection. Each wetland was sampled on seven occasions from April 2010 to February 2011. In each visit, samples of surface and interstitial water, sediment, aquatic invertebrates, carrion flies, and water bird feces were collected. Samplings were performed once per season in spring (April), autumn (November), and winter (February) and monthly during summer (June to September), when botulism outbreaks are more likely. An additional sampling visit to Navaseca lake was performed at the end of July during the onset of a botulism outbreak (see Fig. S2 in the supplemental material).

Water and sediment samples were collected in 12 sampling stations in TDNP, 6 in Navaseca, and 6 in Veguilla. Sampling stations were broadly distributed within each wetland shore to obtain wide coverage. Sediment samples were collected from the upper 5 cm at three random positions with a metal core sampler 7 cm in diameter, and pooled subsamples were kept in plastic bags with zip closure. Interstitial water samples were collected in three 15-ml vacuum tubes, each one with Rhizom soil moisture samplers (Eijkelkamp Agrisearch Equipment). Approximately 1,500 ml of surface water was collected per site, and 300 ml of it was filtered in the field with Whatman GF/C filters (GE Healthcare Ltd.) in order to preserve its stability against biogeochemical changes until processing in the laboratory was performed. Filtered material was weighed to calculate the seston (total and volatile solids), and filtered water was used for the analysis of nutrients (NO_2^-, NO_3^-, NH_4^+, PO_4^{3-}), total organic carbon (TOC), and major ions (SO_4^{2}, Cl^-, Ca^{2+}, Na^+, K^+, Mg^{2+}), while gross surface water was used for 5-day biochemical oxygen demand (BOD_5) and alkalinity determinations. Besides, three replicate measurements of temperature, redox potential (Eh), pH, dissolved oxygen, chlorophyll *a*, turbidity, and conductivity of surface water were taken *in situ* with a Hydrolab DS5X multiprobe (Hach Hydromet). Water depth was measured with a stiff meter (see Fig. S2 in the supplemental material). Water and sediment samples were maintained under dark conditions at 4°C until processing in the laboratory so as to avoid alterations. During the summer, water samples could not be collected from some stations within TDNP and Veguilla because they were dry.

Aquatic invertebrates and carrion flies were collected in the same sampling stations. Aquatic invertebrates were captured from the water with a sieve of 0.5-mm-pore-size mesh and kept alive in sterile plastic containers. Carrion flies were captured using homemade cone traps that were hung on the shore vegetation of the wetlands for 24 h. After that time, the traps were closed, taken to the laboratory, and frozen to kill the trapped flies. Aquatic invertebrates and flies were identified and pooled according, at least, to the taxonomic family. Samples of aquatic invertebrates included specimens of nonbiting midge larvae (*Chironomidae*), water boatmen (*Corixidae*), backswimmers (*Notonectidae*), crustaceans (*Ostracoda*, *Copepoda*, and *Cladocera*), bladder snails (*Physidae*), and beetle larvae (*Coleoptera*). Samples of flies included specimens of the families *Calliphoridae*, *Sarcophagidae*, *Ephydridae*, *Anthomyiidae*, *Muscidae*, and *Ulidiidae*.

Fresh water bird feces ($n = 30$) were collected on each wetland per sampling visit. Before samples were taken, the distribution of water birds on the shore of the wetlands was observed in order to assign the feces collected in each site to a taxonomic group. The samples were collected under aseptic conditions using sterile swabs and plastic bags. Additionally, gull feces were also collected in a landfill beside Veguilla on May 2010 (see Fig. S2 in the supplemental material). The sampled families included *Anatidae* (ducks and geese), *Rallidae* (rails), *Scolopacidae* (waders), *Laridae* (gulls), *Ciconiidae* (storks), *Phoenicopteridae* (flamingos), and *Ardeidae* (herons).

Chemical analysis of water and sediment samples. Sediment samples were analyzed for water and organic matter content (loss on ignition [LOI]), pH, and conductivity following standardized procedures (20). BOD_5 was measured with an OxiTop system (WTW). Surface water samples were analyzed for total and volatile solids, BOD_5, total alkalinity, NO_2^-, NH_4^+, PO_4^{3-}, SO_4^{2-}, Cl^-, Ca^{2+}, Na^+, K^+, and Mg^{2+} following colorimetric methods and NO_3^- with an ion-selective electrode (Metrohm), and inorganic, organic, and total carbon levels were measured with a total organic carbon analyzer (TOC-VCSN; Shimadzu) (21, 22). The same parameters were determined in interstitial water samples, except total and volatile solids, alkalinity, and BOD_5 (see Fig. S2 in the supplemental material).

Microbiological analysis. Microbiological analysis included three avian bacterial pathogens associated with wastewater and *C. botulinum* type C/D. The avian bacterial pathogens were avian pathogenic *Escherichia coli* (APEC), *Salmonella* spp., and *C. perfringens* type A (6, 16). For APEC detection in sediment and feces, samples were streaked directly onto MacConkey agar plates (Scharlau) and incubated at 37°C for 24 h. For surface water, 100-ml samples were filtered through a 0.45-μm-pore-size filter (Millipore) using a filter holder manifold and glass filter holders (Nahita; Auxilab), and then the filter was placed into 9 ml of buffered peptone water (BPW) broth (Scharlau) and incubated at 37°C for 24 h; afterward, 1 ml was streaked onto MacConkey agar plates. Isolation and identification of APEC were performed by multiplex PCR following a method previously described (23, 24). Briefly, an initial gross screening of APEC was done in the growth from the first streaking area of the culture plate so as to discard negative samples. The presence of APEC was tested by multiplex PCR for the following five virulence genes highly conserved

among APEC isolates: the aerobactin siderophore receptor gene (*iutA*), the episomal increased serum survival gene (*iss*), the episomal outer membrane protease gene (*ompT*), the putative avian hemolysin gene (*hlyF*), and the salmochelin siderophore receptor gene (*iroN*). In accordance with Johnson et al. (24), *E. coli* isolates can be considered APEC when they contain at least 4 of these 5 genes. DNA was extracted by boiling 10 μl of the first streaking area in 200 μl sterilized ultrapure water for 5 min, the solution was centrifuged at 12,000 × *g* for 5 min, and the supernatant was used as the PCR template. Afterward, for each PCR-positive culture, 10 individual *E. coli*-like colonies obtained from the MacConkey agar plates were tested again for the presence of the 5 virulence genes and were considered putative APEC if they harbored at least four. The resulting putative APEC isolates were confirmed biochemically as *E. coli* by the API 20E system (bioMérieux) (see Fig. S2 in the supplemental material).

Detection of *Salmonella* spp. was done according to international official standardized procedures (25). Briefly, samples of sediment and feces were preenriched in 9 ml of Rappaport-Vassidialis (RV) broth (Oxoid) and incubated aerobically for 48 h at 42°C. Surface water was analyzed using the same filtered water used for APEC, and 1 ml of the BPW broth was passed to 9 ml of RV broth. At 24 and 48 h, 1 ml of the culture in RV broth was streaked onto XLT4 agar (Biokar Diagnostics) plates and incubated for 24 h at 37°C. The resulting suspected isolates were confirmed biochemically to be *Salmonella* spp. by the use of an API 20E system (bioMérieux) (see Fig. S2 in the supplemental material).

For the detection of *Clostridium perfringens* type A (containing the *cpa* alpha-toxin gene), sediment and feces samples were directly streaked onto tryptose sulfite cycloserine agar (TSCA) (Scharlau). For surface water, 100-ml samples were filtered through a 0.45-μm-pore-size filter (Millipore) which was placed directly onto TSCA plates. All the samples were then incubated anaerobically in an anaerobe container system (BD GasPakTM 129 EZ) at 37°C for 48 h. Suspected individual black colonies were streaked onto a new TSCA plate and incubated as described before. Then, six toxin genes (*cpa* alpha-toxin, *cpb* β-toxin, *cpb2* β2-toxin, *etx* e-toxin, *iap* ι-toxin, and *cpe* enterotoxin) were detected using the PCR assay described by van Asten et al. (26) and colonies containing the alpha-toxin gene were classified as *C. perfringens* type A. Only the samples collected during the first three sampling visits were tested for *C. perfringens*.

Sediments, surface water (as processed for APEC), aquatic invertebrates, fly pools, and bird feces were processed for detection of *C. botulinum* type C/D as previously described by Vidal et al. (27). Briefly, samples were cultured in 9 ml of commercial cooked meat broth supplemented with vitamin K_1, glucose, and hemin (BD BBL cooked meat medium with glucose, hemin, and vitamin K) using an anaerobe container system (BD GasPakTM 129 EZ) over a period of 3 to 5 days at 40°C. DNA was extracted by boiling the pellet obtained from 1 ml of the culture broth in 300 μl of distilled water. The solution obtained after centrifugation at 12,000 × *g* for 5 min was used as the PCR template. Real-time PCR was performed according to the method of Sánchez-Hernández et al. (28) (see Fig. S2 in the supplemental material). This PCR assay amplifies genes encoding both type C and type C/D mosaic toxins, but as we have confirmed the presence in the study area of the mosaic type C/D whereas we have not detected type C (11, 15), we assumed that type C/D is the causative agent. Furthermore, the mosaic type prevails in avian botulism outbreaks in Europe (14) and the botulism outbreak that occurred in Navaseca was confirmed as type C/D by the mouse bioassay.

Statistical analysis. Mean values of physicochemical parameters from sediments and water were compared among wetlands with one-way analysis of variance (ANOVA) tests. *Post hoc* differences were studied with the least significant difference (LSD) test. The frequencies of detection of the four pathogens in sediment, water, aquatic invertebrates, fly pools, and bird feces from the three wetlands were compared by chi-square tests or Fisher's exact probability tests. Associations between the presence of the APEC, *C. perfringens*, and *C. botulinum* and the physicochemical characteristics of water and sediments were studied using generalized linear models (GLM) with a binary logistic distribution. The number of samples positive for *Salmonella* spp. in sediments and water was too low for those samples to be included in this analysis. The absence or presence of the bacteria (negative or positive) was used as the dependent variable, while the physicochemical characteristics of sediments and water as predictors and zone (wetland) and season were used as fixed factors. Physicochemical characteristics that had less than 150 observations for models of *C. botulinum* and APEC and less than 70 for *C. perfringens* were not included in the GLM models so as to guarantee a minimum sample size. Nonnormally distributed variables were transformed to logarithm ($\log_{10}$) values to fulfill the normality requirements of parametric tests. The distribution of the four pathogens (including *Salmonella* spp.) in bird feces was also studied with GLM using "wetland," "season," and "bird family" as fixed variables and the presence of each pathogen as the dependent variable. The corrected Akaike's information criterion (AICc) was used to compare alternative models (29). To select the best model, all possible combinations of fixed effects were compared by using the "dredge" function (30), and models with differences of less than 2 AICc points from the best value ($\Delta AICc = 0$) were considered to have the same empirical support (29). The level of significance of the tests was established at $P < 0.05$. The analyses were conducted with IBM SPSS Statistics 19 software and R 2.12.2 (R Core Team 2010; used only for AICc).

RESULTS

Physicochemical parameters of water and sediment. Most of the physicochemical parameters measured in surface water significantly differed among wetlands (Table 1). Navaseca and Veguilla showed signs of eutrophication and degradation due to the input of treated wastewaters as indicated by higher turbidity, total and volatile solids, BOD_5, total carbon, and NH_4^+ and PO_4^{3-} concentrations. In addition, Navaseca showed higher levels of chlorophyll *a* and dissolved oxygen and lower water Eh. Veguilla revealed the highest values of conductivity, Cl^-, SO_4^{2-}, K^+, Na^+, and Mg^{2+} due to the natural salinity present in this area. Analogous trends were observed for the parameters measured in interstitial water and sediment (Table 2). In addition, the NO_3^- concentration in interstitial water was higher in the lakes receiving treated wastewater.

Prevalence of avian pathogenic bacteria and *C. botulinum* type C/D. The prevalences of APEC, *C. perfringens* type A, and *C. botulinum* type C/D observed in most environmental samples were considerably higher in Navaseca and Veguilla than in TDNP (Table 3). APEC was mainly present in water bird feces (16.9% to 35.6%); *C. perfringens* was widely distributed in water (16.7% to 62.5%), sediment (11.1% to 83.3%), and water bird feces (59.9% to 90.4%); and *C. botulinum* mainly appeared in sediment samples (2.4% to 20.8%) and *Salmonella* spp. in a small number of feces samples collected in Veguilla (3.6%). In the gull feces collected from the landfill close to Veguilla, we detected the highest prevalences of *Salmonella* (30%) and APEC (37%) and the lowest prevalence of *C. perfringens* type A (30%), while *C. botulinum* type C/D was absent. The observed prevalences of *C. perfringens* and APEC in water bird feces from the three wetlands significantly differed among bird taxa. The highest prevalence of *C. perfringens* was detected in feces of *Laridae* (80.5%), *Anatidae* (70.5%), and *Rallidae* (70%) and the highest prevalence of APEC in feces of *Ciconidae* (61.9%). Finally, though the differences were not statistically significant, *Salmonella* appeared more frequently in feces of *Laridae* (3.5%) and *Ciconiidae* (9.5%) and *C. botulinum* in feces of *Rallidae* (2.9%; see Table S1 in the supplemental material).

Parameters associated with bacterial occurrence. Regression models with $\Delta AICc < 2$ revealed an association between several environmental factors and the presence of the studied bacteria in

TABLE 1 Physicochemical parameters of surface water from three wetlands differing in levels of wastewater effect (where Navaseca lake was the most affected and TDNP the least affected)[a]

Parameter	Wetland value									
	Navaseca			Veguilla			TDNP			
	n	Mean	SD	*n*	Mean	SD	*n*	Mean	SD	*P*
Maximum depth (cm)	12	29.5^{A}	3.53	18	25.2^{B}	3.29	29	41.0^{A}	3.92	0.01
Temp (°C)	48	21.8^{A}	8.14	38	17.6^{B}	7.41	78	19.1^{AB}	7.37	0.03
Conductivity (μS/cm)	48	3662^{B}	917	38	9876^{A}	14470	78	2447^{B}	833	<0.01
pH	45	8.29^{A}	0.39	36	8.23^{A}	0.56	78	7.84^{B}	0.82	<0.01
Redox potential (Eh) (mV)	48	281	96.22	38	327	98	78	309	117	0.13
Chlorophyll *a* (μg/liter)[b]	48	128^{A}	111	37	27.3^{B}	46.1	76	19.8^{B}	44.4	<0.01
Turbidity (NTU)[b]	42	108^{A}	157	32	83.2^{A}	102	67	56.1^{B}	104	<0.01
Oxygen saturation (%)	48	158^{A}	109	38	120^{B}	80.9	77	95.7^{B}	61.7	<0.01
Volatile solids (mg/liter)	24	46.5^{A}	33.5	24	51.1^{A}	92.5	39	7.12^{B}	6.72	<0.01
Total solids (mg/liter)[b]	24	58.1^{A}	41	24	137^{A}	271	39	13.3^{B}	12.2	<0.01
BOD_5 (mg/liter)	18	35.2^{A}	16.9	12	44.9^{A}	37.1	22	6.45^{B}	9.91	<0.01
Inorganic carbon (mg/liter)	21	109^{A}	45.6	25	103^{A}	52	39	46.9^{B}	18.1	<0.01
Total organic carbon (mg/liter)	21	20.9	29.2	25	27.7	37.3	39	15.5	13.8	0.21
Total carbon (mg/liter)	21	124^{A}	26.4	25	129^{A}	41	39	61.3^{B}	17.1	<0.01
Carbonate alkalinity (meq/liter)	13	0.82^{B}	0.14	13	1.23^{A}	0.61	11	0.35^{C}	0.17	<0.01
Total alkalinity (meq/liter)	24	9.12^{A}	1.11	25	8.71^{A}	3.25	39	3.72^{B}	0.91	<0.01
$N\text{-}NH_4^+$ (mg/liter)[b]	24	2.95^{A}	3.08	25	36.6^{A}	90.3	39	0.43^{B}	1.68	<0.01
$N\text{-}NO_2^-$ (mg/liter)[b]	24	0.02	0.02	25	0.07	0.11	39	0.04	0.08	0.64
$N\text{-}NO_3^-$ (mg/liter)[b]	21	4.91^{A}	4.6	21	5.65^{A}	7.76	34	3.03^{B}	3.55	<0.01
$P\text{-}PO_4^{3-}$ (mg/liter)[b]	24	1.64^{A}	0.95	25	0.60^{B}	0.74	39	0.03^{C}	0.11	<0.01
SO_4^{2-} (meq/liter)[b]	24	9.28^{C}	5.83	23	69.3^{A}	80.9	39	22.7^{B}	8.31	<0.01
Cl^- (meq/liter)	24	17.3^{B}	8.68	25	38.1^{A}	62.7	37	3.35^{B}	2.33	<0.01
Ca^{2+} (meq/liter)	9	9.94^{B}	2.38	12	11.2^{B}	3.39	18	14.7^{A}	3.72	<0.01
Na^+ (meq/liter)	9	12.8^{AB}	8.56	12	23.9^{A}	36.0	18	2.16^{B}	0.69	0.02
Mg^{2+} (meq/liter)	9	9.51^{B}	4.86	12	102^{A}	153	18	13.8^{B}	7.46	0.02
K^+ (meq/liter)	9	0.42^{B}	0.19	12	2.09^{A}	1.50	18	0.07^{B}	0.04	<0.01

[a] The values are based on data collected during 8 visits throughout 1 year. Means sharing a superscript capital letter represent values that were not significantly different between wetlands.

[b] Factors were transformed into logarithm values for ANOVA.

the wetlands (Table 4). Taking into account just the significant values ($P < 0.05$), APEC was less present in feces from TDNP. Moreover, the presence of APEC in feces was higher from spring to autumn, and it was more frequent in *Ciconiidae* feces than in other bird families. The observed prevalence of *Salmonella* spp. was also lower in bird feces from TDNP. Furthermore, *C. perfringens* was less frequent in water samples from TDNP, while in sediment samples it was positively associated with SO_4^{2-} levels. In feces, *C. perfringens* prevalence was lower during the summer than in spring and it was more present in Navaseca than in TDNP. In sediment samples, *C. botulinum* was less frequent in TDNP. Also, it was positively associated with chlorophyll *a* levels and negatively correlated with conductivity of surface water (Table 4).

Physicochemical parameters associated with the botulism outbreak in Navaseca. Before the onset of the outbreak, we observed a period of maintained high water temperatures (28.5 ± 0.3°C) associated with high levels of chlorophyll *a* concentrations and high sediment BOD_5. At the same time, we observed abrupt changes affecting several environmental parameters that may have triggered the outbreak (Fig. 1). One of the first changes observed was a decrease in water Eh, followed by an increase in the concentration of inorganic carbon in interstitial water. Then, the chlorophyll *a* concentration decreased sharply along with the sulfate concentration in interstitial water and sediment BOD_5 (Fig. 1). Coinciding with these changes, *C. botulinum* type C/D was first detected in sediments and its occurrence in bird feces experienced a peak; later, during the botulism outbreak, it was also detected in surface water samples and *Calliphoridae* flies and, after the peak of the outbreak, in aquatic snails, coleopteran larvae, and *Ephydridae* flies (Fig. 2).

DISCUSSION

The spill of treated wastewater has produced changes in the studied wetlands that can facilitate the onset of avian botulism outbreaks. These changes included eutrophication (i.e., Navaseca and Veguilla) due to the regular input of nutrients which leads to the development of anaerobic environments that favor the presence of *C. botulinum* type C/D. Moreover, the wetlands receiving treated wastewater showed higher prevalences of avian pathogenic bacteria (i.e., APEC and *C. perfringens* type A) that can kill a small number of birds and therefore provide carcasses which are an optimum substrate for the initial growth of *C. botulinum* prior to an outbreak. In addition, these eutrophic wetlands have high phytoplankton and zooplankton productivity from spring to autumn that attracts a great amount of water birds, which increases spatial aggregation and the risk of epidemics (16, 31). The result of this has been a higher frequency of botulism outbreaks in Navaseca (4 summer outbreaks in the last 5 years) and Veguilla (5 recorded outbreaks since 1978) than in TDNP (only 1 large outbreak in

TABLE 2 Physicochemical parameters of interstitial water and sediment from three wetlands differing in levels of wastewater effect (where Navaseca lake was the most affected and TDNP the least affected)[a]

Parameter	Wetland value									
	Navaseca			Veguilla			TDNP			
	n	Mean	SD	*n*	Mean	SD	*n*	Mean	SD	*P*
Inorganic carbon (mg/liter)	48	103^{B}	59	38	134^{A}	101	79	83.7^{B}	56.5	0.02
Total organic carbon (mg/liter)	48	77.9	78.2	38	72.2	49.2	79	72.4	49.8	0.86
Total carbon (mg/liter)	48	181^{AB}	94.7	38	204^{A}	93.6	79	155^{B}	84.9	0.02
$N\text{-}NH_4^+$ (mg/liter)[b]	48	7.71^{A}	10.5	37	6.64^{AB}	10.5	79	3.33^{B}	6.91	<0.01
$N\text{-}NO_2^-$ (mg/liter)[b]	48	0.02	0.02	38	0.03	0.05	78	0.02	0.02	0.94
$N\text{-}NO_3^-$ (mg/liter)	36	8.11^{A}	4.46	28	9.97^{A}	8.39	56	4.42^{B}	4.74	<0.01
$P\text{-}PO_4^{3-}$ (mg/liter)[b]	48	2.92^{A}	3.08	38	2.59^{A}	2.79	79	0.42^{B}	0.58	<0.01
SO_4^{2-} (meq/liter)	48	8.35^{C}	4.39	36	80^{A}	82.7	79	28.4^{B}	14.2	<0.01
Cl^- (meq/liter)[b]	41	22.8^{A}	5.69	35	35.0^{A}	35.2	78	7.31^{B}	10.3	<0.01
Ca^{2+} (meq/liter)	18	9.59^{C}	2.12	18	17.23^{B}	7.46	36	22.2^{A}	9.22	<0.01
Na^+ (meq/liter)	18	21.6^{B}	6.73	18	40.2^{A}	35.9	36	4.43^{C}	6.58	<0.01
Mg^{2+} (meq/liter)	18	13.0^{B}	7.46	18	120^{A}	166	36	25.5^{B}	27.5	<0.01
K^+ (meq/liter)	18	0.46^{B}	0.20	18	2.45^{A}	1.72	36	0.14^{B}	0.16	<0.01
pH[c]	48	8.22^{A}	0.42	44	8.25^{A}	0.32	84	7.89^{B}	0.27	<0.01
Conductivity (μS/cm)[b,c]	42	558^{B}	323	38	2233^{A}	2764	72	909^{B}	818	<0.01
BOD_5 (mg/g)[c]	33	14.4^{A}	10	28	11.5^{AB}	8.83	56	8.39^{B}	3.91	<0.01
Water content (%)[c]	47	31.1^{A}	37.9	44	15.2^{B}	12.1	83	15.9^{B}	9.61	<0.01
LOI (%)[c]	46	1.42^{B}	0.9	44	1.79^{A}	1.13	83	1.87^{A}	0.74	0.02

[a] The values are based on data collected during 8 visits throughout 1 year. Means sharing a superscript capital letter represent values that were not significantly different between wetlands.
[b] Factors were transformed into logarithm values for ANOVA.
[c] Parameters of the sediment.

1999 and another smaller outbreak in an exhibition pond in 2007) (11, 32).

Wastewater discharges in wetlands modify their hydroperiod, soil pH and Eh, nutrient load, and plant communities (33–36). In our study area, we have observed higher turbidity, chlorophyll *a*, total and volatile solids, BOD_5, total carbon, NH_4^+, and PO_4^{3-} concentrations and lower water Eh in the wetlands with an input of treated wastewater. The issue is how these changes can increase the risk of botulism outbreaks. Our results suggest that high water temperatures (especially during the summer) and eutrophication

TABLE 3 Presence of three avian pathogens and *C. botulinum* type C/D in environmental samples taken from three wetlands differing in levels of wastewater effects (where Navaseca lake was the most affected and TDNP the least affected)[a]

Avian pathogen	Sample[b]	Result for indicated wetland								
		Navaseca			Veguilla			TDNP		
		Total no. of samples	No. of positive samples	%	Total no. of samples	No. of positive samples	%	Total no. of samples	No. of positive samples	%
C. botulinum type C/D	Sediment	48	10	20.8*	42	8	19.0*	84	2	2.4
	Water	47	3	6.4*	38	0	0	79	0	0
	Feces	215	8	3.7*	192	1	0.5	248	0	0
	Aquatic invertebrates	26	2	7.7	26	0	0	51	0	0
	Flies	42	2	4.5	20	0	0	72	0	0
C. perfringens type A	Sediment	18	14	77.8*	18	15	83.3*	4	36	11.1
	Water	17	8	47.0*	16	10	62.5*	6	36	16.7
	Feces	83	75	90.4*†	76	47	61.8	110	56	59.9
E. coli (APEC)	Sediment	48	5	10.4*	42	2	4.76	84	1	3.8
	Water	47	6	12.8	38	1	2.63	79	3	1.2
	Feces	205	73	35.6*	192	59	30.7*	248	42	16.9
Salmonella spp.	Sediment	48	0	0	42	0	0	84	1	1.2
	Water	47	1	2.1	38	0	0	79	0	0
	Feces	205	2	0.9	192	7	3.6*	248	1	0.4

[a] An avian botulism outbreak occurred in Navaseca lake during the study period. *, prevalence significantly higher than in TDNP ($P < 0.05$); †, prevalence significantly higher than in Veguilla ($P < 0.05$).
[b] Aquatic invertebrates and flies were grouped in pools.

TABLE 4 Regression models of the presence of the four avian pathogens in different samples based on AICc[a]

Avian pathogen	Sample	Model
C. botulinum type C/D	Sediment	Zone (**TDNP** [−**3.43**]) + season + **water conductivity** (−**0.001**) + **chlorophyll *a*** (+**1.13**) + PO_4^{3-} (−1.02)
	Feces	Zone + season
C. perfringens type A	Sediment	Zone + inorganic carbon (−0.03) + organic carbon (+0.04) + LOI (−2.70) + pH (+9.56) + **SO_4^{2-}** (+**0.21**) + NH_4^+ (+2.58) + PO_4^{3-} (−1.58)
	Water	Zone (**TDNP** [−**3.01**]) + season + conductivity (-5×10^{-3}) + temp (+0.19) + chlorophyll *a* (−1.13) + turbidity (+1.34) + pH (+0.90)
	Feces	Zone (**Navaseca** [+**1.48**] + **TDNP** [−**0.96**]) + season (**summer** [−**1.26**])
E. coli (APEC)	Sediment	Zone + inorganic carbon (−0.02) + organic carbon (+0.001) + LOI (−1.3)
	Water	Zone (**TDNP** [−**1.94**]) + temp (−0.18) + chlorophyll *a* (−0.81)
	Feces	Zone (**TDNP** [−**1.28**]) + season (**autumn** [+**1.37**] + **spring** [+**1.16**] + **summer** [+**0.82**]) + bird family (**Ciconiidae** [+**0.93**] + **Laridae** [−**1.07**] + **Scolopacidae** [−**0.79**] + **Rallidae** [−**0.69**])
Salmonella spp.	Feces	Zone (**TDNP** [−**2.23**])

[a] Models with differences of less than 2 AICc points from the best result (ΔAICc = 0) were considered to have the same empirical support. Factors and variables that have a significant effect ($P < 0.05$) are indicated in bold; values in parentheses represent the average estimates of the effects. Full model for *C. botulinum* type C/D and APEC in sediment: zone plus season plus water temperature plus water conductivity plus water redox plus log water chlorophyll *a* plus log interstitial water PO_4^{3-} plus interstitial water SO_4^{2-} plus interstitial inorganic carbon plus interstitial organic carbon plus sediment pH plus sediment LOI. The reference zone is Navaseca lake. Full model for APEC in water: zone plus season plus water temperature plus water conductivity plus water redox plus log water chlorophyll *a* plus water dissolved oxygen. Reference zone is Navaseca lake. Full model for *C. perfringens* in sediment: zone plus season plus log interstitial water NH_4^+ plus log interstitial water PO_4^{3-} plus interstitial water Cl^- plus interstitial water SO_4^{2-} plus interstitial water Mg^{2+} plus interstitial inorganic carbon plus interstitial organic carbon plus sediment pH plus sediment LOI. Full model for *C. perfringens* in water: zone plus season plus water temperature plus water conductivity plus water redox plus water pH plus log water chlorophyll α plus log water turbidity. Reference zone is Navaseca lake, and reference season is spring. Full model for feces: zone (wetland) plus season plus bird type. Reference zone is Veguilla, reference season is winter, and reference bird type is anatidae.

in Navaseca lake caused an overgrowth of phytoplankton before the outbreak, as revealed by high concentrations of chlorophyll *a* recorded in June and early July. Later, we observed an overgrowth of floating duckweed (*Lemna minor*), which covered the surface of the lake during the second half of July. The lack of light and resources resulting of this overgrowth may have caused the death of the phytoplankton, as indicated the sharp decreases of Eh and chlorophyll *a*. Moreover, the dead phytoplankton could sink into the sediments and the resulting organic matter may have encouraged the overgrowth of microorganisms depleting the oxygen, as reported elsewhere (37). Further supporting evidence for this conjecture are the high inorganic carbon concentration and the decrease of the SO_4^{2-} concentration recorded in interstitial water between July and August which may have been a consequence of the metabolism of microorganisms (i.e., methanogenesis) and the activity of anaerobic sulfate-reducing bacteria (38, 39). Also, the sharp decrease on the BOD_5 levels reflected that microorganisms had consumed organic matter with consequent oxygen depletion. This anoxic environment could be suitable for the growth of *C. botulinum* type C/D, which was first detected in the sediments of Navaseca in July coinciding with the cited changes. Later, *C. botulinum* was also detected in water and in necrophagous invertebrates (i.e., *Calliphoridae* flies and beetle larvae), probably due to its ability to grow in other compartments such as carcasses. Moreover, we found a significant association between sampling sites with high concentrations of chlorophyll *a* and the presence of *C. botulinum* type C/D in the sediments, which may indicate that this pathogen prevails better in eutrophicated sites. Other authors have also observed links between changes in environmental characteristics of wetlands that can be due to eutrophication and botulism. Murphy et al. (40) found that a change in the water Eh in Whitewater Lake (Canada) might have been a significant factor in the development of *C. botulinum* type C in its sediments. Rocke et al. (41) found that increasing temperature and biomass may influence the initial phase of botulism outbreaks and that these were more frequent in wetlands with lower Eh. More recently, Chun et al. (42) found that the macrophytic green alga *Cladophora* provides a habitat for *C. botulinum* type E and that its accumulation in waters near shores coincided with a high incidence of avian botulism at the shoreline.

The proliferation of *C. botulinum* type C/D in sediments can increase the chances of birds ingesting spores either directly from the sediments (43) or along with detritivorous aquatic invertebrates, such as snails. In our study, this hypothesis is supported by the increase in the detection of *C. botulinum* type C/D in bird feces before the occurrence of the outbreak in July. After the death of any of these carrier birds, their carcasses may become substrates for further proliferation and toxinogenesis of *C. botulinum* type C/D prior to an outbreak, which would later propagate by the carcass-maggot cycle (44). The presence of avian pathogens in wetlands can facilitate this process by causing sudden mortalities of water birds and may be one of the reasons that explain the regular botulism outbreaks that occur in wastewater lakes where the prevalence of pathogens is higher. Bird mortalities have already been described as the major initiating factor of outbreaks in Eyebrow Lake, Canada (18). In our study, APEC and *C. perfringens* type A, both capable of producing bird mortalities (6, 9, 45), were significantly more frequent in sediment and bird feces from the two wetlands that receive effluents from wastewater treatment plants (i.e., Navaseca and Veguilla). In addition, *C. perfringens* type A is a good indicator of sewage discharges (35, 46), so the high prevalence observed indicates the presence of poorly treated wastewater in Navaseca and Veguilla which probably contains other pathogens not included in this study, such as *Campylobacter* spp., *Aeromonas* spp., or *Pseudomonas* spp. (6, 47). As an example, *E. coli* and *Proteus mirabilis* were isolated from the heart of a white-headed duck collected during the outbreak in Navaseca. Also, in a recent bird mortality that occurred in another lake receiving wastewater, *Pseudomonas aeruginosa* was isolated from the heart of a coot (*Fulica atra*) (our own unpublished data [IREC]). As further evidence, a discharge of wastewater in the Salada lagoon (south of Spain) was associated with a water bird mortality due to *Pasteurella anatipestifer* (48). Finally, *C. botulinum* type C/D was

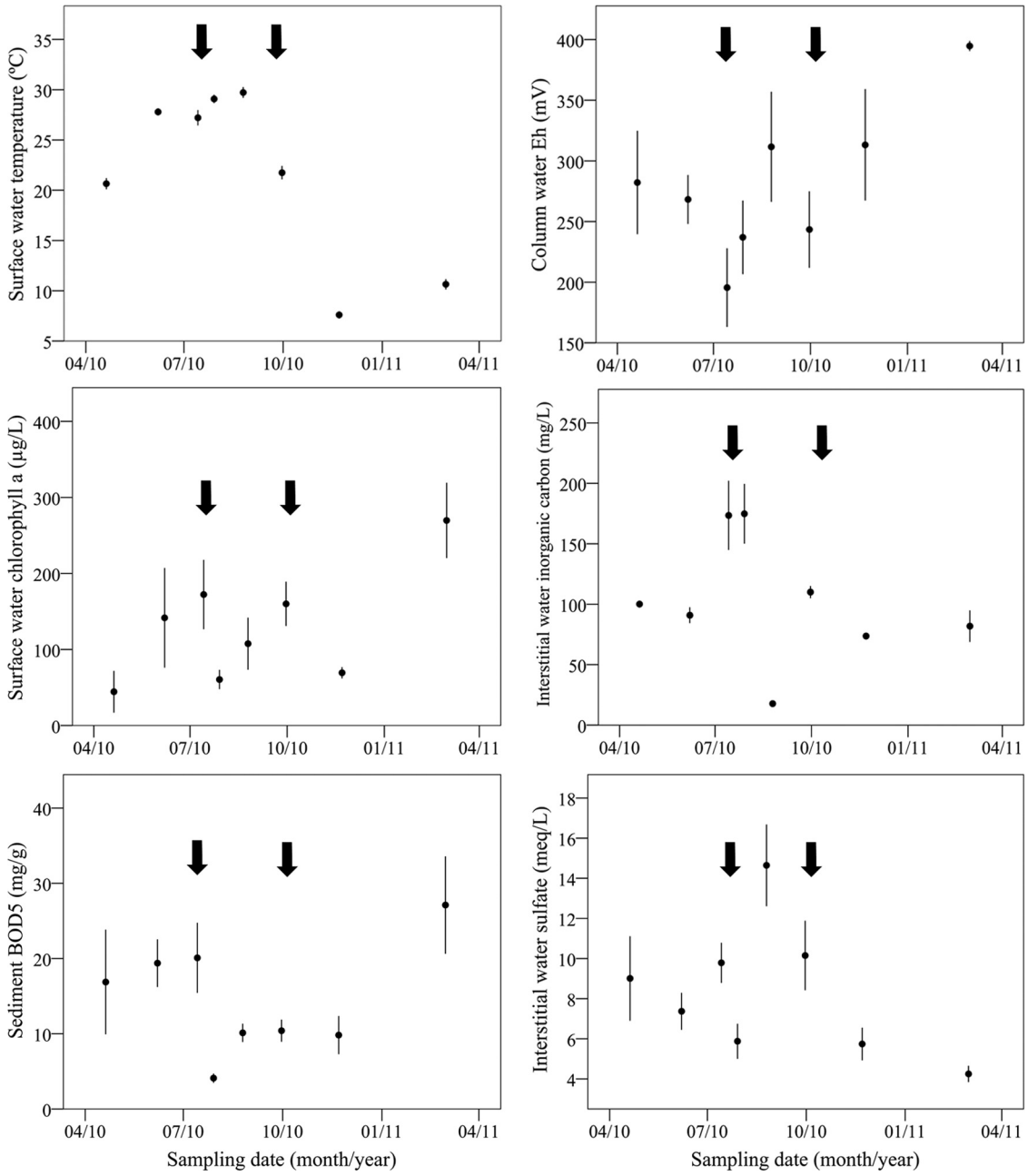

FIG 1 Changes in environmental parameters in Navaseca lake that could have favored the avian botulism outbreak in summer 2010. The arrows indicate the beginning and the ending of the botulism outbreak; data are presented as means ± standard errors of data from 6 sampling points. 04/10, April 2010; 07/10, July 2010; 10/10, October 2010; 01/11, January 2011; 04/11, April 2011.

also more frequent in the sediments collected from Navaseca and Veguilla, which was probably related to the higher frequency of outbreaks in these wetlands (11, 32). In this sense, Wobeser et al. (49) found a strong association between a prior history of botulism in a wetland and the proportion of soil samples containing *C. botulinum* type C. Our regression analysis showed that, more than the physicochemical parameters, the season, or the type of bird sampled, the most important factor explaining the presence of the avian pathogens was the zone (wetland). It is probable that other variables not studied here, such as bird aggregation, the amount of wastewater entering the wetlands, or the water retention time, had an effect on the prevalence of pathogens.

The proximity of landfills to wetlands may present an additional risk for the appearance of outbreaks because opportunistic birds feeding on urban waste, such as gulls or storks, may spread pathogens such as *Salmonella* spp., *Campylobacter* spp., or *C. botulinum* from the landfills where they feed (6, 50, 51) to the wetlands where they roost. Accordingly, we observed the major prevalence of *Salmonella* spp. in bird feces (mainly in gulls and storks) of Veguilla, which can be related to the high prevalence of *Salmo-*

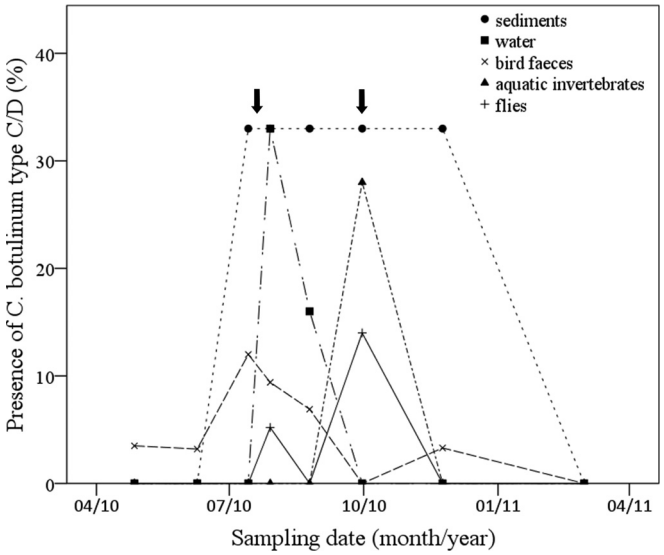

FIG 2 Presence of *C. botulinum* type C/D in different environmental samples collected in Navaseca lake during 8 sampling visits. The arrows indicate the beginning and the ending of the botulism outbreak.

nella spp. detected in gull feces in a nearby landfill (30%). The higher prevalence of APEC observed in feces of *Ciconiidae* (white storks) also supports this statement.

Conclusions. Inefficiently treated effluents from wastewater treatment plants represent a risk for the conservation of wetland ecosystems. During the dry season, these wetlands still maintain water with high phytoplankton and zooplankton productivity, thus attracting a great amount of water birds, including endangered species such as the white-headed duck. The eutrophication and high loads of avian pathogens that are found in these wetlands may increase the risk of botulism outbreaks which, in this situation of aggregation, can have a severe impact on water bird populations due to the high mortality rates produced in short periods of time (12, 52). Water birds attracted by these eutrophic wetlands may be exposed to diverse pathogens and urban or industrial pollutants, with unknown effects for them (8, 16). Improvements of water-treatment plants and the elimination of "bypass" systems that directly pour wastewater on wetlands are measures urgently needed to guarantee the conservation of water birds.

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