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OCCURRENCE OF CLOSTRIDIUM BOTULINUM IN WETLANDS OF THE GUADALQUIVIR RIVER BASIN, SPAIN*

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RESUME :*Nous avons étudié la distribution et les sérotypes des spores de Clostridium botulinum dans le bassin de la rivière Gadalquivir, en Andalousie (Espagne), en 1986-1987. 332 échantillons de boues provenant de 47 zones humides ont été analysés. 62 échantillons (soit 18,67 p. cent) de boues contenaient des spores de C. botulinum. De plus, 17 des 21 terrains humides du parc national Doñana et 10 des 26 terrains humides du Betic Endorrhoeic System contenaient également des spores de cette clostridie. Une analyse de tendance canonique a montré que la concentration en spores de C. botulinum est plus élevée près de l'embouchure du Guadalquivir et qu'elle décroît en amont.*

SUMMARY : *We studied the distribution and serotypes of Clostridium botulinum spores present in the Guadalquivir River Basin, Andalusia (Spain), in 1986-1987. 332 mud samples from 47 wetlands were analized. It was found that 62 mud samples contained C. botulinum spores (18,67 %). In addition, 17 of the 21 wetlands in the Doñana National Park and 10 of 26 wetlands in the Betic Endorrhoeic System were found to contain botulism spores. Canonical trend analysis suggested that concentration of C. botulinum spores are higher near mouth of Guadalquivir and it decreases as the Basin geographically goes up.*

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INTRODUCTION

In Spain, waterfowl mortality from botulism (*Clostridium botulinum*) has been documented in the Ebro River Delta [Nadal, 1974] and in the Guadalquivir River Basin. In the Doñana National Park of Guadalquivir Marshes (D.N.P.) the disease has occasionally caused large die-offs of waterfowl [Bernis, 1974 ; Haagsma and Koeman, 1974 ; Hidalgo, 1974 ; León Vizcaíno et al., 1979]. Minor outbreaks of botulism have been documented for other wetlands (Marchena, Seville) of the Guadalquivir Basin [Contreras et al., 1987]. Large die-offs of waterfowl, that have occurred in other wetlands within a Guadalquivir River Basin, may be botulism but their aetiology remains unconfirmed : Laguna de Medina (Jerez de la Frontera, Cadiz) [Bernis, 1974 ; Smith, 1982], Laguna del Taraje (Las Cabezas de San Juan, Seville) [Smith, 1982].

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It has been hypothesized that outbreaks of avian botulism are related to a complex set of ecological factors [Cobourn and Quortrup, 1938 ; Bell et al., 1955], although many of the aspects of the epidemiology of the disease remains speculative [Wobeser, 1987]. The necessary factors for an outbreak of avian botulism include : a susceptible population of birds, the presence of toxigenic *C. botulinum*, a suitable macro and microenvironment, conditions for bacterial growth and toxin production, and a delivery system of transferring toxin from substrate to birds. Thus, wetlands containing large numbers of botulism spores are especially prone to the outbreak of avian botulism [Smith and Moryson, 1975 ; Marion et al., 1983 ; Wobeser et al., 1987]. This paper describes the geographical distribution and typology of *C. botulinum* present in wetlands of the D.N.P. and the Betic Endorrhoeic System (B.E.S.) within the Guadalquivir River Basin.

MATERIAL AND METHODS

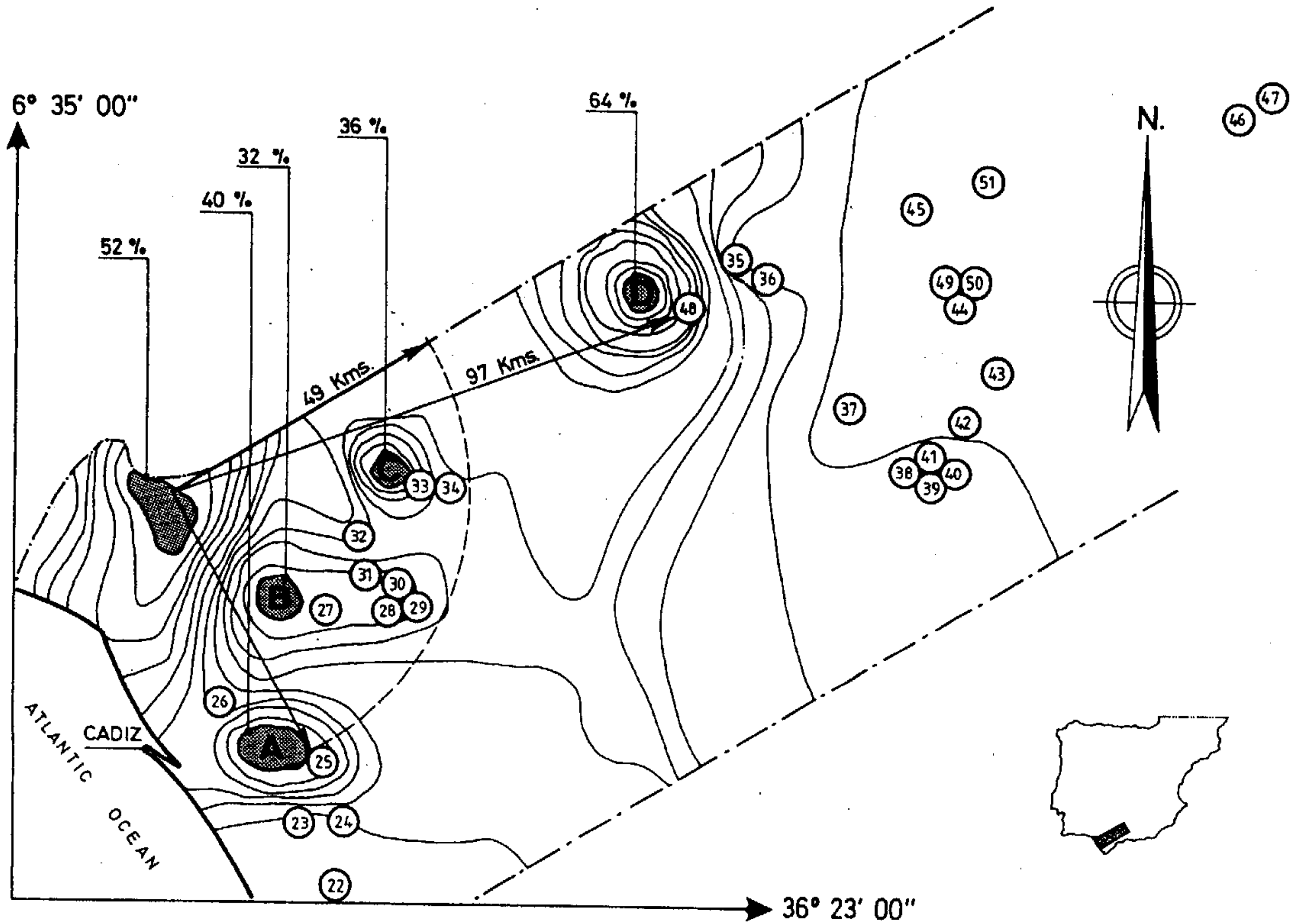
We studied 47 wetlands in the Guadalquivir River Basin (fig. 1), 21 in the summer of 1986 (table 1) and 26 in the spring of 1987 (table 2). These wetlands consisted of 45 lagoons, 1 channel and 1 river. Following the recommendations of Smith and Moryson [1975, 1977], we collected the mud samples by hand from 30 cms at shoreline, each sample was placed in a plastic bag and held frozen (-20°c) until examination. We collected three hundred and thirty two samples.

According to the recommendations of Itoh et al. [1978] and Smith and Moryson [1975], 20 g of mud was suspended in 50 ml sterile phosphate buffered saline -PBS- (pH 7,0) during 10 min, their supernatant was centrifuged (30 min, 8.000 g) and resuspended in 5 ml sterile physiological saline solution, heated (20 min, 60°C) prior to inoculation into Cooked Meat Medium (Difco). The mixture was then incubated (35°C) for 5 days and frozen (-20°C, 24 h) [Marion et al., 1983]. Under sterile conditions, the liquid fraction was succesively centrifuged (8.000 g, 4°C, 30 min), filtered (0,22 µm), diluted (50 %) in physiological saline solution and treated with 0,05 ml of 0,25 % trypsin (pH 6,5).The resulting mixture was incubated at 35°C for 1 h. Presence of botulism toxin and it type was determined by inoculation and neutralisation with know types of antiserum (Institut Pasteur Productions, Marne-La-Coquette, France) in two young adult white mice (weigth 30 g) [Wobeser et al., 1987]. When a toxic sample was not neutralized by any serym type, we attemped to neutralise it with combinations of the botulism antitoxins.

To determine the geographical distribution of positive mud samples in the wetlands studied, a canonical trend surface analysis [Wartemberg, 1985] was used to correlate percentages of positive mud samples to geographical coordinates in the wetlands (X, Y, XY). Canonical trends surface analysis usually involves the analysis of spatial variation in a set of biologically relevant variables. To this end, the multivariate method of canonical correlation is applied, with the set of variables of interest used as "criteron" (dependent) variables, and geographical coordinates (plus their squares and cross-product) as "predictor" (independent) ones. In this way, it is possible to determine if there is an overall relationship between the dependent variables and geographical location of samples. In the particular case of this study, a single criterion was used, hence the problem reduced to a case of multiple regression, in wich geographical coordinates, their squares and cross-product, were the independent variables. The results of this analysis essentially reduce to fitting a response surface to the data. Significance of the regression coefficients associated with the various terms in the equation will inform us about the prevailing trends of spatial variation in botulism occurrence in the study area. Data were transformed according to the equation

$$\text{Freq} = \text{Arcsin}\sqrt{\text{Percent}/100}$$

Figure 1 : Situation of the study area in the Guadalquivir Basin. Numbering of the wetlands studied in the Betic Endorrhoeic System (BES). Representation by trènd curves of positive sampling points (Canonical trend surface analysis, data untransformed) in the whole of the study area.



Geographical coordinates were determined in cm from a 1:200.000 scale map of the area betwen 36° 35' 00" longitude West. Multiple regression was computed using procedure MGLH in SYSTAT [Wilkinson, 1986]. We used results from Contreras de Vera et al. [1987] (table 3) to expand the geographic area of consideration.

RESULTS

In the Guadalquivir Basin 27 of the 47 (54,4 %) wetlands studied and 62 of 332 (18,65 %) mud samples tested contained *C. botulinum* spores. Seventeen of 21 (80,9 %) wetlands examined in DNP were positive for *C. botulinum* (table 1) whereas only 10 of 26 (38,4 %) wetlands in the BES were positive (table 2). Types B, C, D and E botulism were detected in DNP wetlands whereas only types B and C were detected in BES wetlands.

Table 1 : Occurrence and types of *Clostridium botulinum* in the mud samples from wetlands in Doñana National Park.

N°	Wetland	Province	Samples with toxin/ samples examined	N° samples with types				
				B	C	D	E	C+E
1	Dulce	Huelva	0/3	-	-	-	-	-
2	Sanguijuela	Huelva	0/2	-	-	-	-	-
3	Santa Olalla	Huelva	1/9	-	1	-	-	-
4	Sopetón	Huelva	1/3	-	1	-	-	-
5	El Palacio	Huelva	0/3	-	-	-	-	-
6	Caballero	Huelva	1/4	-	1	-	-	-
7	Hondón	Huelva	1/5	-	1	-	-	-
8	Aguas Rubias	Huelva	1/3	-	1	-	-	-
9	El Membrillo	Huelva	4/9	1	2	1	-	-
10	Vetalengua	Huelva	1/2	-	1	-	-	-
11	Largo	Huelva	1/5	-	1	-	-	-
12	Guadiamar	Seville	1/7	-	1	-	-	-
13	El Lobo	Seville	1/3	-	1	-	-	-
14	Mari López	Seville	3/5	-	1	-	2	-
15	Mal Tiempo	Seville	2/4	1	1	-	-	-
16	Los Ansares	Seville	4/8	1	1	-	1	1
17	Leo Biagi	Seville	2/4	-	2	-	-	-
18	Cherry	Seville	4/12	1	1	-	1	1
19	Brazo La Torre	Seville	4/4	-	1	-	3	-
20	Cangrejochico	Seville	0/2	-	-	-	-	-
21	Cangrejogrande	Seville	2/3	-	1	-	1	-
			34/100	4	19	1	8	2

Table 2 : Occurrence and types of *Clostridium botulinum* in mud samples from wetlands in Betic Endorrhoeic System.

N°	Wetland	Province	Samples with toxin/ samples examined	N° samples with types B	C
22	Paja	Cádiz	0/3	-	-
23	Taraje	Cádiz	1/6	-	1
24	Comisario	Cádiz	3/11	-	3
25	Medina	Cádiz	4/10	-	4
26	Salada	Cádiz	5/10	-	5
27	Tollos	Cádiz	0/10	-	-
28	Salada Zorilla	Cádiz	2/10	-	2
29	Dulce Zorilla	Cádiz	0/2	-	-
30	Hondilla	Cádiz	0/8	-	-
31	Cigarrera	Seville	1/9	-	1
32	Taraje	Seville	4/10	-	4
33	Arjona	Seville	4/9	1	3
34	Zarracatin	Seville	0/10	-	-
35	Ballestera	Seville	2/10	-	2
36	Calderona Ch.	Seville	0/9	-	-
37	Gosque	Seville	0/9	-	-
38	Salada	Málaga	0/10	-	-
39	Capacete	Málaga	2/8	-	2
40	Cerero	Málaga	0/8	-	-
41	Dulce	Málaga	0/8	-	-
42	F. de Piedra	Málaga	0/14	-	-
43	Ralosa	Málaga	0/9	-	-
44	Jarales	Córdoba	0/8	-	-
45	Tíscar	Córdoba	0/11	-	-
46	Conde	Córdoba	0/10	-	-
47	Honda	Jaén	0/10	-	-
			28/232	1	27

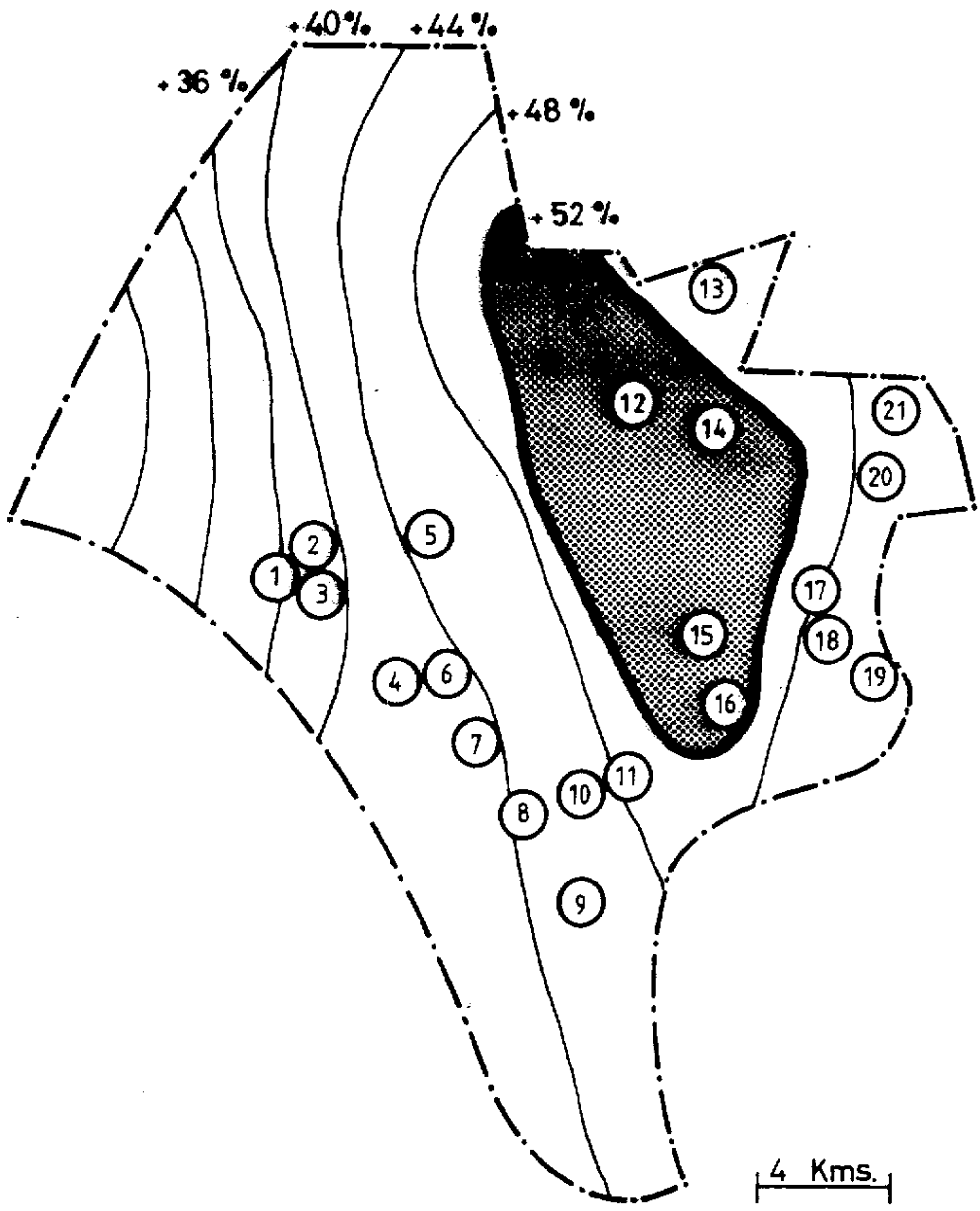
Type C botulism was the most common and was present in 48 of 62 (77,4 %) of the positive mud samples from all wetlands. Type B was found in a BES wetland and in 4 DNP wetlands. Type D was present only in a DNP wetland and type E in 5 DNP wetlands. The joint presence in a single sample of 2 types (C and E) was noted in 2 DNP wetlands (table 1).

Table 3 : Occurrence and types of *Clostridium botulinum* in the mud samples from others wetlands in Betic Endorrhoeic System [By Contreras de Vera et al., 1987].

N°	Wetland	Province	Samples with toxin/ samples examined	Type
48	La Coronela	Seville	7/10	C - E
49	Zoñar	Córdoba	0/17	-
50	Rincón	Córdoba	0/4	-
51	Amarga	Córdoba	0/8	-

Canonical trend surface analysis (data untransformed) indicated that nearly all DNP wetlands were encompassed within the line corresponding to the 40 % positive sampling points (psp) (fig. 2). A small number of wetlands had a higher proportion of positive mud samples (52 % psp). Three wetlands had a small number of positive mud samples and were included within the 36 psp line. The BES had 2 wetlands near to the 64 % psp line (fig. 1), a wetland included in the 40 % psp line, a xetland in the 36 % psp line and 2 near the 32 % psp line. The high presence of positive samples in the DNP conditioned a light deviation from itself to the others psp lines.

Figure 2 : Location of the wetlands studied and representation by trend curves of the positive sampling points (Canonical trend surface analysis, date untransformed) in the Doñana National Park.



Areas of BES with epicenters in A, B and C (fig. 1), were near DNP (in a radius of 49 km from the Mari López wetland, in the center of 52 % psp line). Area D was more distant from this wetland (97 km). Provinces of Córdoba, Jaén and Málaga were free of *C. botulinum* spores, except for 1 wetland that contained few toxigenic *C. botulinum* spores (4 % psp).

Canonical trend surface analysis (data transformed) indicated that higher presence *of C. botulinum* spores had concentrated in SW zone, near the Guadalquivir mouth, including DNP. *Clostridium botulinum* spores presence decreased by a gradient in direction as the Basin geographically goes up.

DISCUSSION

The DNP contains a large number of wetlands with *C. botulinum* spores. High spore counts may result from recurring outbreak of avian botulism in the area [Leon Vizcaino et al., 1983]. Botulism spores persist in wetlands for many years [Smith et al., 1982] and may contribute to outbreaks of avian botulism in the future [Wobeser et al., 1987]. Although since 1980 healthy control (based on removing affected or death birds) have limited avian botulism, type C of *C. botulinum* spores persist in mud and it has been found in 21 (19 alone and 2 with type E) of 100 (21 %) samples tested (table 1). Recently Villalba et al. [1989] studied mud and dry soil from 12 different points in DNP and found 6 positive samples with *C. botulinum* type C. The area studied by them corresponds to the area of maximum psp line found by us (52 %).

Canonical trend surface analysis showed that occurrence of botulism spores was lower in the BES than in DNP wetlands (figures 1 and 3). It is noticeable that we found type C. botulism in all wetlands where previous die-offs of waterfowl had occurred ; some of which had been diagnosed as avian botulism (table 4). On the other hand, we found *C. botulinum* type C in 6 BES wetlands that had no previous history of avian botulism. Several studies suggest that only 1-5 % of mud samples from wetlands without history of avian botulism may contain type C spores [Wobeser et al., 1987]. However, we found 11 %, 17 %, 17 % and 33 % samples with type C botulism from these wetlands. One explanation for this may be that light botulism outbreaks had not been detected in these wetlands. In other study, we are found a low occurrence of *C. botulinum* in wetlands from the Odiel Marses (near to the Guadalquivir Basin but geographically abroad), and it is possible water regime could be an explanation for this low occurrence [Contreras de Vera et al., 1991].

Other types of botulism may be implicated in waterfowl die-offs, above all type E [Brand et al., 1983 ; Haagsma, 1987]. Type E botulism was uncommon in our study (10 samples) but it may be responsible for the outbreak of the disease in waterfowl. Type E has detected in mud and in intoxicated birds from a BES wetland by us [Contreras de Vera et al., 1987].

Type F is less common in avian botulism, while types A, B, D and G are not know to affect birds [Clark, 1987]. Type B botulism appears to be of little presence in our study (5 samples) (in Europe, this type has also been reported to be present in Great Britain and Ireland [Borland et al., 1977 ; Smith et al., 1982], France [Smith et al., 1977] and Holland [Haagsma, 1987]). Type D botulism was detected in only 1 sample in our study. This serotype is rare in the northern hemisphere [Smith and Moryson, 1975] and has been reported in Great Britain and Ireland [Smith and Moryson, 1977 ; Smith et al., 1978], Holland [Haagsma, 1987] and the USSR [Krauchenko and Shishulina, 1967].

Figure 3 : Location of the wetlands studied. Distribution of presence of **Clostridium botulinum** spores
(Canonical trend surface analysis, data transformed). It decreased by a gradient in direction SW-NE (as the basin geographically goes up).

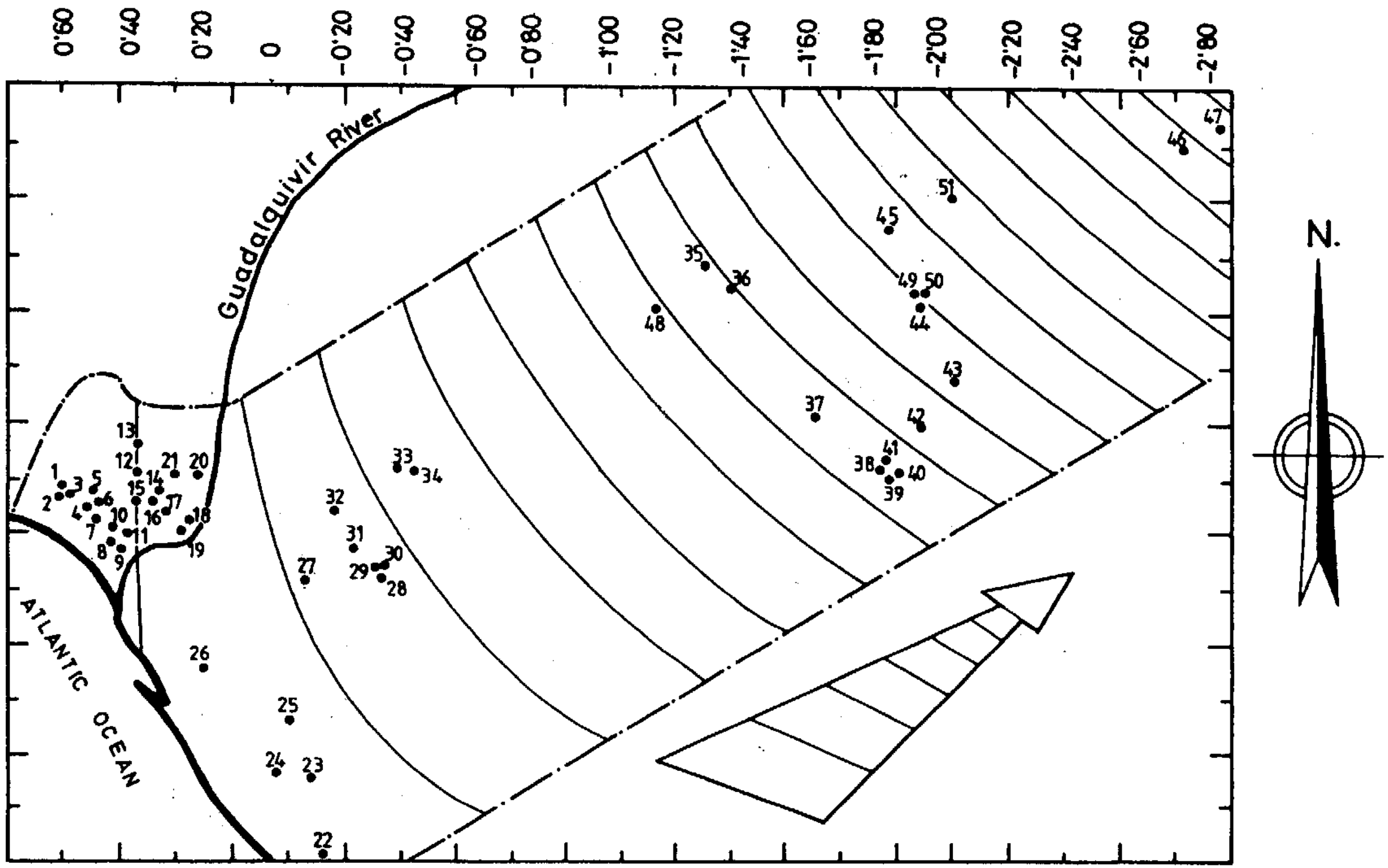


Table 4 : Occurrence of *Clostridium botulinum* in mud samples from wetlands in Betic Endorrhoeic System, conected with histories of botulism or other waterfowl mortalities.

Wetland	Province	History [reference]	% positive samples	Type
Salada del Puerto	Cádiz	Avian mortality [Gomez, 1987]	50 %	C
Medina	Cádiz	Botulism [Bernis, 1974 ; Smith, 1982]	40 %	C
Salada de Zorilla	Cádiz	Avian mortality [Contreras de Vera, 1989]	20 %	C
Taraje	Seville	Botulism [Smith, 1982]	40 %	C
Coronela	Seville	Botulism [Contreras de Vera et al., 1987]	70 %	C-E
Río Corbones	Seville	Botulism [Contreras de Vera et al., 1987]	20 %	C
Comisario	Cádiz	Unknown	17 %	C
Taraje	Cádiz	Unknown	17 %	C
Cigarrera	Seville	Unknown	11 %	C
Ballestera	Seville	Unknown	20 %	C
Arjona	Seville	Unknown	44 %	C-B
Capacete	Málaga	Unknown	25 %	C

We present now a few recommendations both connected with the control of avian botulism in wetlands of the Guadalquivir River with history of the disease and the avoidance of spores dispersion in wetlands without history : Firstly, surveillance teams should observe the wetlands during summer and early-fall to detect any mortality. This would allow to remove the affected birds or carcasses previous to larvae maggots proliferation and to send them to Veterinarian Diagnostic Centers. This would be specially useful due to risk of confusion between avian botulism and poisoning by agricultural pesticides and other chemicals. At the same time laboratorial diagnostic is necessary because the clinical signs of botulims are not specific to permit a firm clinic diagnosis [Smith, 1987]. Secondly this team activity should be more intensive in those wetlands with history of botulism and/or presence of C and E *Clostridium botulinum* spores. Finally, in general terms, the following should be done in all wetlands of the Guadalquivir Basin : control indiscriminate hunting, eliminate power transmission lines near the wetlands, keep water as "fresh" as possible (above all avoiding the agricultural pesticide contamination) avoid sudden changes of water level. In addition any other measure aimed to prevent mortality of vertebrates or invertebrates is recommended - because a single descomposing carcass can provide a suitable environment for the bacterial multiplication and toxin production [Malcom, 1982 ; Clark, 1987 ; Wobeser, 1987].

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