CAPÍTOL IV. Avaluació de la Classificació de Zones de Producció de Marisc al Delta de l'Ebre

Relation between current classification of shellfish-growing areas and viral pollution in bivalve molluscan shellfish.

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RESUM DEL CAPÍTOL

L'objectiu del present capítol és examinar l'estabilitat de la classificació de diferents zones de producció de marisc a partir dels nivells d'*Escherichia coli* en diferents espècies de bivalves. També es pretén avaluar els nivells de contaminació vírica en aquestes zones en relació amb els estàndards bacterians.

Els resultats de les anàlisis realitzades durant 18 mesos d'AdH, EV, VHA, NV i *E. coli* en ostres i musclos procedents del Delta de l'Ebre, en total, 84 mostres, s'analitzaren estadísticament. També es mesuraren paràmetres físico-químics com ara temperatura i salinitat per a una millor definició de les àrees estudiades i per a poder establir qualsevol possible influència sobre la contaminació fecal del marisc.

Els valors d'*E. coli* obtinguts durant aquest treball foren comparables amb resultats obtinguts en estudis previs que s'havien fet servir per a classificar les zones d'acord amb les directives europees (91/492/EEC). Aquest resultats s'havien obtingut de l'estudi dels nivells d'*E. coli* en diferents espècies de mol·luscs bivalves durant quatre anys.

D'altra banda, s'analitzaren la distribució estacional de la presència de virus i dels nivells d'*E. coli* en dues de les àrees estudiades. També s'avaluaren els actuals tractaments de depuració, els quals mostraren ser efectius en el cas d'*E. coli* però no pels virus humans.

En la zona A un 31% de les mostres analitzades foren positives per adenovirus humans i un 6% per norovirus. Cap mostra resultà positiva ni per enterovirus ni pel virus de l'hepatitis A. En la zona B, el 35% de les mostres fou positiu per AdH, el 32% per EV, el 4% per VHA i el 24% per NLV.

Les conclusions extretes del treball són:

1. La classificació de les tres zones estudiades es confirmà malgrat la utilització de dues tècniques diferents d'enumeració d'*E. coli*. Aquesta classificació es mantigué estable i dins els límits marcats per la seva classificació original durant els 4 anys estudiats (1998 a 2001).

2. Al comparar els nivells d'*E. coli* en espècies de mol·luscs bivalves amb hàbitats diferents, s'observà que el nombre de mostres amb valors ≤ 230 NMP/100g de carn de mol·lusc era major en les espècies cultivades en suspensió que en les que es cultiven en contacte directe amb el sediment.

3. Els adenovirus humans foren els virus més comunament aïllats en bivalves i presentaren una relació estadísticament significativa amb la presència dels altres virus estudiats. Així doncs, els AdH detectats per PCR podrien ser un bon índex molecular de contaminació vírica en mol·luscs bivalves.

APORTACIÓ PERSONAL AL TREBALL

L'autora d'aquesta tesi realitzà les anàlisis d'*Escherichia coli*, adenovirus humans, enterovirus i virus de l'hepatitis A de les mostres de musclos bivalves durant els 18 mesos de mostreig. L'autora també ha participat activament en la comparació de les dades obtingudes pel Centre d'Aqüicultura de Sant Carles de la Ràpita, així com en la discussió i avaluació de les conclusions del treball. Finalment, ha intervingut en la preparació del manuscrit.

Relation between current classification of shellfish-growing areas and viral pollution in bivalve molluscan shellfish

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ABSTRACT

Viral pollution in relation to *Escherichia coli* standards was studied in oysters (*Crassostrea gigas*-Thunberg, 1973) and Mediterranean blue mussels (*Mytilus galloprovincialis*-Lamarck, 1819) over a 18-month monitoring period. A total of 84 samples were studied and the obtained results were statiscally analyzed. Additionally, physic-chemical parameters such as temperature and salinity were measured to better define the studied areas and to establish any potential influence on fecal-pollution of shellfish. The *E. coli* values obtained over this study were compatible with data from previous studies, which had been used to classify these areas following EU directive (91/492/EEC). These data were obtained from the examination of *E. coli* in various species of bivalve molluscan shellfish (*Tapes* sp., *M. galloprovincialis* and *C. gigas*) over four years to establish the stability in the classification of shellfish-growing areas according to bacterial parameters. Furthermore, the seasonal distribution of viral presence and *E. coli* levels in shellfish were analyzed. The depuration treatment currently applied was also examined and shown to be correct for *E. coli*, but ineffective for the reduction of viral contaminants. Besides, in A area 31 % of samples were positive for human adenovirus (Adv) and 6 % for Norwalk-like virus (NLV). None sample was neither positive for enterovirus (EV) nor for hepatitis A virus (HAV). In B area, 35 % of samples were positive for Adv, 32 % for EV, 4 % for HAV and 24 % for NLV. Regarding the statistical analysis, it showed that in clean areas such as the studied here the presence of human pathogenic viruses do not correlate with the presence

of *E. coli* and so, shellfish from such zones may contain human viruses. Moreover, it seems that in the present case, human adenovirus detected by PCR would be a better index of viral pollution in bivalve mollusks.

KEY WORDS: seafood, human enteric viruses, *Escherichia coli*, fecal contamination, shellfish-growing areas

INTRODUCTION

Shellfish harvesting areas are classified according E.U. legislation (91/492/EEC) in three categories based on the concentration of *E. coli* or fecal coliforms, briefly: A areas with less than 230 *E.* coli/100g, B areas must not exceed in 90% of samples the limit of 4,600 *E. coli*/100g and C areas are over the 4,600 *E. coli*/100g limit. Bacterial indicators of fecal contamination used currently in shellfish have limited predictive value for pathogens such as enteric viruses (Gerba et al. 1979). Moreover, they fail to be a good indicator of currently applied depuration processes, which remove bacteria but are not effective to eliminate pathogenic viruses (Desenclos et al. 1990, Halliday et al.1991).

The aim of this study was to examine the stability in the levels of *E. coli* in diverse species of shellfish in regards to the classification from different bivalve shellfish harvesting areas. Furthermore, the presence of human enteric viruses in shellfish was studied at areas classified A and B over 18 months to study the relation between viral contamination and bacterial standards.

MATERIALS AND METHODS

Samples

Crassostrea gigas, Mytilus galloprovincialis and *Tapes* sp. were harvested on a monthly basis from different shellfish growing areas localized at West Mediterranean, in Spain. Once harvested, shellfish were shipped directly to the laboratory via cold storage within a 24 hour period where they were processed immediately. From February 2000 to July 2001, oysters and mussels collected at one B area were sent to a depuration plant and the samples were analyzed before and after the 24-hour purification treatment.

Treatment

Shellfish samples were processed and analyzed for human enteric viruses as described by Pina et al. (1998) and Muniain-Mujika et al. (2000) with some modifications (Formiga-Cruz et al. 2002). Briefly, digestive glands were eluted with glycine buffer 0.25 N at pH 10 and viral particles in the homogenate were concentrated by ultracentrifugation. Nucleic acids were extracted following the method described by Boom et al. (1990) using guanidinium thiocyanate as the principal component of lysis buffer and adsorption of the nucleic acids to silica particles, and then, analyzed by nested PCR or nested RT-PCR.

Routine examination of shellfish for *E. coli* was performed by a two-stage most probable number (MPN) method in series of 5 tubes as a modified version of the prescribed method (Robert et al. 1993). For both first and second stage Brilliant

Green Bile Broth was used. In samples tested for comparative analysis of *E. coli* contamination and presence of human enteric viruses, *E. coli* was analyzed by following the methodology described for Donovan et al. (1998). Briefly, it requires firstly inoculation in minerals modified glutamate broth and further confirmation by subculturing positive tubes onto a chromogenic agar to detect β-glucuronidase activity.

Physic-Chemical Parameters

Temperature was measured with a probe at the same depth at which shellfish was recollected. Salinity was measured with a conductivity-meter (WTW: LF 196) at the same depth where shellfish was collected. The apparatus gives measures as S/cm and to calculate salinity uses "International Oceanographic Tables" from the National British Institute and from UNESCO, with a reference temperature of 20 ° C.

Statistical Analysis

All the statistical tests were computed by using the statistical package SPSS 10.0.7 under a Pentium III machine running MS-Windows 2000 Professional. In order to perform the statistical analysis the variable *E. coli* was transformed by the $\log_{10}(x+1)$ function.

For the first part of the analysis, a bivariate regression model with *E. coli* and the variables salinity and temperature as explicative variables was carried out. The collected data had a classical factorial structure, with two levels on the type of culture (suspended and bottom cultures) and two levels on the bay factor. The temperature and salinity were considered as covariates. The analysis was performed separately in each of the four groups.

In the second part, the dependence of every pair of viruses was tested by using the Fisher exact test to evaluate any significant dependency. Additionally, a logistic regression model to measure the predictive capacity of *E. coli* on the presence of human enteric viruses (Adv, EV, HAV, NLVI and NLVII) was performed.

RESULTS

Evaluation of the stability in the classification of shellfish growing areas

Values of *E. coli* for the 1998-2001 period is presented in Fig. 1 as a percentage of samples falling into the A, B or C categories for each month. One A area and two B areas were studied. The *E. coli* values obtained were, overall, consistent with the recommended limits, although one sample from the A area presented *E. coli* values over the 230 MPN/100g limit. In the B category areas, some samples gave *E. coli* levels within the C category, but they did not exceed the 10 % allowed in the EU Directive.
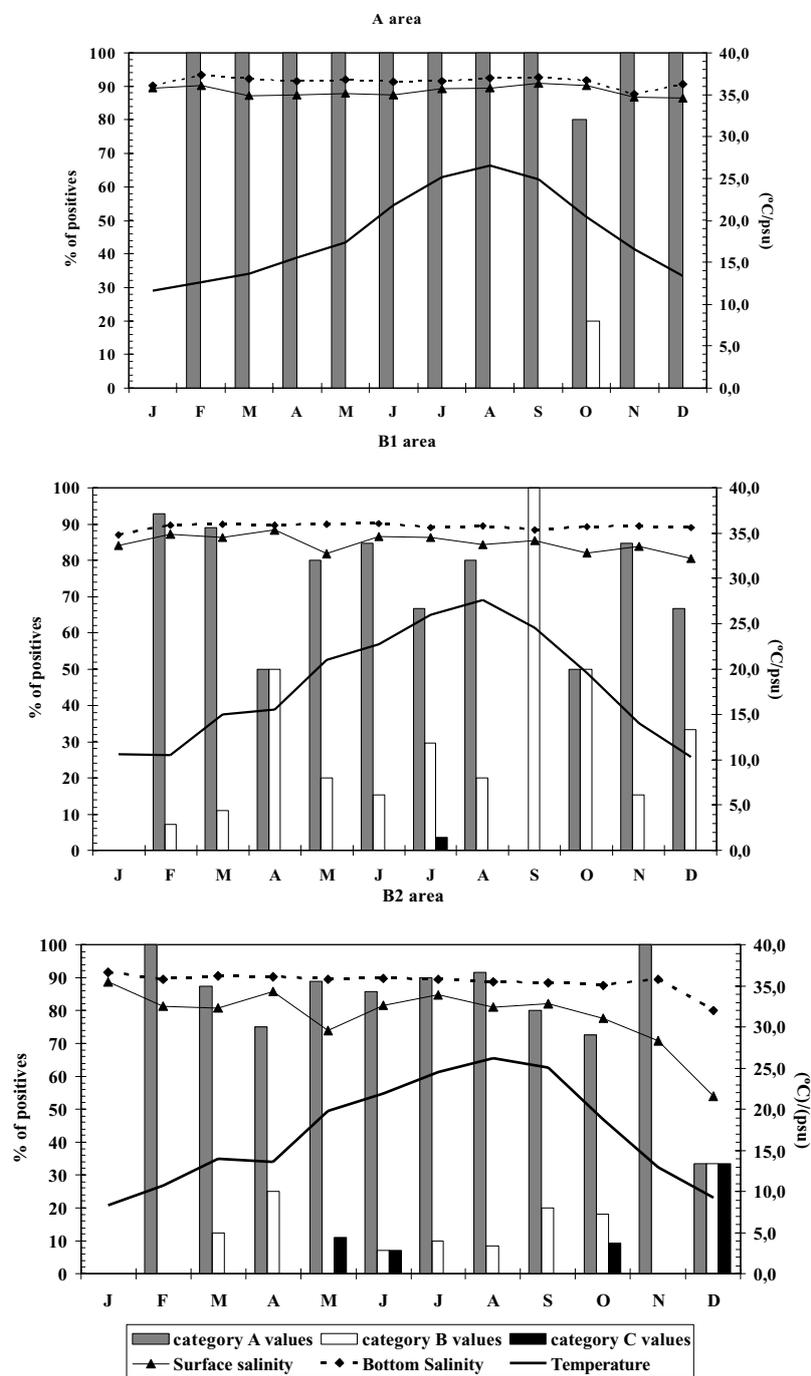
Fig. 1. *Escherichia coli* values and physic-chemical parameters in bivalve molluscan shellfish sampled during 1998 to 2001.

A bivariate regression model with *E. coli* and the variables salinity and temperature as explicative variables was performed separately in each group. The results showed no significant linear relation between *E. coli* and salinity and temperature.

From the different analysis performed in these areas, it was observed that suspended and bottom cultures presented differences related to bacterial contamination even when harvested from the same shellfish-growing area (Fig. 2). Thus, a statistical analysis was performed to assess whether the observed differences were significant. Hence, Fig. 3 shows that suspended cultures presented homogeneous *E. coli* levels in the two bays, both in variance and mean. In contrast, bottom cultures showed homogeneous values in mean, but a significant heterogeneity between both bays (p-value of the Levene test < 0.001). Thus, the means between the two species showed a significant difference at bay B1, but not at bay B2.
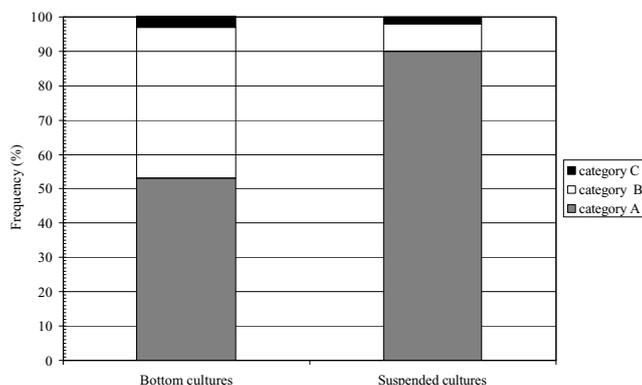

Fig. 2. Comparison of *Escherichia coli* levels in a B area between shellfish grown on suspended cultures (*Crassostrea gigas, Mytilus galloprovincialis*) or bottom cultures (*Tapes sp.*).
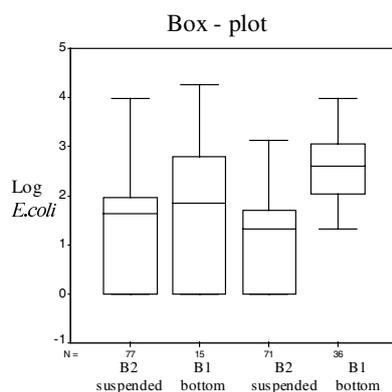

Fig. 3. Box-plot of the mean values and variances of *Escherichia coli* values between suspended and bottom cultures in the two B areas.

Comparative analysis of bacterial and viral contamination

From February 2000 to July 2001, a comparative analysis of bacterial and viral contamination in two shellfish-growing areas was carried out. For this study, the previous studied A and B2 areas were selected. Shellfish from these areas were always within the limits established by the EU Directive for each category. Furthermore, 83 % of the samples from the B area presented values lower than 230 *E. coli*/100g (data not shown). Besides, human adenovirus were the most prevalent group of viruses in both areas (Table 1). In the A area, neither EV nor HAV were found over the monitoring and only 6% of the samples contained NLVI and NLVII. In fact, the samples positives for NLV were predominantly harvested in the winter months as observed in the B area (Fig. 4). Thus, NLV was the only virus that presented a distribution pattern affected by seasonality all through the sampling.
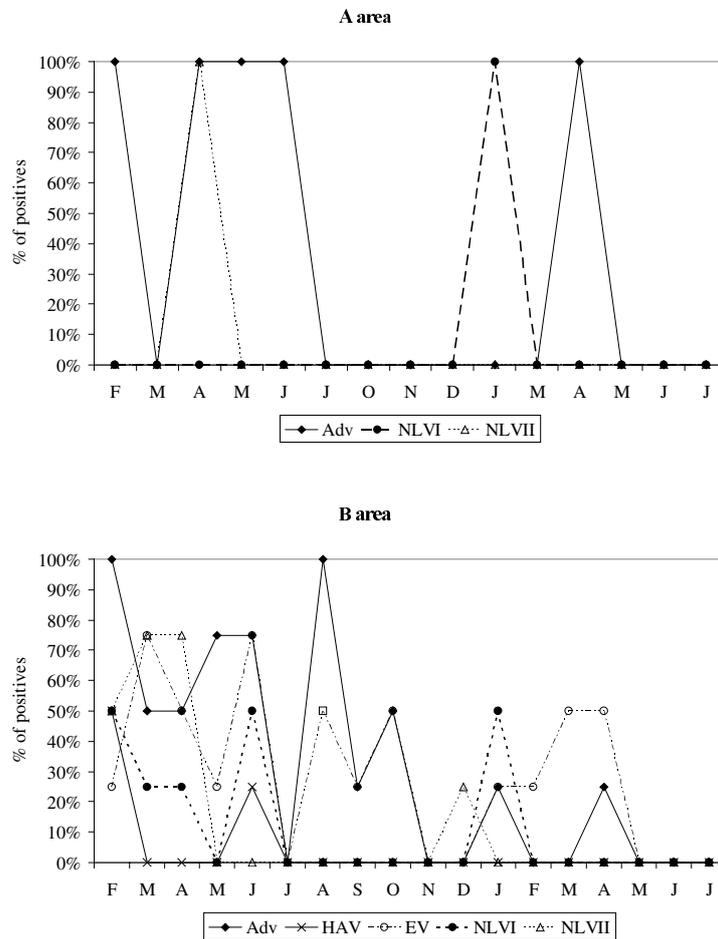

Fig. 4. Viral contamination of an A and a B area over 18 months (2000-2001).

Table 1. Percentages of shellfish samples positive for human enteric viruses at each studied area. In brackets, number of tested samples.

		Adv	EV	HAV	NLVI	NLVII
A area	*Crassostrea gigas* (16)	31	0	0	6	6
B area	*Crassostrea gigas* (34)	27	27	6	21	21
	M. galloprovincialis (34)	44	38	3	3	6
	TOTAL B area	35	32	4	12	13

Concerning the microbiological contamination before and after applying a commercial purification treatment in the samples from the B area, *E. coli* was efficiently removed after the depuration treatment and all the purified shellfish had less than 230 *E. coli*/100g, as required by current legislation (EEC/492/91), whereas viruses were not affected by this process (Table 2).

Table 2. Percentages of positive samples for *E. coli* and enteric viruses in bivalve shellfish from B area before and after a depuration treatment*.

	E. coli		Adv		HAV		EV		NLVI		NLVII	
	ND	D	ND	D	ND	D	ND	D	ND	D	ND	D
C. gigas	61	6	28	25	6	6	28	25	17	25	22	19
M. galloprovincialis	61	6	39	39	0	6	44	31	6	0	6	6
TOTAL	61	6	33	31	3	6	36	28	11	13	14	13

ND: non-depurated sample; D: depurated sample
E. coli values were within limits allowed by current legislation for human consumption in all depurated samples.

Regarding the statistical analysis carried out, it showed that *E. coli* did not present any statistical relation with the viral contaminants. However, the studied A area showed a lower level of viral contamination than the studied B area (Table 1). In addition, the Fisher exact test showed a significant dependence ($\alpha = 0.05$ level) between human adenovirus and the other viruses except for NLVI (Table 3).

Table 3. Statistical analysis of the dependence between every pair of viruses carried out by applying an independence Fisher exact test. General p-value is indicated for every pair of virus. When a significant statistical relation is found, (*) is marked.

	HAV	EV	NLVI	NLVII
Adv	.043(*)	<.001(*)	.214	.032(*)
HAV		.164	.333	.355
EV			.315	.186
NLVI				.687

DISCUSSION

Results obtained during the 1998-2001 period, showed that, in the studied area, the official shellfish water classifications of A and B were consistent. Seasonal variation of water parameters (salinity and temperature) did not have any influence on the microbiological results regarding classification in the studied areas.

When *E. coli* data (MNP/100 g) was compared by shellfish species habitat in one shellfish-growing area, it was observed that the total number of *E. coli* values falling into the category A level (≤230 MNP /100 g) was significantly higher for species cultured in suspended systems than in those living in direct contact with the sediment (bottom cultures). However, both culture system values were consistent with a B classified area.

Characterization of fecal contamination of bivalve shellfish performed from February 2000 to July 2001 showed that *E. coli* did not present any statistical relation with the viral contaminants. However, the studied A area showed a lower level of viral contamination than the studied B area.

The effectiveness of commercial depuration to reduce *E. coli* levels to below those required by European legislation, i.e. <230 *E. coli* per 100 g of mollusk flesh and intravalvular fluid, was confirmed. However, depuration as currently commercially practiced was shown not to significantly affect the occurrence of human pathogenic viruses.

NLV appeared to be the only group of virus that presented seasonal variation with lower numbers in the warm months and presented a significant relation with temperature.

Adv was the most commonly isolated human virus in shellfish and presented a statistically significant relation with the presence of other viruses. These findings and the specific human origin of the Adv detected in the PCR assay indicated that the Adv detected by PCR may be a useful molecular index of viral contamination of human origin in the environment and in shellfish as proposed in previous studies (Pina et al. 1998, Muniain-Mujika et al. 2000).

ACKNOWLEDGEMENTS

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ANNEX I. Descripció de les àrees de mostreig analitzades

En aquesta secció s'ofereix una breu descripció de les tres zones de mostreig situades al Delta de l'Ebre complementària a la ja comentada en el Capítol I d'aquesta tesi. Així, la Figura I.1. mostra la situació de la zona A estudiada, localitzada a Alcanar; la Figura I.2. mostra la zona B1, a la badia dels Alfacs, i la Figura I.3. la zona B2, a la badia del Fangar.

El Delta de l'Ebre és un sistema natural molt productiu on les interaccions entre sediments, aigua dolça i aigua de mar tenen un paper molt important. Hi coexisteixen diverses activitats com pesca i aqüicultura, cultiu d'arròs, àrees naturals protegides i turisme. L'activitat pesquera i l'aqüicultura es dóna principalment en dos estuaris, les badies dels Alfacs i del Fangar (Figures I.2 i I.3). Com s'ha mencionat al Capítol I, s'hi cultiven *Mytilus galloprovincialis, Crassostrea gigas, Ruditapes semidecussatus, Tapes decussatus, Bolinus brandaris* i *Cardium edule*. També hi ha piscifactories amb una activitat cada cop més important.

La badia del Fangar es localitza al nord del delta, presenta una fondària màxima de 4,5 m, una extensió de 12 km^2 i una capacitat de 1,6 × 10^7 m^3. La badia dels Alfacs és a la part sud del delta, amb una fondària màxima de 6,5 m, una extensió de 50 km^2 i una capacitat de 2 × 10^8 m^3. Ambdues badies es consideren estuaris (Camp i Delgado, 1987) i reben aportacions d'aigua dolça procedents dels canals d'irrigació del delta, propietat de l'associació d'agricultors.

Bàsicament, s'hi diferencien dues estacions en termes de producció primària que es veuen afectades pel cicle anual de l'arròs: l'estació més productiva va d'abril a novembre gràcies a les aportacions d'aigua dolça i nutrients associats a través dels canals d'irrigació. L'estació menys productiva va de desembre a març, quan es reben aportacions discontínues d'aigua dolça (majoritàriament associades a pluges) i hi ha una menor renovació d'aigua.

A la badia del Fangar els *blooms* algals són pràcticament inexistents, probablement perquè el seu flux d'aigua és més ràpid en comparació amb el de la badia dels Alfacs, on aquests *blooms* són més comuns. Es considera que a la badia del Fangar els canvis hidrogràfics es donen en períodes de temps molt més curts que a la badia dels Alfacs (10-20 dies), excepte per aquells provocats per tempestes (Camp i Delgado, 1987).

Figura I.1. Zona A localitzada a Alcanar, al Delta de l'Ebre. Es tracta d'un polígon de cultiu en *long-lines* o fileres on es cultiven ostrons.
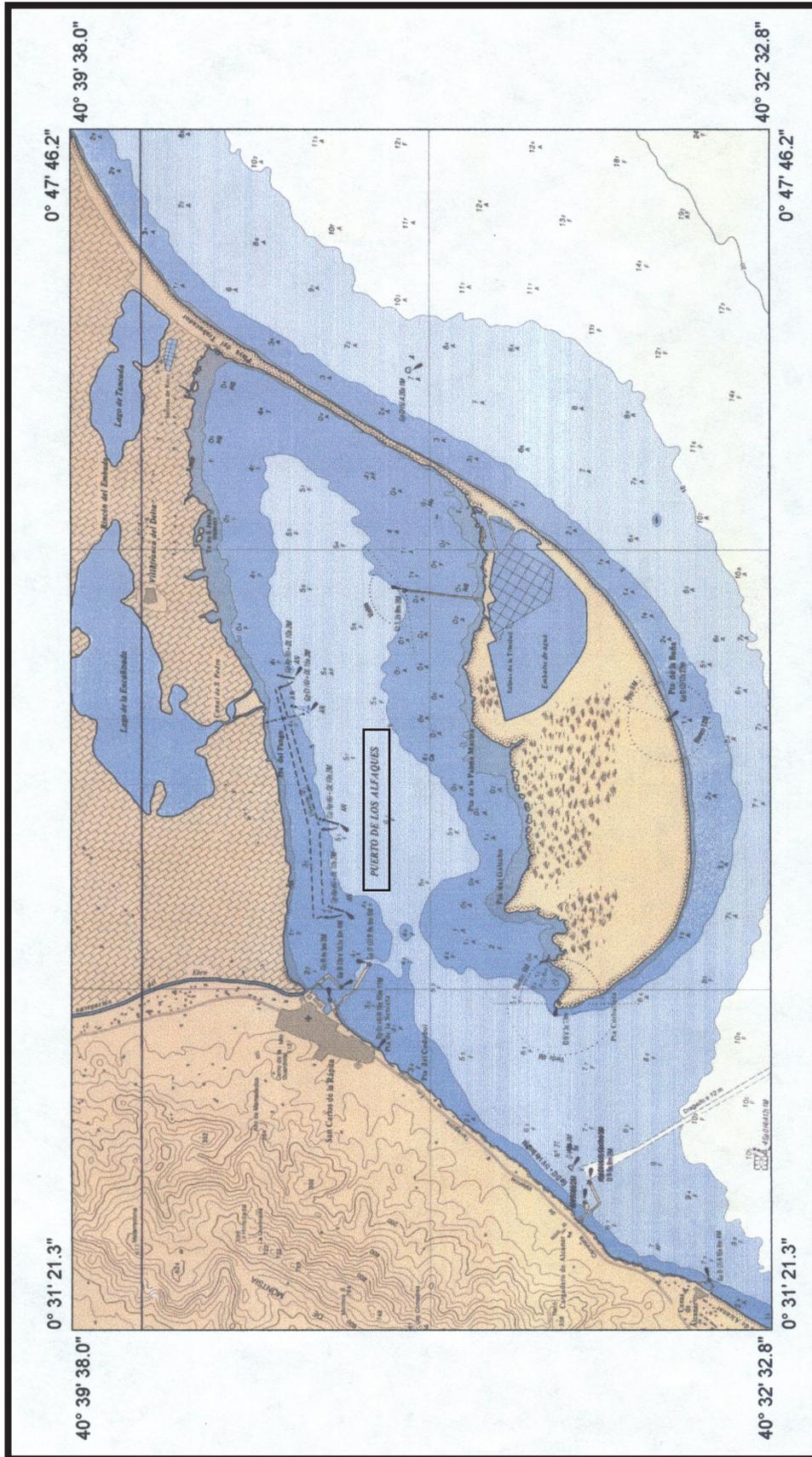
Figura I.2. Zona B localitzada a la badia dels Alfacs, al Delta de l'Ebre. Es tracta d'un polígon de cultiu en vivers de suspensió. A l'article apareix codificada com zona B1.

Figura I.3. Zona B localitzada a la badia del Fangar, al Delta de l'Ebre. Es tracta d'un polígon de cultiu en vivers de suspensió. A l'article apareix codificada com a zona B2. M-17 és la musclera on es recolliren les mostres d'ostra i musclo durant el període entre febrer del 2000 i juliol del 2001.