CAPÍTOL II. Avaluació de Possibles Indicadors de Contaminació Vírica en Bivalves de Diferents Àrees Geogràfiques

Evaluation of Potential Indicators of Viral Contamination in Shellfish with Applicability to Diverse Geographical Areas.

Meritxell Formiga-Cruz, Annika K. Allard, Ann-Christine Conden-Hansson, Kathy Henshilwood, Bodil E. Hernroth, Joan Jofre, David N. Lees, Francisco Lucena, Maria Papapetropoulou, Rachel E. Rangdale, Andromachi Tsibouxi, Apostolos Vantarakis, Rosina Girones.

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RESUM DEL CAPÍTOL

En el capítol anterior s'analitzava la distribució de diversos virus humans en mol·luscs bivalves de 4 països pertanyents a la Unió Europea. L'objectiu d'aquest segon capítol es centra en l'estudi dels nivells de diferents microorganismes proposats com a indicadors de contaminació fecal d'origen humà en relació amb virus patògens humans. Així, s'analitzaren les àrees de producció de marisc ja esmentades al capítol I per a diferents paràmetres físico-químics i microbiològics (*Escherichia coli*, bacteriòfags F-específics d'ARN, colifags somàtics i fags de la soca RYC2056 de *Bacteroides fragilis*).

Els resultats obtinguts mostraren que la temperatura i la salinitat de l'aigua de mar de les zones mediterrànies eren, com s'esperava, superiors a les de les zones situades a l'Oceà Atlàntic i al mar de Skagerrak. Pel que fa als diferents bacteriòfags estudiats, els colifags somàtics foren detectats generalment a nivells més alts que els altres grups. Cal destacar que en les àrees mediterrànies es detectaren, en general, nivells més baixos d'*E. coli*, fags F-ARN i fags de *Bact. fragilis*. També destaca el fet que els fags F-específics d'ARN van mostrar una distribució estacional similar a la dels norovirus.

En total, es recolliren dades d'un total de 475 mostres que posteriorment s'analitzaren estadísticament. D'acord amb l'anàlisi estadística, la presència de virus humans sembla estar relacionada amb la presència de tots i cadascun dels indicadors proposats en aquelles àrees més fortament contaminades, on *E. coli* seria probablement l'indicador d'elecció. Els fags F-ARN, presents en nivells més alts al nord europeu, semblen estar significativament relacionats amb la presència de contaminació vírica en bivalves, amb una capacitat predictiva molt dèbil pels virus de l'hepatitis A, adenovirus humans i enterovirus i una de major pels norovirus. Tanmateix, és important remarcar que els bivalves procedents de zones A o B netes poden contenir virus humans de forma esporàdica, fins i tot, en absència d'*E. coli* i/o fags ARN F-específics.

Les conclusions extretes del treball són:

1. Els actuals tractaments de depuració no redueixen de forma significativa els nivells de bacteriòfags de bacteris entèrics.

2. Els fags F-específics d'ARN són l'únic grup d'indicadors que mostren una relació estadísticament significativa amb tots els virus humans estudiats. Tanmateix, la utilització d'aquests fags en zones poc contaminades (amb nivells baixos d'*E. coli*) no proporciona informació addicional sobre la presència de virus patògens en mol·luscs bivalves donada la presència de virus humans en absència d'aquests bacteriòfags.

3. Els bacteriòfags F-ARN poden ser útils com a paràmetre complementari a *E. coli* per a l'avaluació de certs tractaments i en determinades localitzacions com ara els processos de depuració i norovirus al Regne Unit.

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 mol·lucs bivalves procedents de les zones de producció d'Espanya durant els 18 mesos de mostreig i també col·laborà

en la detecció de norovirus i dels diferents bacteriòfags. A més, participà en el disseny i el desenvolupament del programa de control de qualitat en els diferents laboratoris all llarg del projecte, el qual incloïa els estudis d'intercalibratge del mètode d'enumeració d'*Escherichia coli*, i en la preparació de les cartes de control per a l'avaluació de la detecció dels diferents fags estudiats a tots i cadascun dels laboratoris participants. Finalment, l'autora ha participat activament en l'anàlisi dels resultats obtinguts i en la preparació del manuscrit.

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Evaluation of Potential Indicators of Viral Contamination in Shellfish and Their Applicability to Diverse Geographical Areas

M. Formiga-Cruz,[1] A. K. Allard,[2] A.-C. Conden-Hansson,[2] K. Henshilwood,[3] B. E. Hernroth,[4] J. Jofre,[1] D. N. Lees,[3] F. Lucena,[1] M. Papapetropoulou,[5] R. E. Rangdale,[3] A. Tsibouxi,[5] A. Vantarakis,[5] and R. Girones[1]*

Biology School, University of Barcelona, Barcelona, Spain[1]; Umeå University Hospital, Umeå,[2] and Kristinebergs Marine Research Station, Fiskebäckskil,[4] Sweden; Centre for Environment, Fisheries and Aquaculture Science, Weymouth, United Kingdom[3]; and School of Medicine, University of Patras, Patras, Greece[5]

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The distribution of the concentration of potential indicators of fecal viral pollution in shellfish was analyzed under diverse conditions over 18 months in diverse geographical areas. These microorganisms have been evaluated in relation to contamination by human viral pathogens detected in parallel in the analyzed shellfish samples. Thus, significant shellfish-growing areas from diverse countries in the north and south of Europe (Greece, Spain, Sweden, and the United Kingdom) were defined and studied by analyzing different physicochemical parameters in the water and the levels of *Escherichia coli*, F-specific RNA bacteriophages, and phages infecting *Bacteroides fragilis* strain RYC2056 in the shellfish produced, before and after depuration treatments. A total of 475 shellfish samples were studied, and the results were statistically analyzed. According to statistical analysis, the presence of human viruses seems to be related to the presence of all potential indicators in the heavily contaminated areas, where *E. coli* would probably be suitable as a fecal indicator. The F-RNA phages, which are present in higher numbers in Northern Europe, seem to be significantly related to the presence of viral contamination in shellfish, with a very weak predictive value for hepatitis A virus, human adenovirus, and enterovirus and a stronger one for Norwalk-like virus. However, it is important to note that shellfish produced in A or clean B areas can sporadically contain human viruses even in the absence of *E. coli* or F-RNA phages. The data presented here will be useful in defining microbiological parameters for improving the sanitary control of shellfish consumed raw or barely cooked.

Shellfish are filter-feeding organisms that accumulate and concentrate pathogenic microorganisms present in the water, which remain infectious for a certain period (5). The role of shellfish in the epidemiology of fecally-orally transmitted infectious diseases is well known (5, 11). Traditionally, coliform bacteria and *Escherichia coli* have been used as indicators of the sanitary quality of shellfish, and this has led to success in the prevention of shellfish-borne infections by fecal bacteria. However, it has been clearly established that bacterial standards do not always reveal the presence of viruses or the presence of members of the genus *Vibrio* (9, 24). Hence, there is a need for indicators of viral fecal pollution in order to improve the microbiological control of shellfish. Somatic coliphages (24), bacteriophages infecting *Bacteroides fragilis* (16, 17, 20), and F-specific RNA (F-RNA) bacteriophages (12, 13, 16) have been proposed as potential indicators of infectious viruses. Additionally, the detection of human adenovirus by PCR has been proposed as a molecular index of viral contamination of human origin (23).

The main objective of this study was to analyze the distribution of the proposed indicators of viral contamination in shellfish produced in highly diverse geographical areas. For this purpose, shellfish samples collected in shellfish-growing areas in the Atlantic Ocean, the Skagerrak Sea, and the eastern and western Mediterranean Sea were analyzed for *E. coli*, somatic coliphages, F-RNA bacteriophages, and phages infecting *B. fragilis* RYC2056. In addition, physicochemical parameters of the shellfish-growing areas were measured. The values obtained for potential indicators through 18 months of sampling in the four areas were also compared with the presence of human adenovirus (ADV), enterovirus (EV), hepatitis A virus (HAV), and Norwalk-like viruses, including genogroup I (NLVI) and genogroup II (NLVII), in shellfish samples, described in more detail in a previous article (8). The information obtained in the study is highly valuable for improving microbiological control of shellfish and increasing the level of safety for the population.

MATERIALS AND METHODS

Sampling. Bivalve molluscan shellfish were collected in shellfish-growing areas with different levels of fecal pollution in Greece, Spain, Sweden, and the United Kingdom on a monthly basis over 18 months. These areas are classified in accordance with European Union legislation as A areas (<230 *E. coli* organisms/100 g of shellfish flesh and liquor), B areas (<4,600 *E. coli* organisms/100 g in 90% of samples), and C areas, which exceed the mentioned limits. In Greece, *Mytilus galloprovincialis* from two B areas and four A areas and *Crassostrea gigas* from two B areas were harvested. In Spain, *M. galloprovincialis* and *C. gigas* from an A area and a B area located in the western Mediterranean Sea and *Ostrea edulis* from a nonclassified area in the Atlantic Ocean was harvested. In Sweden, *M. galloprovincialis* from an A area, a B area, and a nonclassified area were collected. Finally, *C. gigas* from an A area and a B area, *Mytilus edulis* from a B area, and *C. gigas* and *M. edulis* from a B, a C, and a prohibited area in the United Kingdom were harvested.

Once collected, the shellfish were shipped directly to each laboratory via cold storage within 24 h, and *E. coli* and bacteriophages were measured immediately. Processed samples were stored at −70 ± 10°C and later used for human enteric virus detection by PCR as described by Formiga-Cruz et al. (8). The sampling

* Corresponding author. Mailing address: Biology School, University of Barcelona, Av. Diagonal, 645, 08028-Barcelona, Spain. Phone: 34-93-402-14-83. Fax: 34-93-411-05-92. E-mail: rosina@bio.ub.es.

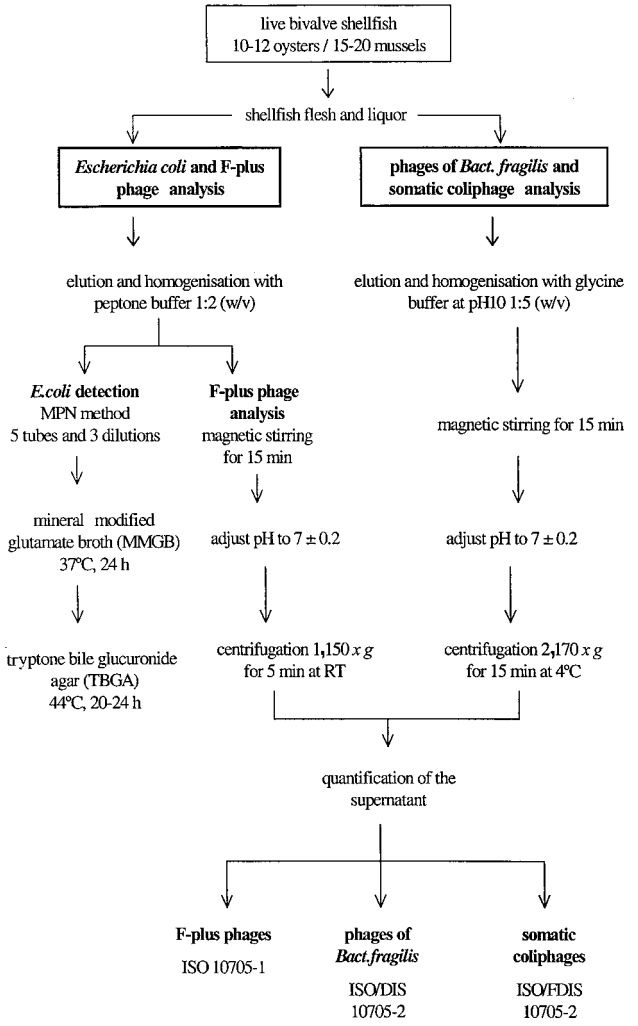

FIG. 1. Procedure for quantification of bacteriophages and *E. coli*.

regimen also included paired samples directly from B harvesting areas (1), and after the depuration treatment the end product was tested to provide information on the effectiveness of commercial depuration processes for virus removal.

Shellfish processing. Shellfish were washed, scrubbed under clean running water, and opened with a sterile shucking knife. For the analysis of phages infecting *B. fragilis*, somatic coliphages and F-RNA phages, 10 to 12 oysters and 15 to 20 mussels were selected. Shellfish flesh and liquor were collected into a sterile beaker and diluted with glycine buffer, pH 10 (1:5, wt/vol), for the detection of somatic coliphages and bacteriophages infecting *B. fragilis* (21, 23). For F-plus bacteriophages, shellfish meat was eluted with peptone water (1:2, wt/vol), since these phages have been shown to be more stable at neutral pH (7). Once eluted, they were homogenized in a blender and magnetically stirred for 15 min. After the pH was adjusted to 7.2 ± 0.2, the homogenate was centrifuged at 2,170 × *g* for 15 min at 4°C, and the supernatant was used for phage enumeration by the procedures outlined below (Fig. 1).

Performance assessment program. Concentrated suspensions of bacteriophages ϕX174, MS2, and B56-3 were distributed to all the laboratories to be used as reference suspensions in internal controls. Each bacteriophage was cultured by the appropriate method described in the corresponding ISO protocol (2, 3, 4). The bacteriophage suspension obtained was treated with chloroform at a 2.5:1 (vol/vol) culture ratio and centrifuged at 3,000 × *g* for 20 min. This high-titer phage suspension was divided into 2.2-ml volumes and stored at −70 ± 10°C. Prior to reference material preparation, each frozen vial was defrosted at room temperature to prepare 10-fold dilution series in peptone saline. Before distribution into vials in 2.2-ml volumes and storage at −70 ± 10°C, a first titration was carried out to assess homogeneity between samples. Further titrations were carried out during a long enough period to correctly assess homogeneity.

Commercially preprepared lenticules (National Collection of Type Cultures, Central Public Health Laboratory, London, United Kingdom) for *E. coli* were analyzed in order to evaluate the level of performance of the selected procedure by each laboratory. Lenticules are plano-convex discs containing biologically active material in a solid water-soluble matrix. They are easily transported and robust and can be used to provide a reproducibly countable number of CFU. Upon receipt, the lenticule contents were rehydrated in 100 ml of peptone (producing a 1:10 dilution, from which a further serial dilution was prepared) and analyzed within 1 h. Once prepared, the samples were analyzed by all partners using a two-stage, five-tube, three-dilution most-probable-number (MPN) method.

Control charts for standard suspensions of the above-mentioned phages and *E. coli* were prepared by all laboratories during the study, and sporadic discrepancies were detected and immediately corrected.

***E. coli* analysis.** The procedure for detection of *E. coli* was, with little modification, that described by Donovan et al. (6), which consists of a two-stage, five-tube, three-dilution MPN method. In brief, it requires inoculation into mineral-modified glutamate broth and further confirmation by subculturing the contents of positive tubes onto a chromogenic agar to detect β-glucuronidase activity.

Analysis of bacteriophages. All phages were quantified by the double-agar-layer method. *E. coli* WG5 grown on modified Scholten's broth was used as the host strain for the quantification of somatic coliphages. *Salmonella enterica* serovar Typhimurium WG49 (14) grown on tryptone-yeast extract-glucose broth was used as the host strain for F-specific bacteriophages. *B. fragilis* RYC2056 grown on *Bacteroides* phage recovery medium broth was used as the host strain for the quantification of *B. fragilis* bacteriophages. All procedures are described in the corresponding standardized protocol (2, 3, 4).

Human enteric virus detection. Prior to detection of enteric viruses (ADV, EV, HAV, and NLV) by nested PCR, shellfish samples collected in Greece, Spain, and Sweden were processed by the method based on elution with 0.25 N glycine buffer at pH 10 (1:5, wt/vol) described by Pina et al. (23) and Muniain-Mujika et al. (21), with some modifications (8). The laboratory in the United Kingdom analyzed bivalve mollusks by a procedure based on direct nucleic acid extraction (8, 19). The methods exhibited equivalent sensitivities in viral standard suspensions at the PCR level, and the method used in the United Kingdom laboratory also produced a high number of positive results for human viruses in shellfish.

Physicochemical parameters. The parameters of the shellfish-growing waters that were studied were temperature, salinity, pH, and dissolved oxygen content. The temperature was measured at the depth at which shellfish were collected. The pH was determined upon arrival at the laboratory with an Orion SA250 pH meter with a temperature-compensatory system and calibrated with pH 7 and 4 buffers. Salinity was measured with a conductivity meter (Wissenschaflich Technische Werkstätten; LF 196) at the depth at which shellfish were collected. Dissolved oxygen was measured with a mobile potency meter that gives measurements as saturation percentage. Calibration in air (100% saturation) was done before every measurement.

Statistical analysis. In order to perform the statistical analysis, the variables for *E. coli*, bacteriophages infecting *B. fragilis*, somatic coliphages, and F-RNA phages were transformed by the $\log_{10}(x + 1)$ function. All statistical tests were done with the statistical package SPSS 10.0.7 in a Pentium III machine running MS Windows 2000 Professional. Values that fell below the level of detection (31 PFU/100 g for phages and 20 [MPN]/100 g for *E. coli*) were considered zeroes in the statistical analysis.

The first block of the analysis included two logistic regression models. It was intended to measure the predictive capacity of a set of classificatory variables and covariates on the binary variables ADV, EV, NLVI, and NLVII. The first logistic model had the mollusk type and the country as classificatory variables and phages infecting *B. fragilis*, somatic coliphages, F-RNA phages, *E. coli*, and temperature as covariates. The second logistic model did not include the temperature as a covariate, because some samples were collected under conditions that did not allow measurement of the water temperature, i.e., low tides and other technical difficulties. It is important to note that the two models must be carefully compared because of the differences between the sample sizes. *P* values were obtained at the last step of each stepwise regression procedure. Stepwise regression is an iterative procedure that explores the statistical significance of the relations between a set of prediction variables and a response variable. In our case, the method was backward stepwise: it started with a logistic model that included all the classificatory variables (the temperature and all the phages above) and in several steps discarded the nonsignificant variables. The procedure stopped when the model was reduced to significant variables, showing a final equation that related the response and the prediction variables. This method requires a probability level, the POUT value, in order to discard nonsignificant relations along

TABLE 1. Physicochemical parameters measured

Country	Site	Classification[a]	Temperature (°C)	pH	Salinity (‰)	Dissolved oxygen content (% saturation)
Spain	1	A	13.2–25.3	8.1–8.32	34.4–37.8	8.1–102
	2	B	8.8–27.2	8.01–8.58[b]	17.3–37.6	90–105
Greece	1	B	13–27	7.1–8.2	35.6–38.13	72–103
	2	B	12–26.8	7–8.35	35.7–37.6	91–105
	3	A	12–26.7	7.2–8.2	35–38.2	88–103
	4	A	13–27	7.1–8.3	35.6–37.52	89–102
	5	A	12–26.8	7–8.35	35.7–38.2	76–105
	6	A	11–23	6.87–8.3	34.7–39.15	6–107
	7	A	11.5–25.6	6.9–8.47	34.5–37.69	52–104
	8	A	10.7–26	6.1–8.58	33.67–37.76	54–107
United Kingdom	1	A	5–21.5	6.9–8.1	14.8–34	66–102
	2	A/B	3.9–12.3	6.6–7.5	30–34	6–100
	3	B1	NT[c]	7.2–7.8	20–26	83–105
Sweden	1	B	1–18.1	8	19.9–26.8	NT
	2	NC[d]	0.8–18.2	8	19.4–26.9	NT
	3	A	1.2–19.9	8	13.8–26.4	NT

[a] According to European Union guidelines.
[b] Measured at 12 noon.
[c] NT, not tested.
[d] NC, nonclassified.

the iterative process (POUT = 0.10). The second block of the analysis was a standard test of nonparametric regression for every pair of parameters: somatic coliphages, phages infecting *B. fragilis*, F-RNA phages, *E. coli*, and temperature.

RESULTS

Level of fecal contamination in the studied areas according to *E. coli* standards. Bacteriological results have been described elsewhere (8). Briefly, areas in northern Europe yielded in general higher values than those in the south. Thus, 83% of the tested samples from the B area in Spain yielded *E. coli* values lower than 230 per 100 g. In Greece, 76 to 94% of the samples from the B areas analyzed yielded similar values. In the United Kingdom, 56 to 76% of samples presented the same levels of fecal contamination. In Sweden, 94% of samples from a B area had values below 230 *E. coli* organisms/100 g, whereas in the other B area examined these levels were detected in 46% of the samples. Regarding areas in the Skagerrak Sea, an increase of *E. coli* numbers occurred in the three Swedish areas

TABLE 2. Levels of *E. coli* and phages at each sampling site

Country	Site	Classification[a]	No. of samples tested	Level (geometric mean ± SD) of[b]:			
				E. coli (MPN/100 g)	Somatic coliphages	F-RNA phages	Phages infecting *B. fragilis*
Spain	1	A	16	23 ± 18	1,239 ± 2,567	33 ± 10	33 ± 2
	2	B	68	34 ± 355	1,132 ± 10,366	35 ± 10,554	36 ± 34
	3	NC	20	105 ± 238	6,943.74 ± 8,299.55	31 ± 0	39 ± 67
Greece	1	B	17	56 ± 174	72 ± 1,072	123 ± 716	58 ± 499
	2	B	17	26 ± 310	277 ± 1,668	55 ± 340	61 ± 803
	3	A	17	71 ± 924	70 ± 1,105	80 ± 1,201	48 ± 478
	4	A	18	100 ± 1,506	241 ± 37,714	158 ± 726	54 ± 757
	5	A	18	37 ± 559	206 ± 3,116	127 ± 873	34 ± 39
	6	A	15	94 ± 849	244 ± 1,635	60 ± 4,383	196 ± 1,678
	7	A	18	85 ± 763	576 ± 1,608	136 ± 571	87 ± 990
	8	A	18	127 ± 2,302	1,416 ± 89,163	165 ± 971	158 ± 585
Sweden	1	~~A~~ B	18	227 ± 37,558	2,018 ± 2,089	479 ± 592	168 ± 1,108
	2	~~A/B~~ NC	18	228 ± 37,618	1,157 ± 718	238 ± 413	1,108 ± 884
	3	~~B1~~ A	18	61 ± 37,700	1,872 ± 6,810	138 ± 434	57 ± 1,219
United Kingdom	1	~~B~~ A	68	93 ± 207	1,202 ± 5,907	167 ± 6,411	32 ± 9
	2	~~NC~~ A/B	17	185 ± 552	1,256 ± 4,233	70 ± 516	45 ± 50
	3	~~A~~ B	18	133 ± 2,910	3,410 ± 29,175	979 ± 4,857	42 ± 465
	4	~~B~~ B	35	67 ± 615	1,302 ± 5,711	237 ± 4,857	41 ± 98

[a] According to European Union guidelines. NC, nonclassified.
[b] Values for phages are in PFU per 100 g of shellfish flesh.

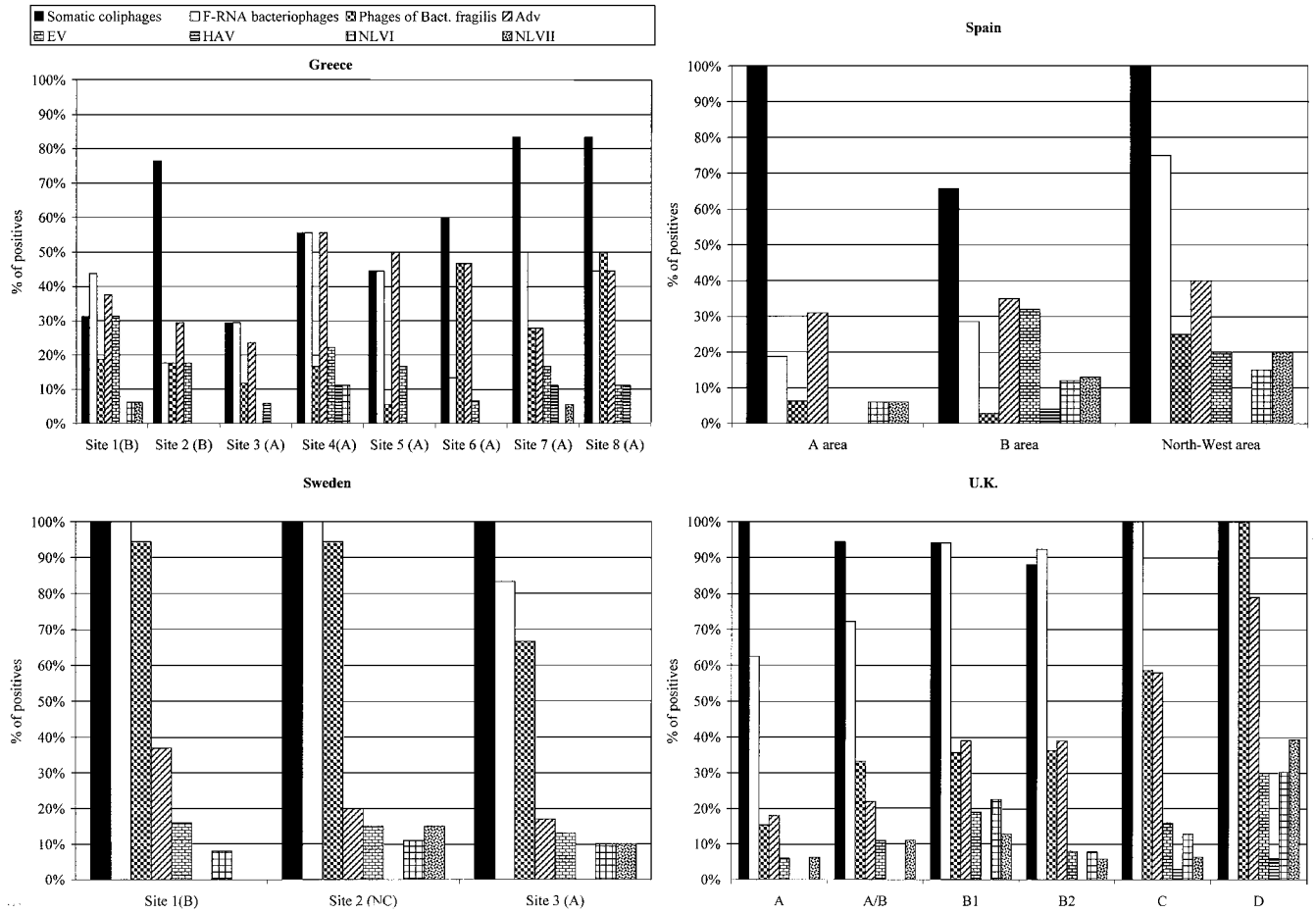

FIG. 2. Percentage of shellfish samples positive for phages and viruses in shellfish harvested in different shellfish-growing areas of four countries.

due to flooding and ice (thawing of the water in the ground) to more than 4,600 *E. coli* organisms per 100 g of shellfish flesh in the samples collected in March to April 2001.

Physicochemical parameters. Temperatures in the Mediterranean Sea shellfish-growing areas were higher than those in the areas located in the Atlantic Ocean and the Skagerrak Sea (Table 1). Regarding salinity, the values were also higher in the areas of the Mediterranean Sea, and the Swedish shellfish-growing areas presented the lowest salinities. The differences may be explained by the diverse source of water influents affecting the shellfish-growing area. In contrast, pH and dissolved oxygen content remained similar in all areas (Table 1).

Distribution of the bacteriophage levels in shellfish. In general, somatic coliphages were detected at higher levels than the other groups of phages tested (Table 2) as well as in a higher percentage of samples in all areas (Fig. 2). Additionally, the Mediterranean sampling areas presented generally low levels of *E. coli* and both F-plus phages and phages infecting *B. fragilis*, whereas in the analyzed Atlantic Ocean and Skagerrak Sea shellfish-growing areas, higher values of *E. coli* and a higher number of samples presenting F-plus phages were observed. The numbers of phages infecting *B. fragilis* were more similar across all examined zones (Table 2 and Fig. 2). F-RNA bacteriophages were shown to be the unique group of bacteriophages with a seasonal distribution and presented higher numbers in all areas and regions during the winter months. Figure 3 presents percentages of positive results, which were very high in Sweden almost every month and may be affected by water flow occurring in March to April 2001. Depuration effectively reduced *E. coli* counts but was less effective at decreasing the bacteriophage levels. In fact, in some cases the numbers of phages detected went up substantially after depuration (Table 3).

Comparative analysis of the distribution of indicators and human viruses in shellfish. The results on the presence of human viruses in the shellfish samples analyzed for bacteriophages in this study are described in detail in a previous article (8), and the comparison with the values obtained for the three groups of bacteriophages is described in Fig. 2. From all the studied viruses, only F-RNA bacteriophages and NLV (8) presented a seasonal distribution, with higher numbers during the cool months. In Fig. 2, the percentage of positive samples for all groups of phages and the human viruses analyzed in the diverse shellfish-growing areas studied is presented.

Statistical analysis. For the first block of the analysis, two different logistic regression models were made in order to examine the predictive capacity of different classificatory variables and several covariates. In one model, temperature was

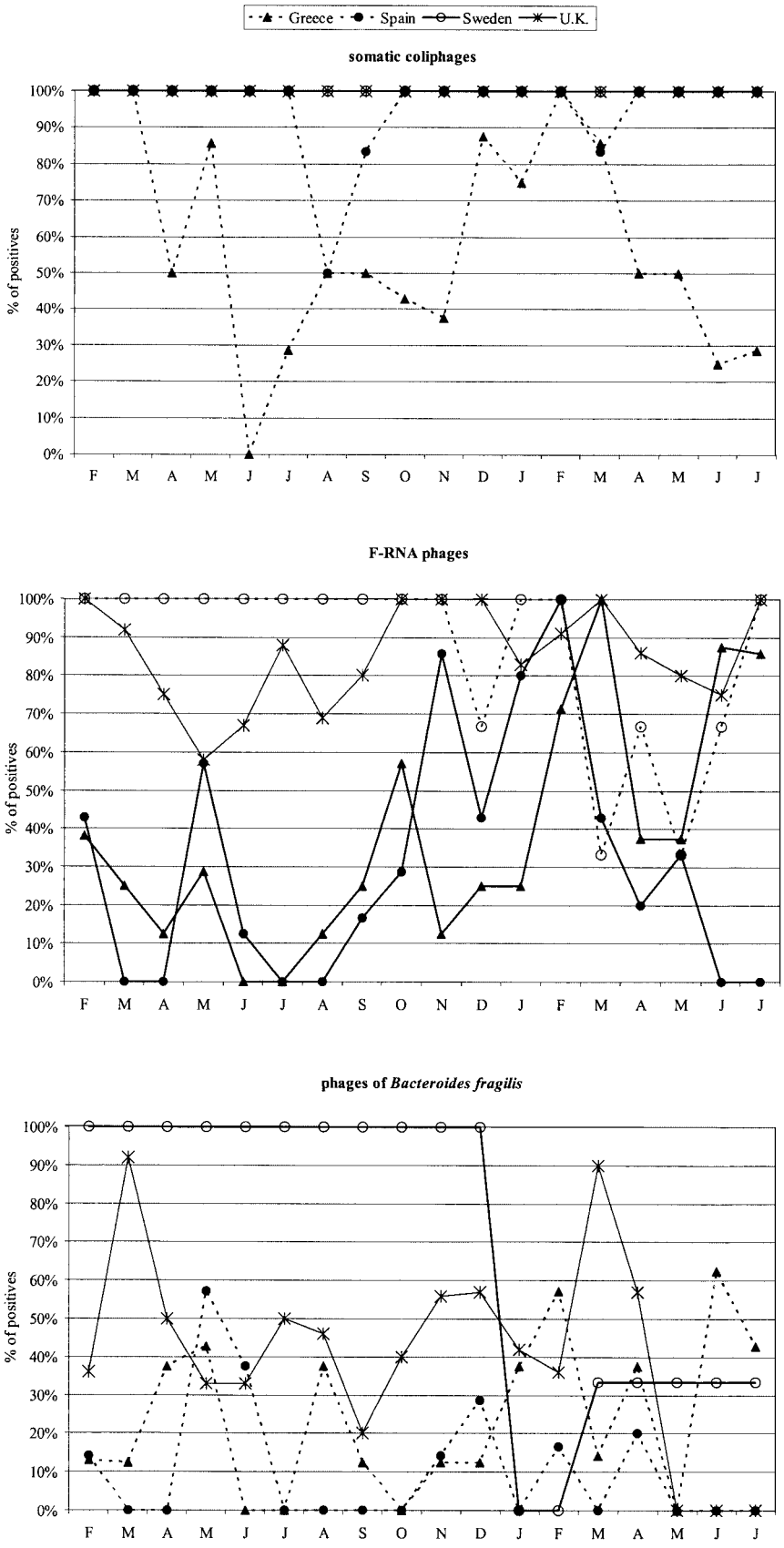

FIG. 3. Distribution of the percentage of positive shellfish samples for three groups of bacteriophages over 18 months of sampling.

TABLE 3. Mean levels of *E. coli* and the three groups of bacteriophages in bivalve shellfish from a B area before and after a depuration treatment

Shellfish (no. of samples)	Mean level[a] of:							
	E. coli		Somatic coliphages		F-RNA bacteriophages		Phages of *B. fragilis*	
	ND	D	ND	D	ND	D	ND	D
C. gigas (34)	83 (61)	1 (6)	5,061 (94)	7,312 (100)	134 (33)	43 (25)	2 (6)	30 (13)
M. galloprovincialis (34)	269 (61)	0 (6)	3,814 (94)	5,768 (88)	99 (28)	53 (19)	0 (0)	17 (25)
Combined data	176 (61)	1 (6)	4,438 (94)	6,540 (94)	116 (31)	48 (22)	1 (3)	24 (19)

[a] ND, nondepurated samples; D, depurated samples. *E. coli* values are MPN per 100 g; phage values are PFU per 100 g. Values in parentheses are percent positive samples.

excluded as a classificatory variable. According to Table 4, F-RNA phages were significantly related to the four viruses. The country classification had significant differences in the variables EV, NLVI, and NLVII. Finally, somatic coliphages had a significant relation to EV, and the mollusk classification showed significant differences for NLVI. Regarding the smaller set of data, when the temperature was included in the model (Table 5) the temperature appeared to be a significantly related variable for EV, NLVI, and NLVII but not for ADV, while F-RNA did not appear to be related to EV, NLVI, or NLVII but was related to ADV.

The differences between Tables 4 and 5 could be interpreted by taking into account the smaller sample size used for Table 5 and also by acknowledging multicollinearity effects when temperature was added in the second model. Multicollinearity appears in regression models when some (or all) prediction variables are related. This undesired situation produces a general instability in the estimation of regression because of the numerical impossibility of accurate estimates of the regression coefficients. A common consequence of multicollinearity is that a few changes in the data set (e.g., dropping several cases) may dramatically change the significance of several variables in a stepwise procedure. It was then reasonable to assume that here multicollinearity played a role in the differences between the two tables because of the significant relations between phages infecting *B. fragilis*, somatic coliphages, F-RNA phages, and temperature (see the interpretation of Table 6 below).

It is important to note the differences observed when the data from each country were examined separately. Thus, whereas F-RNA phages correlate with NLVI and NLVII in the United Kingdom and with NLVI and NLVII in Sweden and Greece, respectively, they correlate only with EV in Spain. Additionally, somatic coliphages correlate well with ADV and EV in Sweden, EV in Greece, and NLVII in Spain, while in the United Kingdom there is no relation of these phages to any of the analyzed viruses.

The second block of the analysis (Table 6) was a standard test of nonparametric regression between pairs formed by the phages infecting *B. fragilis*, somatic coliphages, F-RNA phages, *E. coli*, and the temperature variables. Table 6 shows significant relations between all the pairs, but again, as in the logistic regression analysis, all were weak relations. Notice finally the negative correlation between temperature and the phages and *E. coli*.

DISCUSSION

This is the first report on the predictive efficacy of indicator organisms across a wide range of geographical regions, with the concomitant variations in physicochemical characteristics and social trends.

Indicator microorganisms were analyzed by harvesting whole soft tissues and shell liquor to provide a procedure which is easily applied in routine food control laboratories. On the other hand, human viruses were studied by dissecting out the digestive glands to design a test with the highest sensitivity. This difference in procedures may have some implication for comparison of the results. However, 3 g of the whole animal was tested for indicator microorganisms, whereas the equivalent of 1 to 2 g of the whole animal was analyzed for human viruses.

Depuration as currently commercially practiced did not appreciably reduce the levels of F-RNA bacteriophages, phages of *B. fragilis*, somatic coliphages, or the occurrence of human pathogenic viruses in either of the countries (United Kingdom and Spain) where this was examined, though its effectiveness in reducing *E. coli* levels was confirmed. On the basis of these findings, it is suggested that the current legislative standards

TABLE 4. Logistic regression model without temperature as a classificatory variable (468 cases)[a]

Virus	*P* values for classificatory variable					% of samples		
	Somatic coliphages	F-RNA phages	Phages of *B. fragilis*	Mollusk type	Country	With correct classification	Positive, correctly classified	Negative, correctly classified
ADV		<0.001*				60.5	46.2	69.6
EV	0.027*	0.022*	0.092		0.005*	64.6	54.0	67.0
NLVI		<0.001*		0.004*	<0.001*	75.5	74.3	75.6
NLVII	0.069	0.038*			<0.001*	75.1	67.4	75.8

[a] Asterisks indicate significant values showing dependence between classificatory variables and human viruses. No values were obtained for *E. coli*.

TABLE 5. Logistic regression model with temperature as a classificatory variable (306 cases)[a]

Virus	*P* values for classificatory variable					% of samples		
	Somatic coliphages	F-RNA phages	Mollusk type	Country	Temp	With correct classification	Positive, correctly classified	Negative, correctly classified
ADV		<0.25*				61.8	27.8	80.3
EV	0.021*			<0.001*	0.021*	61.7	58.6	61.1
NLVI			0.030*	0.091	<0.001*	84.0	60.9	85.9
NLVII	0.041*			<0.008*	<0.027*	81.7	63.0	83.5

[a] Asterisks indicate significant values showing dependence between classificatory variables and human viruses. No values were obtained for *E. coli* and phages of *B. fragilis*.

for *E. coli* in postpurification bivalve mollusks do not effectively protect the consumer from the risk of exposure to pathogenic viruses associated with fecal contamination. This finding is supported by numerous reports of viral illness outbreaks following consumption of depurated shellfish (15, 18). It further follows that it is important to reexamine the protection afforded to shellfish consumers by the requirements for depuration in current legislation. A particular issue for examination is the reliance on removal of *E. coli* organisms and fecal coliforms to determine the duration of depuration. A recently published study suggests 5-day depuration treatment under technically well-controlled conditions in order to ensure elimination of viruses in mussels (22).

The quantification of F-RNA bacteriophages, somatic coliphages, and bacteriophages of *B. fragilis* as indicators of viral pollution in shellfish through 18 months of sampling was carried out in four geographical areas, and the relationship between these and both *E. coli* and human enteric viruses was evaluated. Initial analysis revealed a degree of correlation between all indicator organisms, but a high variability was observed in the data, particularly at sites classified as A, A/B, and B. Closer association was demonstrated with less overall variability at more heavily polluted sites (B areas in Northern Europe). However, it should also be noted that in cleaner shellfish areas (with very low levels of *E. coli*, such as A and B areas in Spain), the correlation between all fecal pollution indicators and the presence of human enteric viruses was less robust. Therefore, titers of somatic bacteriophages were highly variable and did not correlate well with occurrence of enteric viral pathogens. In addition, the method for this indicator isolates diverse families of phages, rather than a single family, which markedly complicates the interpretation of the data and the potential setting of standards.

Among the bacteriophages studied, the F-RNA bacteriophages demonstrated the most significant relationship to the presence of human viruses in shellfish, although with very weak predictive capability for ADV, EV, and HAV and a stronger predictive capability for NLV. Phages infecting *B. fragilis* were less frequently detected than the F-RNA phages in all areas studied. Distribution of F-RNA bacteriophages was also shown to be seasonal, with higher numbers recorded in all areas and regions during the winter months; this trend was also observed in the identification of typified NLV but not with detection of ADV, EV, or HAV. However, levels of F-RNA bacteriophages often fell below the limit of sensitivity of the assay (31 PFU/100 g), particularly in shellfish collected from southern European waters, where shellfish samples negative for F-RNA phages contained human viruses. It has been reported previously that the viability and the stability of viral particles in seawater are highly influenced by temperature (10) and by prolonged exposure to higher-intensity UV radiation during the summer months. It has been suggested that somatic coliphages may multiply under specific circumstances in the environment, though F-RNA phages apparently do not (25). This may influence the concentration of the detected phages to a limited extent. The impact of fluctuations in environmental parameters would be particularly notable in shallow waters and may offer a partial explanation for the low phage recovery in some studied areas. It may then be necessary to consider testing specific viral pathogens by RT-PCR or to evaluate by PCR ADVs (DNA viruses), which are exclusively human viruses and the most prevalent human viral parameter in shellfish. In addition, ADVs exhibit a significant relation to the presence of other viruses, as shown by M. Formiga-Cruz et al. (8).

The results of the statistical analysis of the data produced in this study showed that the only group of phages with a significant relationship to all studied viruses was F-RNA phages, but only when all 475 results were analyzed together. However, the utility of this parameter is not clear in the evaluation of the viral contamination in shellfish samples collected in A or clean B areas, especially in countries of southern Europe. In these areas, most of the samples yielding viruses were negative for F-RNA phages, and so this parameter would not provide any additional indication of the presence of human viruses in shell-

TABLE 6. Spearman correlation coefficients between potential indicators

Indicator	Correlation coefficient (significance[a])			
	F-RNA phages	Phages of *B. fragilis*	*E. coli*	Temp
Somatic coliphages	0.527 (<0.001*)	0.091 (<0.001*)	0.401 (<0.001*)	−0.485 (<0.001*)
F-RNA phages		0.340 (<0.001*)	0.447 (<0.001*)	−0.316 (<0.001*)
Phages of *B. fragilis*			0.249 (<0.001*)	−0.190 (0.001)
E. coli				−0.252 (<0.001*)

[a] Asterisks indicate significant values showing dependence between potential indicators.

fish compared with *E. coli* tests. It is important, however, to note that the method used for the quantification of the studied phages was well standardized and was successfully implemented, without significant quality problems or cost, in all laboratories involved in the study, according to a performance assessment program. Quantification of F-RNA phages may be useful as a parameter complementary to *E. coli* and as an indicator for specific parameters in specific locations, for instance, NLV in the United Kingdom. Furthermore, although shellfish ready for human consumption in Europe yield <230 *E. coli* organisms/100 g of shellfish, the addition of a viral indicator ensuring, if possible, the absence of F-RNA phages could improve the microbiological control of the shellfish distributed to be consumed raw or lightly cooked with a reasonable cost for monitoring the final product in specific geographical areas.

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ANNEX I. Control de qualitat de l'anàlisi dels bacteriòfags F-específics d'ARN, colifags somàtics i fags de *Bacteroides fragilis*

IA. Introducció a les cartes de control

Les cartes de control proporcionen els límits d'acceptabilitat i/o tolerància i el seu ús està lligat als processos de control de qualitat i calibratge.

El control intern de qualitat es desenvolupa mitjançant mesures realitzades amb materials referència. Els materials han de ser estables, homogenis i amb una mínima variabilitat vial a vial, raó per la qual ha de mesurar-se l'homogeneïtat en i entre vials.

L'objectiu principal del control estadístic de la qualitat és la reducció sistemàtica de la variabilitat. Amb la introducció de controls estadístics del procés, aquest s'estabilitzarà i la variabilitat es veurà reduïda.

I.A.1. Causes de variació: errors sistemàtics, deguts a l'atzar i incertesa de mesura

En qualsevol procés de mesura sempre existeix un cert grau de variació inherent o natural degut a errors a l'atzar. Poden trobar-se presents altres menes de variabilitat. Aquesta variabilitat és generalment major que la variabilitat natural i normalment suposa un nivell acceptable del funcionament del sistema. Aquestes fonts de variabilitat que no formen part de l'esquema de les causes fortuïtes s'anomenen causes atribuïbles i normalment, són degudes a errors sistemàtics.

La naturalesa sistemàtica o a l'atzar d'un error es determina mitjançant el concepte de mesures repetides i depèn de les condicions en què es realitzen les mesures. Hi ha dos tipus ben definits de condicions: les condicions de repetitivitat i les condicions de reproductibilitat.

- Repetitivitat: s'aconsegueix quan el mateix operador realitza totes les mesures sobre la mateixa quantitat seguint un únic protocol ben definit, en condicions d'operació idèntiques i durant un curt interval de temps. En aquestes condicions tots els errors són sistemàtics.

- Reproductibilitat: diferents operadors realitzen algunes de les mesures sobre la mateixa quantitat seguint un únic protocol ben definit, però en condicions d'operació diferents i en temps diferents. En aquest cas, no tots els errors són de caire sistemàtic.

Quan es realitza una mesura existeix una variabilitat natural resultat de l'efecte acumulatiu de moltes i petites causes, essencialment incontrolables. Quan la variabilitat natural és relativament petita es considera que hi ha un nivell acceptable de funcionament del procés.

En el control de qualitat, a la variabilitat natural se l'anomena sistema estable de causes fortuïtes. Un procés que funcioni només amb causes fortuïtes de variabilitat es considera sota control, però si funciona en presència de causes atribuïbles es considera fora de control.

Un dels objectius del control de qualitat és la detecció ràpida de causes atribuïbles o canvis en el procés per tal de poder-ho investigar i prendre accions correctives.

La finalitat del control de qualitat és l'eliminació de la variabilitat d'un procés o reduir-la al mínim possible. Una de les formes de determinar i eliminar la variabilitat d'un procés és mitjançant l'ús de diagrames o cartes de control.

La incertesa és un paràmetre associat amb el resultat d'una mesura que caracteritza la dispersió de valors que es pot atribuir de forma raonable a allò que es pretén mesurar. Pot representar-se com una desviació estàndard, un interval de confiança, etc.

El concepte d'incertesa no ha de confondre's amb el d'error. Un error de mesura és la diferència entre el valor real i l'obtingut. Pel contrari, la incertesa pren la forma d'un interval dins el qual entra amb una certa probabilitat el valor d'allò que es mesura.

I.A.2. Base estadística del diagrama de control

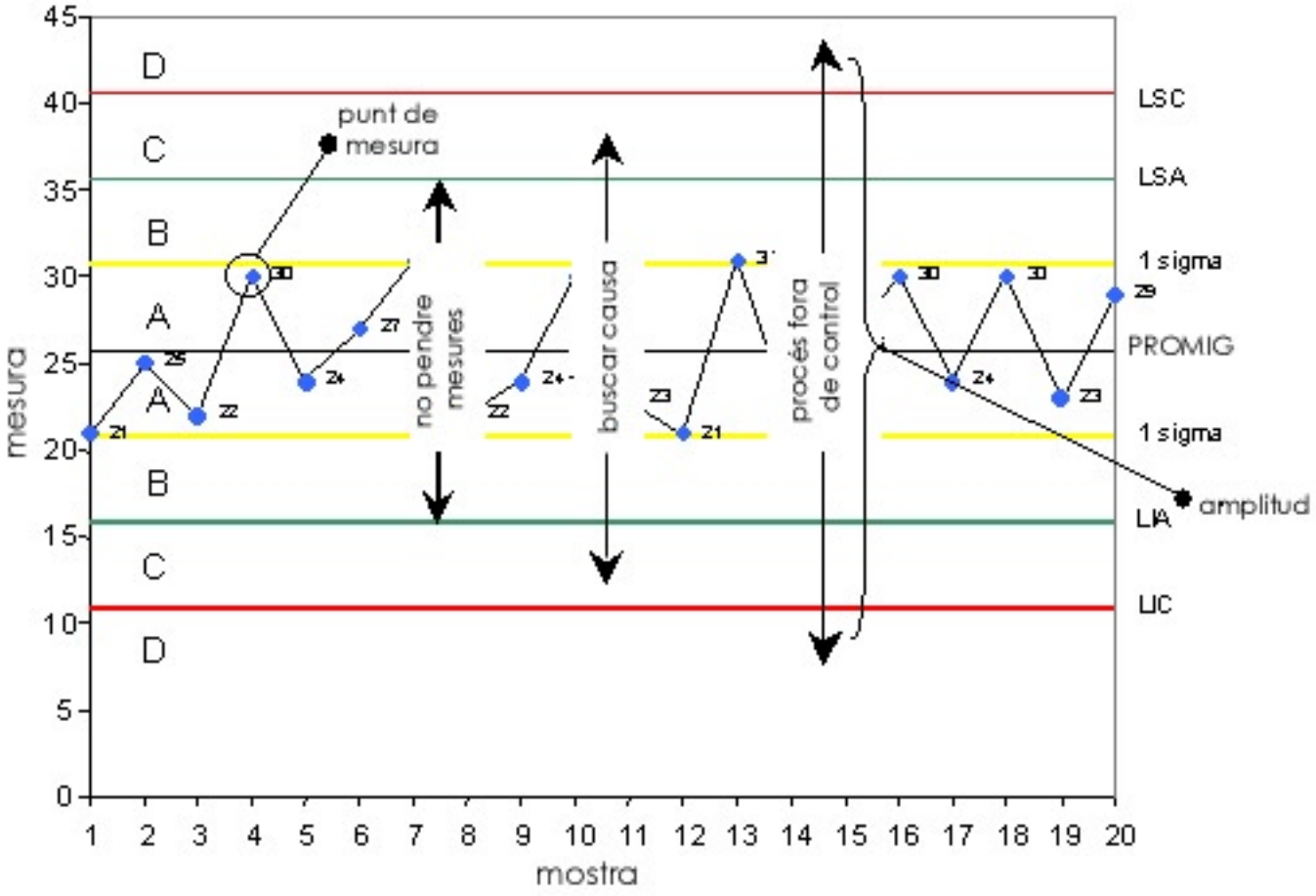
Un diagrama típic de control és una

representació gràfica d'una característica de qualitat o mesura en funció del número de la mostra o del temps.

Els patrons o materials de referència tenen una valor diana mitjà definit, i molt més en microbiologia, on es treballa amb individus. El gràfic, així doncs, presenta una línia central que representa el valor mitjà de la característica de qualitat o mesura. En el gràfic també es mostren dos, quatre o sis línies horitzontals que representen límits de control, d'alerta o desviacions (Figura A1).

Els diagrames de control es classifiquen segons el tipus de característica de qualitat,

Figura A1. Diagrama de control d'individus i rang mòbil. Límits en els cartes de control (modificat de Méndez, 2002)



LSC: límit superior de control; LSA: límit superior d'alerta; LIC: límit inferior de control o d'acció; LIA: límit inferior d'alerta

és a dir, si es pot expressar com una mesura (diagrames de control de variables) o mitjançant un atribut (diagrames de control d'atributs). Tradicionalment, els diagrames de control per a la tendència central i la variabilitat s'anomenen diagrames de control de variables. A partir d'ara, ens referirem a aquest últims.

L'especificació dels límits de control és una de les decisions crítiques que cal prendre al dissenyar una carta de control; allunyar aquests límits de la línia central redueix el risc d'errors de tipus I (on se situarien algunes característiques de qualitat fora dels límits, la qual cosa indicaria una condició fora de control quan no existeix una causa atribuïble), però augmentaria el risc d'errors de tipus II (on se situarien algunes característiques de qualitat dins els límits, que indicaria una condició controlada quan de fet, es trobaria fora de control i existiria una causa atribuïble). Generalment, els límits de control es calculen com el valor mitjà ± 3 sigmes (desviació estàndard) i el límits d'alerta com el valor mitjà ± 2 sigmes.

I.A.3. Interpretació d'un diagrama de control

Una carta de control pot indicar una condició fora de control quan un o més punts es troben fora dels límits o quan els punts exhibeixen un patró de comportament no aleatori.

Una successió d'observacions del mateix tipus o tendència major o igual a 8 punts representa un patró no aleatori i el procés es troba fora de control.

Si es donen un o més dels següents criteris, el procés es troba fora de control:

1. Punts fora dels límits de control.

2. Una tendència de 7 o 8 punts ascendent o descendent a un o altre costat de la línia central.

3. Dos o tres punts fora dels límits d'alerta o advertència però dins els límits de control.

4. Quatre o cinc punts consecutius més enllà dels límits sigma.

5. Patrons anormals o no aleatoris de dades.

6. Un o més punts a prop d'un límit d'advertència o control.

I.B. Diagrames de control dels materials de referència emprats en els estudis d'intercalibratge

En aquesta secció es mostren les cartes de control resultat de les titulacions del material de referència fetes per a assegurar-ne l'homogeneïtat, tal i com s'indica a l'apartat **"Performance assesment program"** de l'article.

Els resultats obtinguts permeten fer una millor interpretació de les dades obtingudes en els estudis d'intercalibratge, ja que estableixen el rang de valors de les suspensions de fags distribuïdes com a material de referència.

Figura B1. Diagrama de control del material de referència del fag ΦX174 (colifag somàtic)

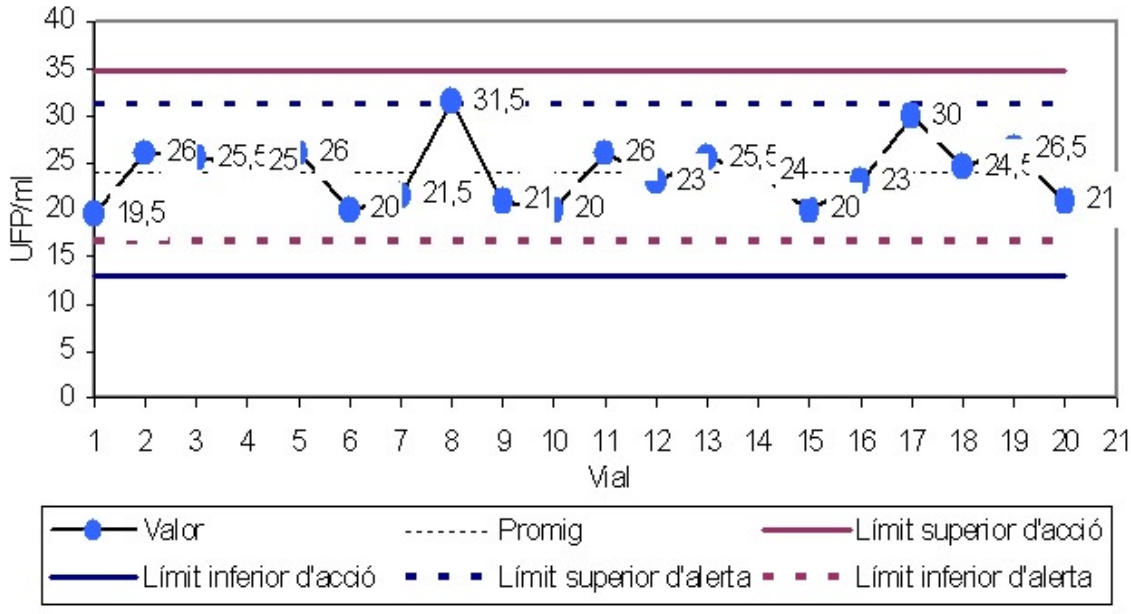


Figura B2. Diagrama de control del material de referència del fag MS2 (fag F-específic d'ARN)

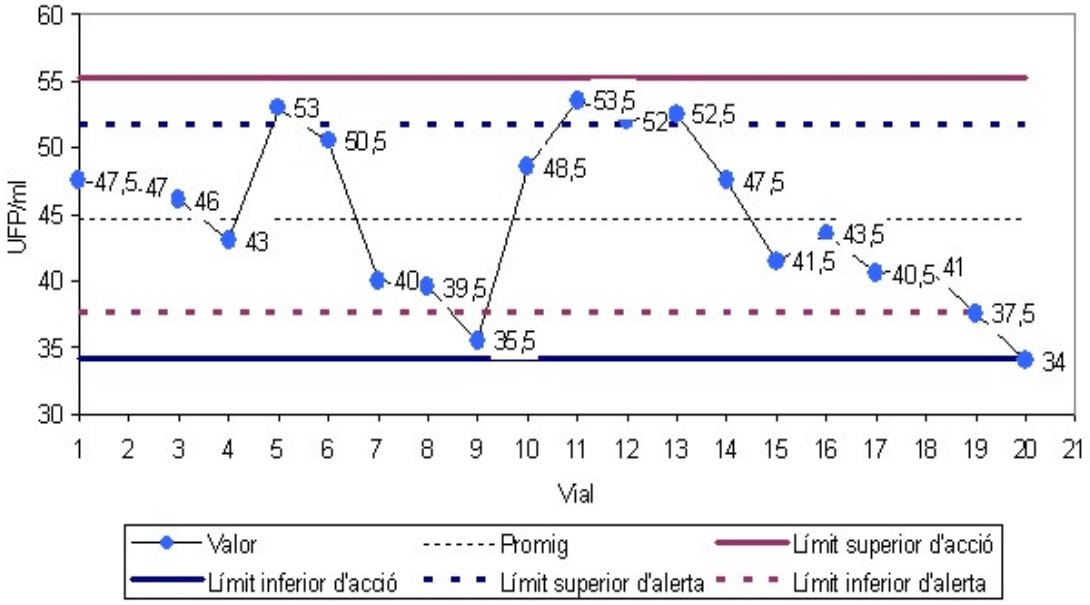
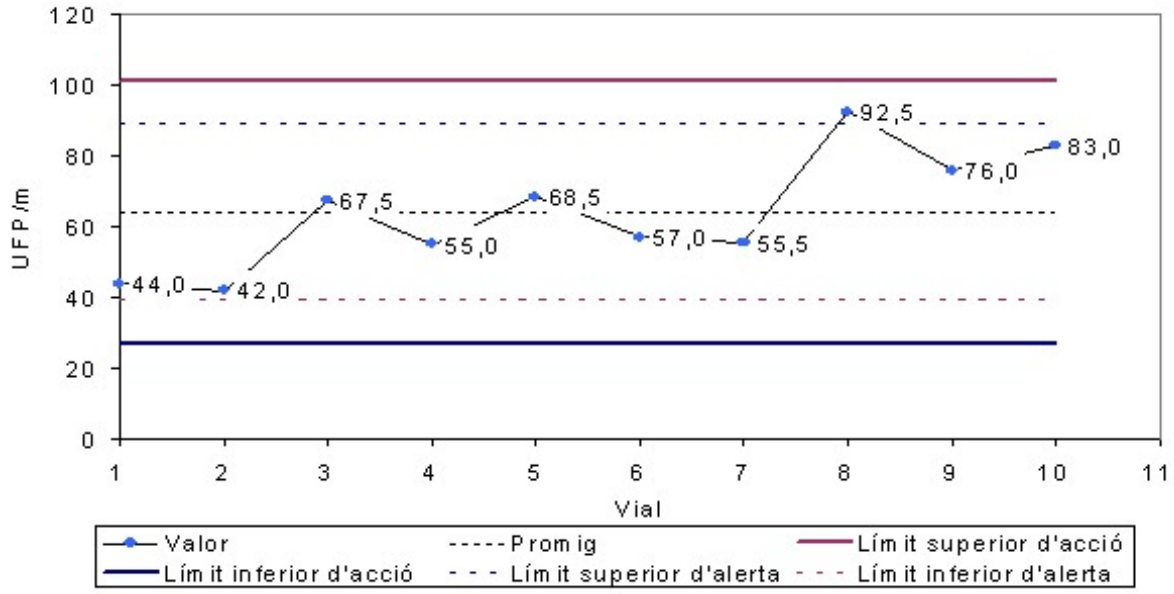


Figura B3. Diagrama de control del material de referència del fag B56-3 (fag de *Bact. fragilis*)



I.C. Diagrames de control dels estudis d'intercalibratge del protocol de quantificació de bacteriòfags de bacteris entèrics

Com s'ha esmentat als Materials i Mètodes de l'article, els laboratoris participants en aquest treball realitzaren estudis d'intercalibratge dels protocols de quantificació de bacteriòfags a partir del material de referència preparat al laboratori 1.

Els resultats obtinguts per a cadascun dels bacteriòfags es presenten en forma dels següents diagrames de control.

Figura C1. Diagrama de control de la quantificació del fag ΦX174 al laboratori 1 (Espanya)

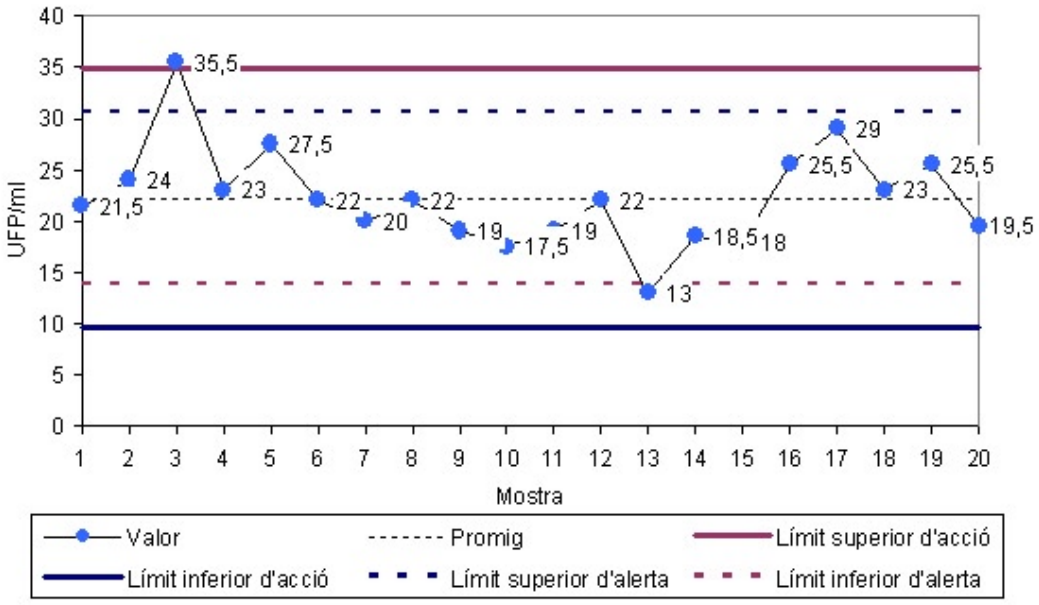


Figura C2. Diagrama de control de la quantificació del fag MS2 al laboratori 1 (Espanya)

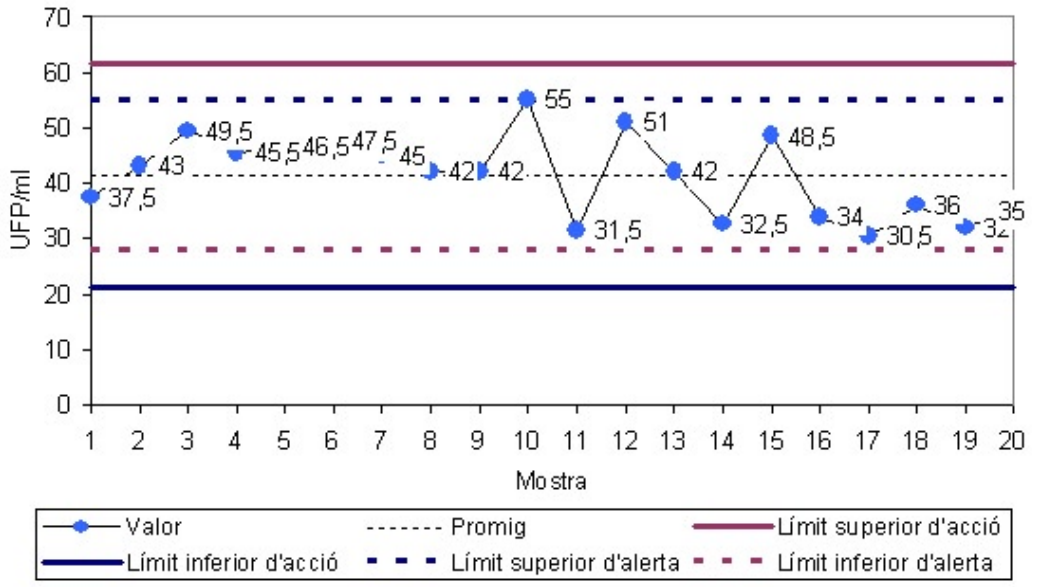


Figura C3. Diagrama de control del prócés de quantificació del fag B56-3 pel laboratori 1 (Espanya)

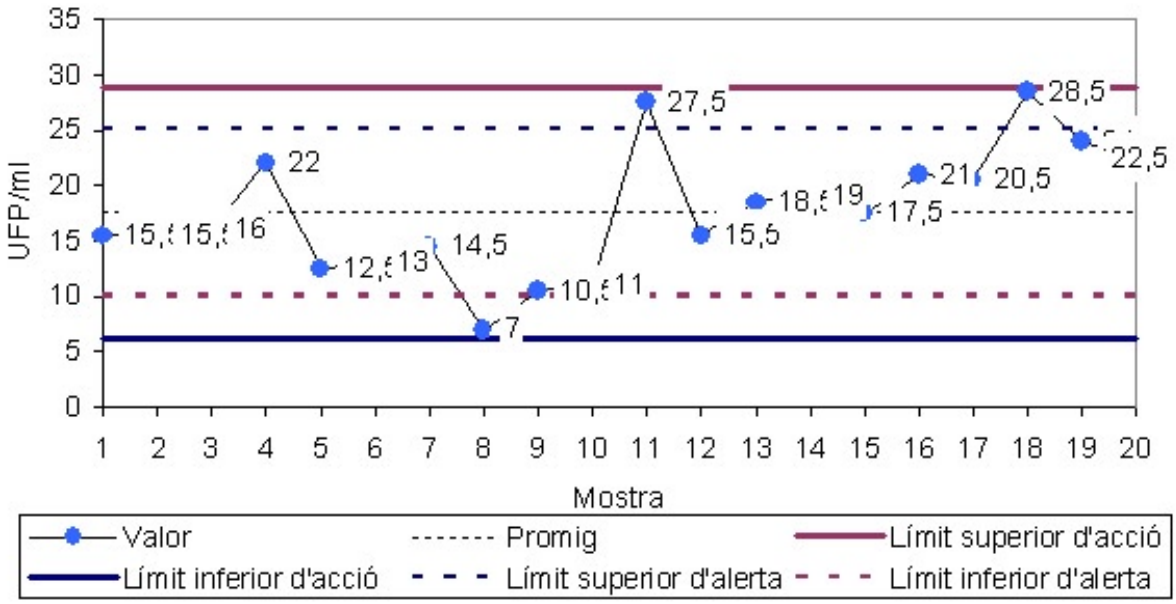


Figura C4. Diagrama de control de la quantificació del fag ΦX174 al laboratori 2 (Grècia)

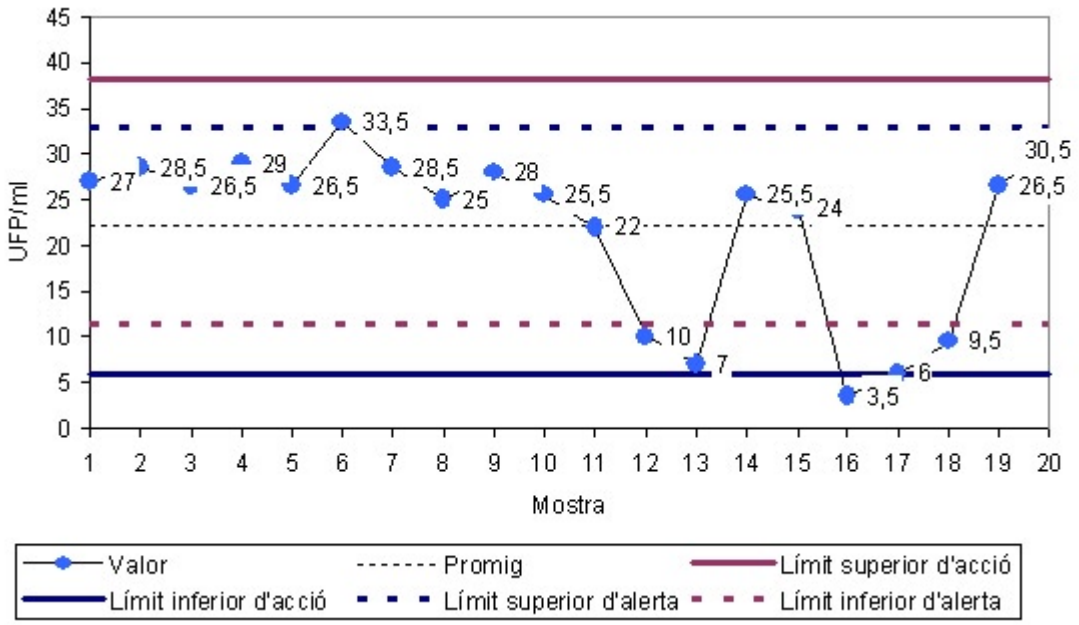


Figura C5. Diagrama de control de la quantificació del fag MS2 al laboratori 2 (Grècia)

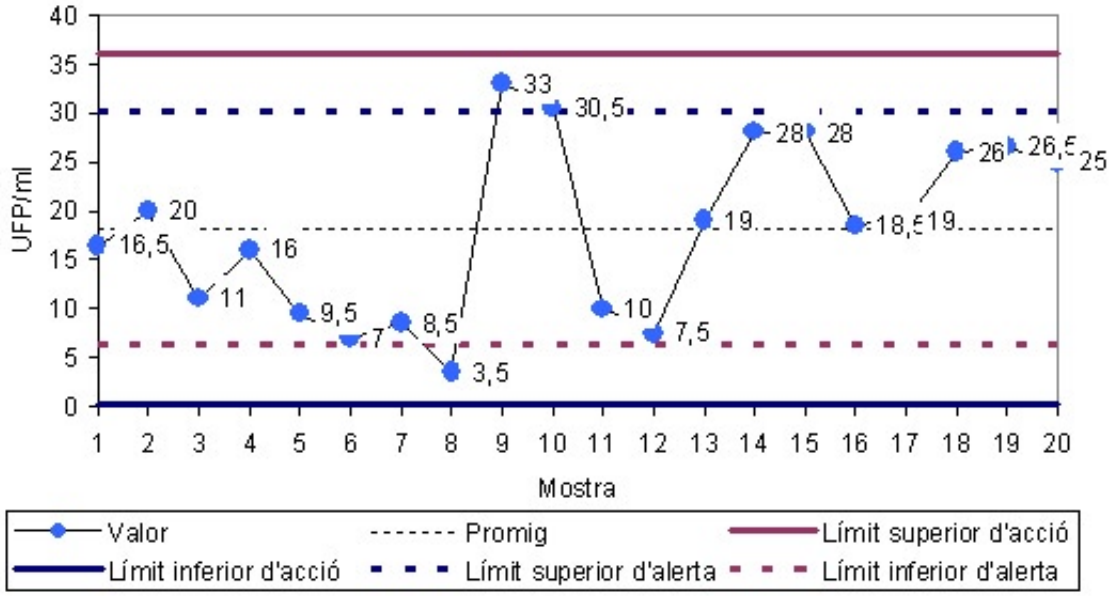


Figura C6. Diagrama de control de la quantificació del fag B56-3 al laboratori 2 (Grècia)

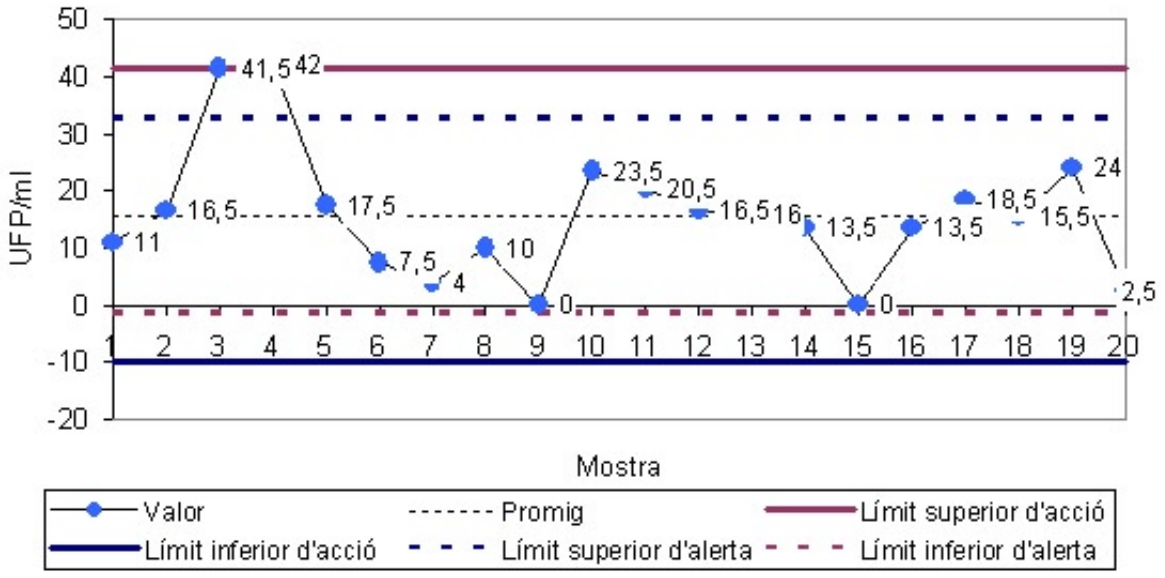


Figura C7. Diagrama de control de la quantificació del fag ΦX174 al laboratori 3 (Regne Unit)

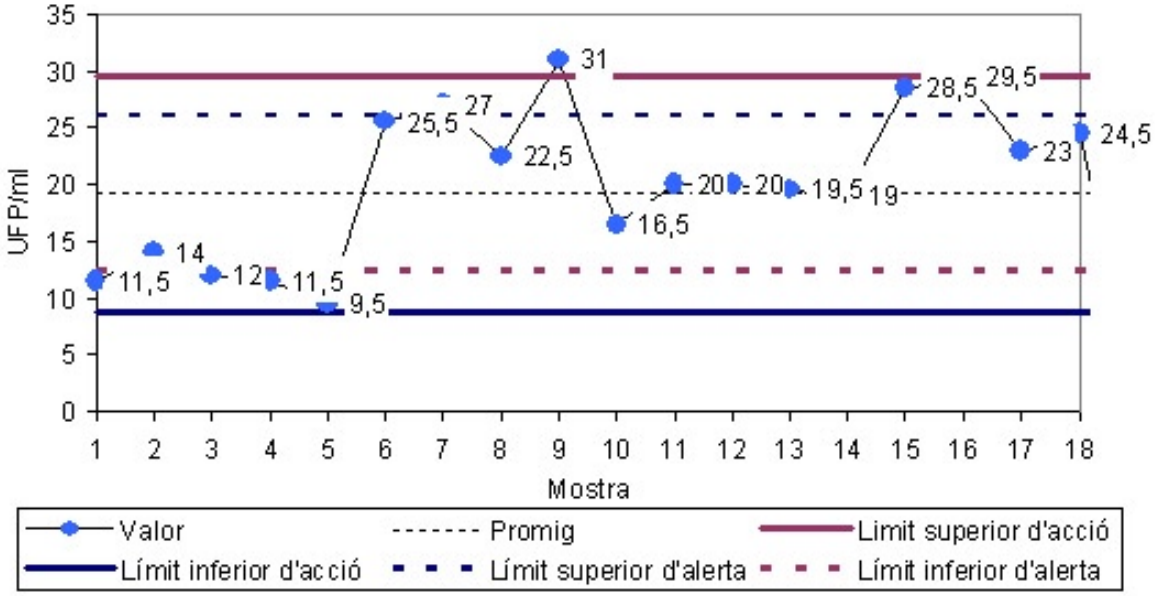


Figura C8. Diagrama de control de la quantificació del fag MS2 al laboratori 3 (Regne Unit)

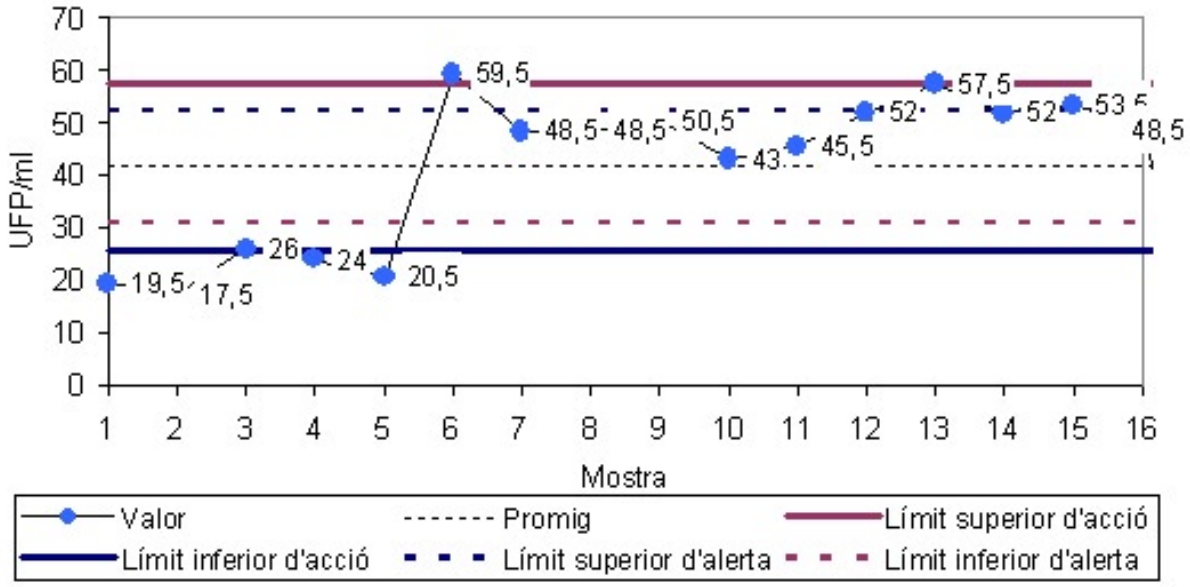


Figura C9. Diagrama de control de la quantificació del fag B56-3 al laboratori 3 (Regne Unit)

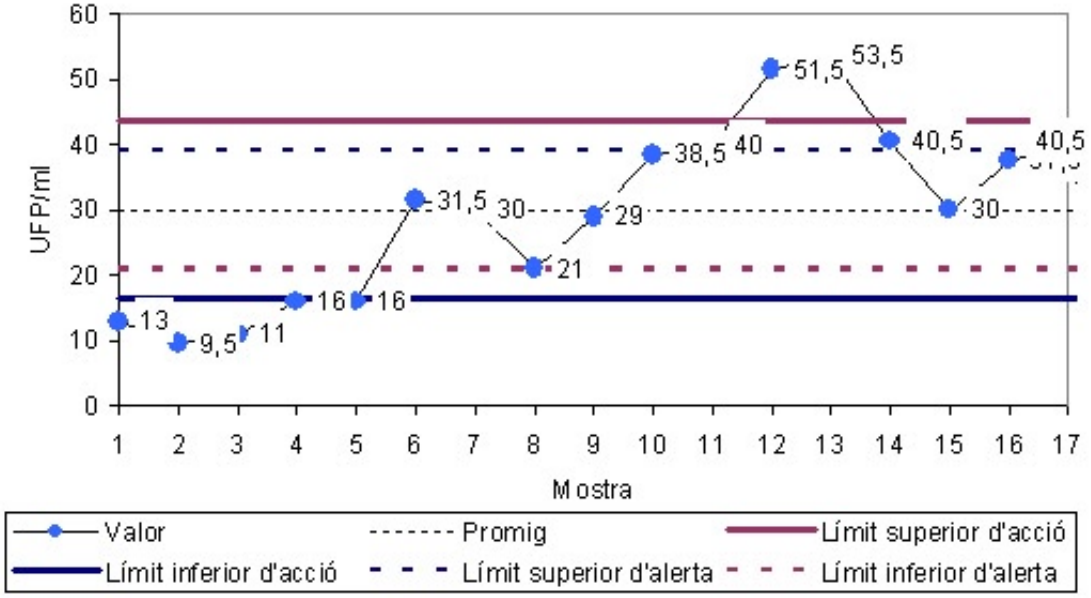


Figura C10. Diagrama de control de la quantificació del fag ΦX174 al laboratori 4 (Suècia)

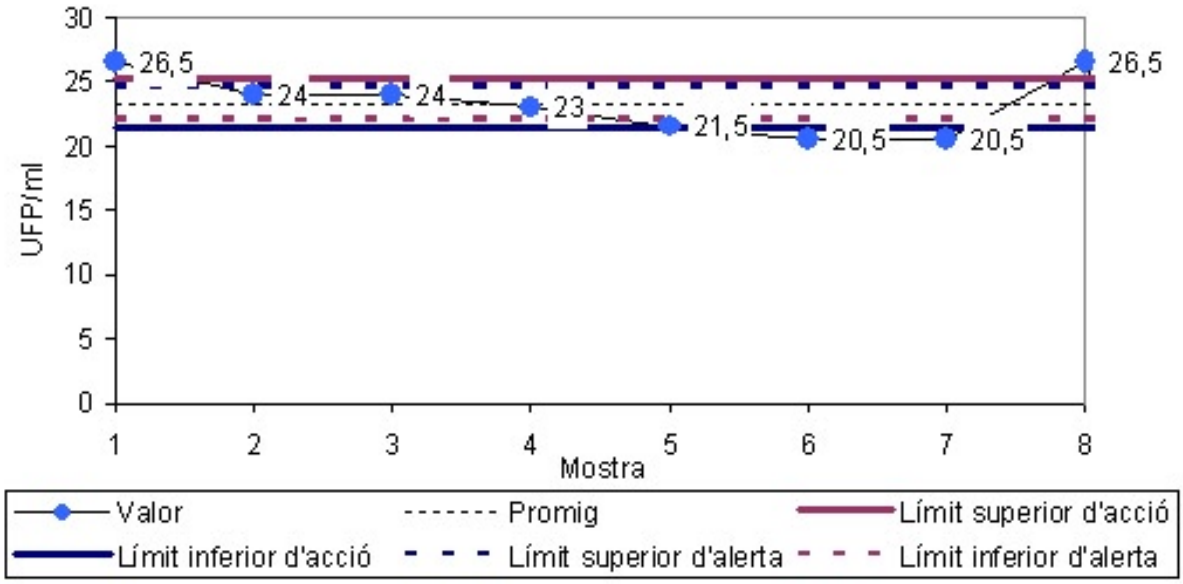


Figura C11. Diagrama de control de la quantificació del fag MS2 al laboratori 4 (Suècia)

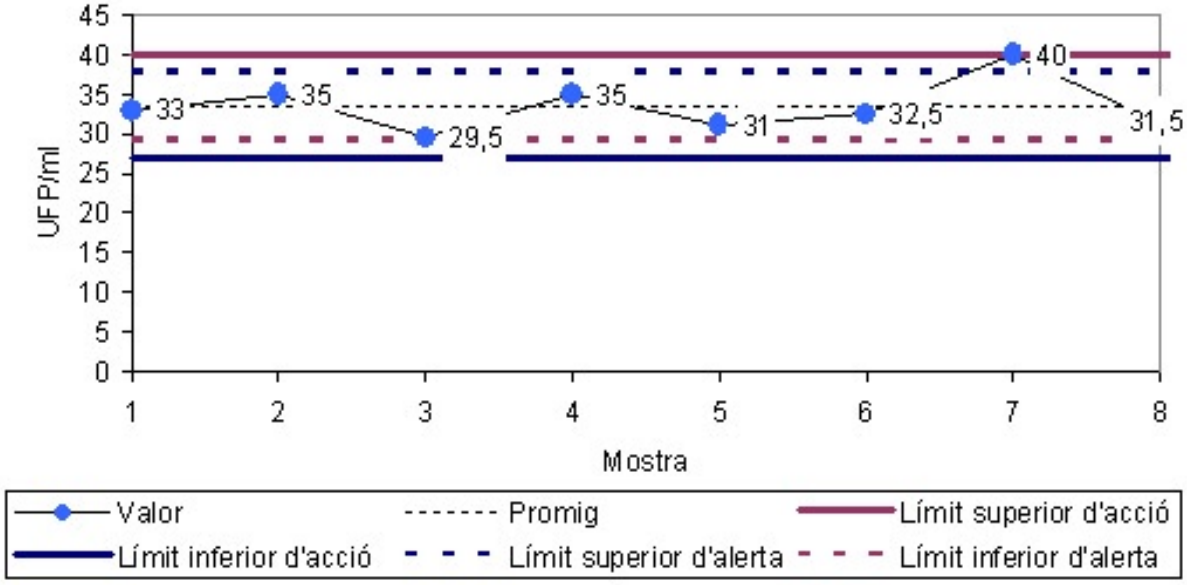
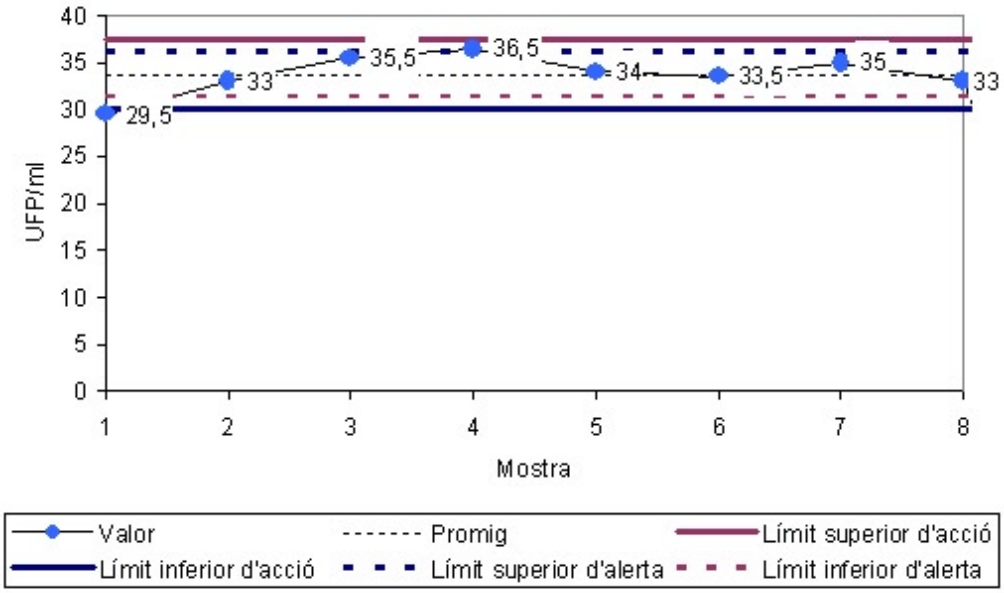


Figura C12. Diagrama de control de la quantificació del fag B56-3 al laboratori 4 (Suècia)



I.D. Interpretació dels resultats obtinguts

Es detectaren alguns problemes metodològics en la realització de les anàlisis dels tres fags per part del laboratori 3 (Figures C7, C8 i C9), ja que s'obtingueren valors baixos inesperats. El problema es solucionà en canviar el medi de cultiu comercial; resultats posteriors a aquest canvi entraren dins el rang de valor acceptables.

Les diferències entre el valors promig obtinguts pels diferents laboratoris s'explica, d'una banda, per la diferència en el nombre de persones de cada laboratori que feren les anàlisis. D'altra banda, la variabilitat del lot distribuït (veure figures B1 a B3) podria ser responsable d'una part d'aquestes diferències, ja que els resultats sempre depenen de l'heterogeneïtat natural de qualsevol mostra. Cal dir que les titulacions fetes per testar l'homogeneïtat dels material de referència foren realitzades per diverses persones, fet que contribueix a augmentar la variabilitat dels resultats obtinguts en les figures B1 a B3. Així doncs, el promig obtingut pel laboratori 2 en les anàlisis dels fags F-ARN i dels colifags somàtics (figures C4 i C5) és inferior a l'obtingut pels altres laboratoris, però és consistent amb la variabilitat dels materials de referència proporcionats. Pel que fa a les anàlisis del fags B56-3, podem observar en les corresponents figures (C3, C6, C9 i C12), que els valors mitjans obtinguts per tots els laboratoris són molt inferiors als observats en la figura B3. Aquests mals resultats són la conseqüència de problemes descongelació durant el transport. A l'analitzar un segon lot, els resultats obtinguts foren correctes.

Finalment, com es pot observar a les figures C1 a C12, el nombre de vials testats per cada laboratori és diferent i va des dels 20 vials analitzats pels laboratoris 1 i 2, als 16 analitzats pel laboratori 3 i als 8 testats pel laboratori 4. Malgrat el baix nombre de vials testat en aquest darrer laboratori, cal fer constar que els resultats obtingut són molt acurats, amb una variabilitat mínima i dins el rang de valors establerts per l'heterogeneïtat del material de referència. Les suspensions de fags estàndards foren utilitzades en els laboratoris 3 i 4 per a seleccionar materials, resoldre problemes tècnics i confirmar alguns resultats.

ANNEX II. Control de qualitat de l'anàlisi d'*Escherichia coli* per una tècnica del número més probable de 5 tubs i 3 dilucions

Tal i com es comenta a l'apartat de Materials i Mètodes de l'article, es va dur a terme un programa de control de qualitat del protocol d'enumeració d'*Escherichia coli*. En aquest procés es distribuiren materials de referència que consistien en tres lots de lentícules comercials la composició de les quals es mostra a continuació:

Taula II.1. Formulació de les lentícules emprades en els estudis d'intercalibratge del protocol de quantificació d'*Escherichia coli*

Lot	Codi	Organisme	Promig UFC/lentícula	Rang
1	NCTC 9001	*Escherichia coli*	64 (BA)- 42 (MLSB)	46-97 (BA); 27-63 (MLSB)
2	NCTC 9528	*Klebsiella aerogenes*	112 (BA)- 48 (MLSB)	99-124 (BA); 37-58 (MLSB)
3	NCTC 9001	*Escherichia coli*	$1,5x10^3$	-

NCTC: National Collection of Type Cultures, Central Public Health Laboratory, Regne Unit.
BA: agar Columbia basat en l'agar sang; és un agar ric.
MLSB: brou lauril sulfat utilitzat en el mètode de filtració estàndard per a *E. coli*.
El rang d'UFC per lentícula només es dóna per aquelles lentícules amb un nombre baix d'UFC. Pels recomptes superior no es dóna rang ja que són promitjos aproximats.

Les Figures II.1, II.2 i II.3 són representacions gràfiques dels resultats obtinguts en l'estudi dels lots 1, 2 i 3, respectivament. Aquests gràfics estan construïts de forma semblant als diagrames de control mostrats en l'Annex I per facilitar-ne la comprensió. Així doncs, els límits inferior i superior d'acció es defineixen com més o menys dues vegades la desviació estàndard del promig, i els límits inferior i superior d'alerta com a tres vegades aquesta desviació estàndard. Com es veu a les figures, cada laboratori va analitzar un nombre de resultats diferents per tal d'avaluar els diversos operadors involucrats en el procés i, també, per a controlar la resolució d'alguns problemes tècnics.

Figura II.1. Resultats, expressats com a $\log_{10}(n+1)$, de l'anàlisi del lot 1. En blau, valors obtinguts pel laboratori suec, en vermell pel britànic, en verd per l'espanyol i en lila pel grec.

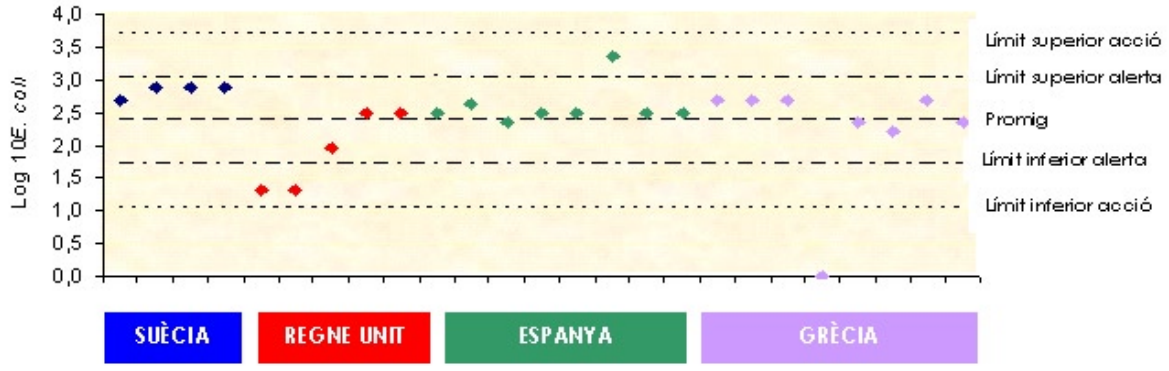
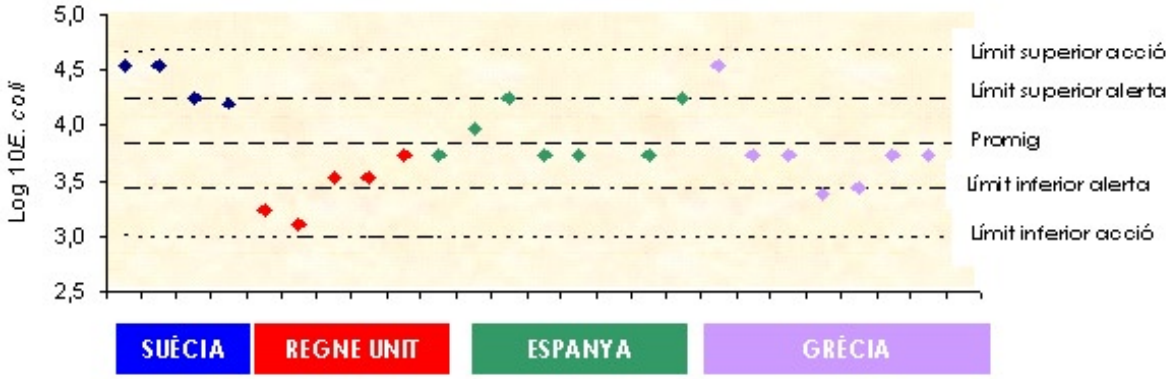


Figura II.2. Resultats, expressats com a $\log_{10}(n+1)$, de l'anàlisi del lot 2. En blau, valors obtinguts pel laboratori suec, en vermell pel britànic, en verd per l'espanyol i en lila pel grec.

Figura II.3. Resultats, expressats com a $\log_{10}(n+1)$, de l'anàlisi del lot 3. En blau, valors obtinguts pel laboratori suec, en vermell pel britànic, en verd per l'espanyol i en lila pel grec.



Els resultats foren molt satisfactoris, i només en dos casos els valors obtinguts caigueren fora dels límits d'acció. El lot 2 donà en un sol cas un resultat positiu. Com es veu a la Taula II.1, aquesta mostra correspon a *Klebsiella aerogenes*, que fermenta la lactosa amb producció d'àcid a 37ºC i, per tant, dóna positiu en el brou glutamat mineral modificat, però que al no presentar activitat β-glucuronidasa a 44ºC, no hauria de confirmar aquest positiu en l'agar triptona bilis glucurònid. Així doncs, aquest valor erroni es deuria a una contaminació de la mostra. Pel que fa als dos valors inesperadament baixos obtinguts pel laboratori anglès en les anàlisis dels lots 1 i 3, es dugué a terme una investigació on es trobà que l'agar cromogènic proporcionat per certa casa comercial disminuïa la recuperació de certes soques d'*E. coli*. Es canvià el medi i s'analitzaren dues mostres més per a confirmar la resolució del problema.