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Coliphages as ecological indicators of enteroviruses in various water systems*

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The occurrence of coliphages and enteroviruses in a variety of water systems in Czechoslovakia was monitored for two years. Two host strains of Escherichia coli *bacteria were used to test 1161 water samples for the presence of bacteriophages. These strains were polyvalent hosts for a broad spectrum of morphologically distinct coliphages, and their use thus gave quantitative data on the degree of viral pollution in any given water sample. Ninety-two water samples were tested in parallel for the presence of enteroviruses, by using a flocculation method to concentrate the viruses followed by isolation in cultures of a buffalo green monkey (BGM) kidney continuous cell line. The enterovirus and coliphage recovery rates showed similar differences when waters with different levels of pollution were compared. Seasonal fluctuations of both the coliphage and enterovirus (mostly poliovirus) levels in river water were demonstrated by statistical analysis of the data collected. The levels increased in the winter and sharply declined in the summer months as the river water temperature increased. Chemical pollution did not seem to influence the survival of either the coliphages or the enteroviruses in the observed rivers.*

Viral contamination of water systems throughout the world is increasing and therefore more attention is being focused on the evaluation of the associated health hazards and their effective control. In this connexion, bacteriological indicators of water quality have proved to be unsatisfactory in respect of viral contamination (*1*). In an effort to find suitable virus indicators, bacteriophages have often been studied (*2–8*), but further research is required (*9*).

The purpose of the present two-year field study was to compare levels of viral pollution through the recovery of coliphages and enteroviruses in different types of water system, and to follow their distribution, survival, and fluctuations throughout the year.

MATERIALS AND METHODS

Water sample collection

Water samples were collected from four types of water system in 28 localities: river water, from 4 points along a 172 km length of the River Danube and from 7 points along a 132 km length of the Small River Danube; surface water, from 6 irrigation channels; subsurface water, from 10 wells; and wastewater, from a town sewerage system—to be used as a control.

All water samples were collected by the grab-sampling method of repeatedly dipping 0.5-litre sterile bottles 30 cm below the surface of the water. At each sampling point 10 litres of water were collected for virological tests and a 0.5-litre sample was taken for the detection of bacteriophages. When the rivers and irrigation channels were flooded, the samples were taken at a point 2 m from the bank. The samples were always taken before midday, 2–4 times a month throughout the two-year period, and immediately transported to the laboratory for examination.

Coliphage assay

Bacterial strains. Escherichia coli strains B-39 and K_{12}Z-2 were obtained from Charles University, Prague.[a]

Media. The blood agar medium consisted of: 10 g of heart infusion, 10 g of peptone, 5 g of sodium chloride and 11 g of agar per litre of distilled water, adjusted to pH 7.2. The nutrient broth contained: 10 g of peptone, 10 g of meat extract, and 5 g of sodium chloride per litre of distilled water, adjusted to pH 6.8.

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Phage counts. The water samples were assayed by a double agar layer method using 100-mm diameter Petri dishes. The base consisted of a solid layer of the above medium (1.5% agar). This was covered with a second layer consisting of: 5 ml of the above medium containing 0.7% agar, 1.5 ml of the water sample, and 0.1 ml of a freshly prepared culture of one of the two *E. coli* strains. The Petri dishes were then incubated for 16 h at 36 °C. The number of plaques was then counted. Each sample was assayed 6–10 times using 3–5 dishes for each strain of *E. coli.*

Virus assay

Virus concentration. The viral contents of the 10-litre water samples were concentrated one thousand-fold by flocculation with aluminium sulfate (*10*) using the elution procedure described by Wallnerova & Šimková (*11*) in connexion with the precipitation method.

Virus isolation. Bottle cultures (12.5 cm^2 of cell monolayer per bottle) of a buffalo green monkey (BGM) kidney continuous cell line (*12*)[b] were inoculated with the viral concentrates. In most cases the virus could be detected in the BGM cultures only after successive passages, and samples were considered to be negative if there was no apparent cytopathic effect after three passages. Successive passages with non-inoculated BGM cells were performed at the same time, to act as controls. Attempts at virus reisolation were made in a similar way to reisolate viruses from the remaining portions of the eluents which were stored at −20 °C.

Virus identification. Isolates were identified in virus neutralization tests, using enterovirus typing pools (and also monospecific sera in several cases). The verification of the identified viral isolates was carried out in two references laboratories.[c] The *rct*$^+$ marker was determined by parallel virus titrations in tube cultures incubated at 36 °C and 40 °C (±0.5 °C). The *rct*$^+$ polioviruses grew at 40 °C while the vaccine strains (*rct*$^-$) did not survive. The difference between the two titres was $> 10^5$. Selected *rct*$^+$ isolates were further characterized by antigenic marker analysis, using a modified McBride test in which the data on the kinetics of viral neutralization were based on two observations at 0 and 10 minutes (*22–24*).

Statistical analysis

The monthly arithmetic means (and their standard deviations) were calculated for the number of coliphage plaques obtained from the water samples taken during each month at each collecting site. Student's *t*-test was applied to evaluate the significance of any seasonal variation in these mean values and the chi-squared (χ^2) test was used to study the significance of seasonal variations in the number of viruses isolated.

RESULTS

Table 1 summarizes the recovery of coliphages and enteroviruses from the different types of water system studied in 1977 and 1978. A total of 1161 water samples were tested for bacteriophages. Of 525 river water samples, 506 (96.6%) contained coliphages (positive), whereas in 406 water samples collected from wells, coliphages were not detected at all. The 214 samples obtained from irrigation channels yielded coliphages in 83 cases (38.8%). The 16 wastewater

[b] Kindly supplied by Dr Gerald Berg, US Environmental Protection Agency, Cincinnati, Ohio, USA.

[c] Reference Laboratory for Enteroviruses, Research Institute of Preventative Medicine, Bratislava, Czechoslovakia; and Reference Laboratory for Enteroviruses, Institute of Hygiene and Epidemiology, Prague, Czechoslovakia.

Table 1. Summary of coliphage and enterovirus recoveries from different types of water systems during a two-year field study (1977–78)

Type of sample	No. of collecting points	Coliphage recovery			Enterovirus recovery			Percentage of samples positive (coliphage/enterovirus)
		No. tested	No. positive	No. negative	No. tested	No. positive	No. negative	
River water	11	525	506	19	66	32	34	96.6/48.4
Irrigation channel water	6	214	83	131	14	1	13	38.8/7.1
Well water	10	406	0	406	9	0	9	0/0
Wastewater	1	16	16	0	3	3	0	100/100
Total	28	1161	605	556	92	36	56	52.1/39.1

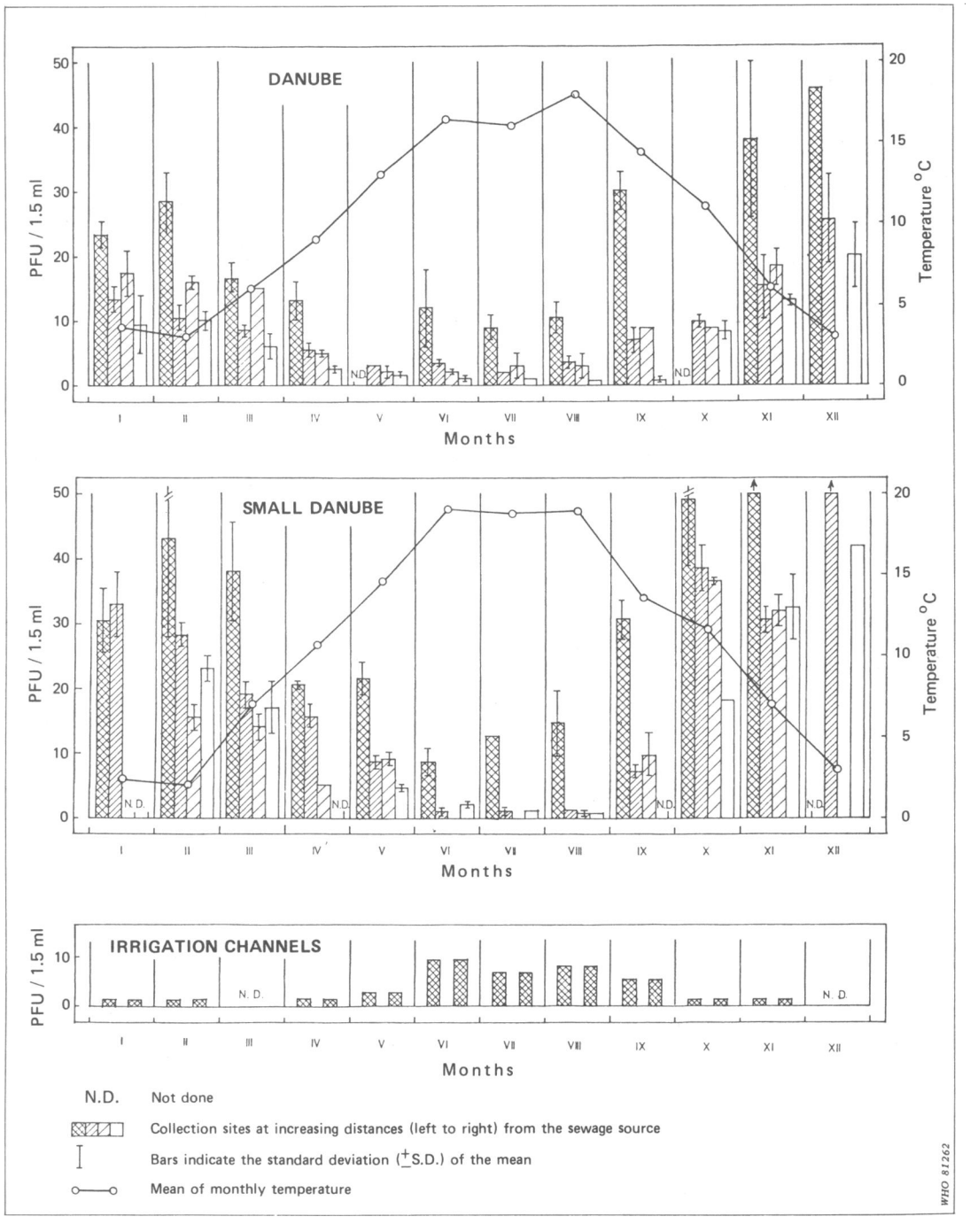

Fig. 1. Seasonal distribution of monthly mean coliphage titres (PFU) in three surface water systems.

samples, which were used as controls, were all positive. For enterovirus recovery, 92 parallel water samples were evaluated. Of 66 river water samples tested, viruses were isolated from 32 (48.4%) whereas in 9 well water samples viruses could not be detected at all. From 14 samples from irrigation channels, enterovirus was isolated in only one case (7.1%), whereas each of three control samples of wastewater yielded enteroviruses. Even when relatively few water samples were examined virologically, the agreement between the isolations of enteroviruses and coliphages was good, when waters known to be different in their levels of contamination were compared.

The monthly averages for the number of coliphages found in samples from the three surface water systems (i.e., the River Danube, the Small River Danube, and irrigation channels), expressed in plaque-forming units (PFU), showed a statistically significant difference between the winter and summer levels in the two rivers ($P<0.001$) (Fig. 1). In general, the coliphage titre was higher in the Small River Danube than in the River Danube; and in both rivers the level fell the greater the distance from the source of pollution. There was a marked inverse relationship between the monthly mean of the water temperature and the corresponding coliphage titres. A different pattern of seasonal variation was found in the coliphage titres in irrigation channel water, although the number of coliphages was much lower. A summer–autumn seasonal increase has also been demonstrated in wastewater (*2*).

The seasonal fluctuations in enterovirus titres in river water samples are shown in Table 2 and Fig. 2. From Fig. 2 it can be seen that whereas, in the samples collected in January, February, March, November, and December, 25 of the 28 samples were positive, in

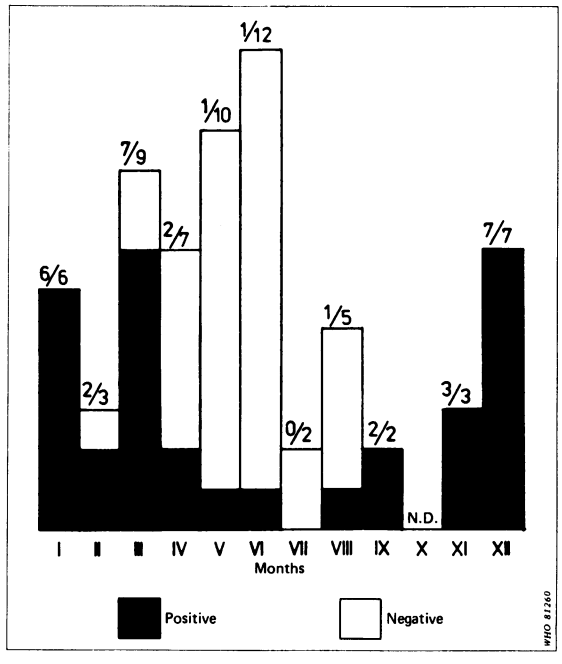

Fig. 2. Seasonal distribution of enterovirus occurrence in river water (No. of samples positive/No. tested).

Table 2. Monthly distribution of number of river water samples found positive, by virus type

Type of virus	Months												Total
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	
Mixture of poliovirus types 1, 2, 3	2	1	4								2	6	15
Mixture of poliovirus types 1, 2, 3 and coxsackievirus	1												1
Mixture of poliovirus types 1, 2			1								1		2
Mixture of poliovirus types 2, 3	1	1											2
Poliovirus type 2	2		2	2	1	1	0	1	1			1	11
Mixture of poliovirus type 2 and echovirus									1				1
Total number of samples positive	6	2	7	2	1	1	0	1	2	ND[a]	3	7	32

[a] Not done.

those collected in April–September only 7 of the 38 were positive. The difference between the summer and winter levels was statistically significant ($P<0.01$).

Among the enteroviruses isolated there was a high frequency of polioviruses, particularly of the type 2 strain. All the poliovirus strains recovered from the river water samples were *rct*$^+$ positive. An *rct*$^+$ poliovirus type 2 strain was also isolated from irrigation channel water. By contrast, among the three virus isolates from wastewater, one contained a mixture of poliovirus types 1, 2, and 3 (*rct* markers not determined) and the other two contained poliovirus type 1 *rct*$^-$ strains. Eight of the *rct*$^+$ poliovirus strains isolated were also tested for a characteristic antigenic (McBride) marker (*23*) and appeared to possess a "vaccine-like" antigen. Similar discrepancies between the *rct*$^+$ and McBride antigen test have been noted by other authors (*21*).

Fig. 3 summarizes the monthly coliphage and enterovirus results at four collection points on the River Danube at different distances from the source of pollution. The coliphage titre decreased with increas-

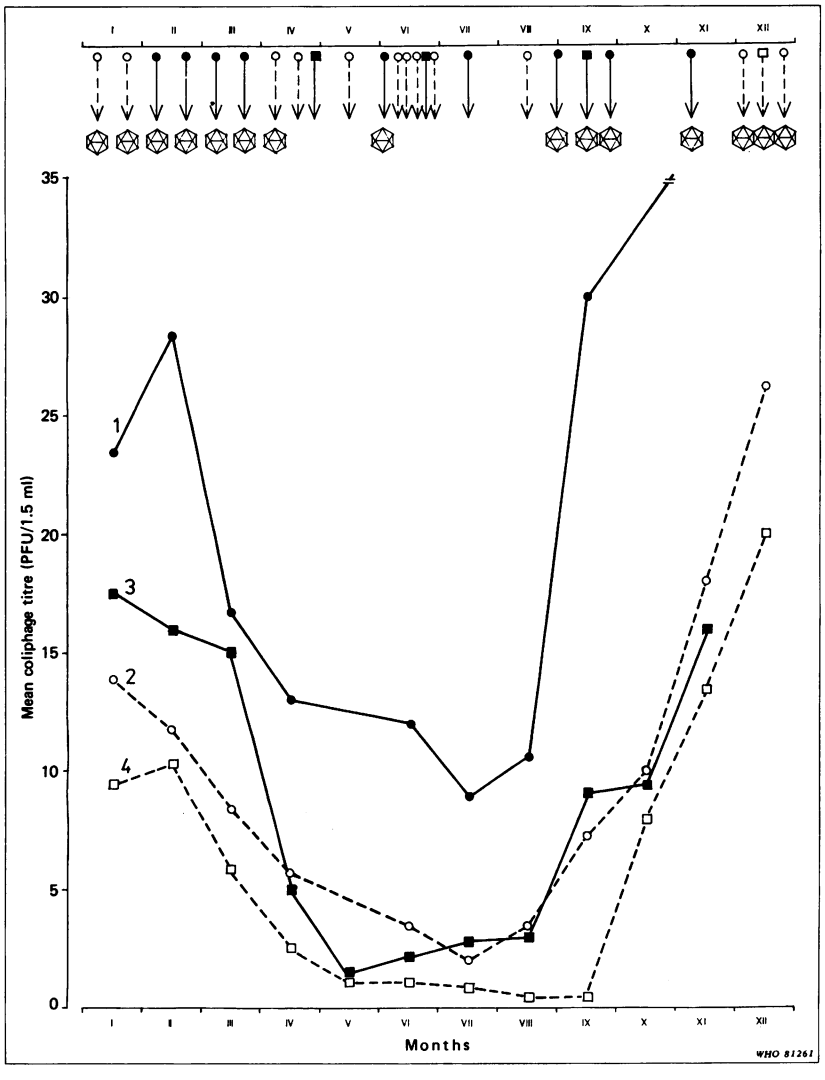

Fig. 3. Coliphage and enterovirus isolations from the River Danube. The curves (1–4) show the coliphage titres at four sites that were situated progressively further downstream from the source of pollution. Arrows indicate attempts at virus isolation at the different sites. The hexagonal symbols represent successful virus isolations.

ing distance from the source (i.e., from collection point No. 1 to collection point No. 4) but the seasonal variation was marked at all four points. Despite repeated attempts to isolate enteroviruses in the summer, only one positive result was obtained, and that was in a sample that contained a relatively high number of coliphages (18 PFUs in a 1.5-ml water sample).

DISCUSSION

This quantitative field study was designed to investigate the occurrence of coliphages and enteroviruses in a variety of "clean water" systems. A broad spectrum of bacteriophages was monitored using two appropriate *E. coli* strains. The coliphage recovery rates and the observed decreases in the coliphage titre with increasing distance from the source of pollution suggest that coliphages are a good indicator of the degree of faecal pollution in water systems. The coliphage numbers increased in winter and dramatically decreased in summer.

An economical and effective method of detecting enteroviruses in these systems was also developed. In this method, the viruses were first concentrated by flocculation with aluminium sulfate, and then isolated by successive passages through 2–3 bottle cultures of BGM cells. Experimental studies with this method showed that it could detect 4 TCD_{50}[d] of a model virus in a 10-litre sample of surface water (Šimková, unpublished results). However, in our opinion, the field study data probably represent the minimum level of virus in each sample.

Unusual seasonal variations in the numbers of enteroviruses were demonstrated in river water. Numerous physical, chemical, and biological factors influence the survival and proliferation of enteroviruses, and of these, water temperature and flow rate appear to be important. In the River Danube and the Small River Danube the water flow is least in the summer months. In a previous study (*13*) of the occurrence of coxsackieviruses in the River Danube between March and October, only those samples collected in March, September, and October gave positive results. In another study, samples from the River Thames were shown to have an enterovirus concentration of about 100 PFU/litre during the winter, and less than 0.1 PFU/litre in the summer (*9*).

Vaughn et al. (*14*, *15*) studied the occurrence of enteroviruses in various water systems on Long Island throughout the year by taking monthly samples, and they demonstrated the long-term survival of enteroviruses in natural waters during the winter and early spring months. The persistence of viruses in surface waters far removed from sources of pollution have been demonstrated in the ice-covered River Tanana of Central Alaska (*16*).

The higher rates of recovery of polioviruses, in comparison with coxsackieviruses and echoviruses, in this study was probably due to selection of the former by the BGM cell line. Similar results have been reported with BGM cells by Schmidt et al. (*17*), in a paper comparing the suitability of several cell culture systems for the isolation of enteroviruses. The higher frequency of isolation of the poliovirus type 2 vaccine strains with the *rct*$^+$ marker from the ecosystems studied, may indicate that these strains are more resistant to environmental factors.

In order to evaluate the effect of chemical pollution on the survival of coliphages and enteroviruses, river water samples were taken from two rivers with different types of pollution. The River Danube is known to contain enteroviruses (*13*, *18*, *19*) but has little chemical pollution. In contrast, the Small River Danube which also contains enteroviruses is heavily polluted with chemicals. Prior to the field studies, a model enterovirus was added to separate samples of water from the two rivers and virus survival was monitored. Small amounts of the virus were shown to survive for 60 days at 4 °C (Šimková, unpublished results).

The field studies reported in this paper confirm the long-term survival of both coliphages and enteroviruses in the water of both rivers and thus indicate that coliphages can be used as long-term indicators of enterovirus contamination, even in the presence of chemical pollution (*20*).

However, it must be stressed that even the best indicator of viral pollution cannot replace precise virological investigation. Nevertheless, observations on coliphages appear to give a good indication of poliovirus survival in a given water system; but further verification is needed to confirm this relationship in respect of other enteroviruses.

[d] TCD—tissue culture dose end point.

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RÉSUMÉ

UTILISATION DE COLIPHAGES COMME INDICATEURS ÉCOLOGIQUES DE LA PRÉSENCE D'ENTÉROVIRUS DANS DIFFÉRENTES EAUX

Dans le monde entier, le contamination virale des eaux est en augmentation et l'on s'intéresse donc davantage à l'évaluation des risques qui en résultent pour la santé ainsi qu'aux moyens d'y remédier efficacement. A cet égard, les indicateurs bactériologiques de la qualité de l'eau se sont montrés décevants en ce qui concerne la contamination virale. Dans le souci de trouver des indicateurs viraux appropriés, des travaux ont souvent été effectués sur les bactériophages.

La présente étude de deux ans, menée sur le terrain en Tchécoslovaquie, vise à comparer les niveaux de pollution virale par collecte de coliphages et d'entérovirus dans différents types d'eaux et à suivre leur répartition, leur survie et leurs fluctuations tout au long de l'année.

Deux souches hôtes d'*Escherichia coli* ont été utilisées pour rechercher la présence de bactériophages dans 1161 échantillons d'eau. Ces souches étaient des hôtes polyvalents d'une large gamme de coliphages morphologiquement distincts, et leur utilisation fournissait ainsi des données quantitatives sur le degré de pollution virale de n'importe quel échantillon d'eau. Sur 525 échantillons d'eau fluviale, 506 (96,6%) contenaient des coliphages (étaient donc positifs), alors que dans 406 échantillons recueillis dans des puits, aucun coliphage n'a été décelé. Les 214 échantillons obtenus dans des canaux d'irrigation ont fourni des coliphages dans 83 cas (38,8%). Les 16 échantillons d'eau usée, qui servaient de témoins, étaient tous positifs.

Parallèlement, on a recherché la présence d'entérovirus dans 92 échantillons d'eau, en concentrant d'abord les virus par floculation puis en les isolant dans des cultures d'une lignée continue de cellules rénales de grivet. Sur 66 échantillons d'eau fluviale, 22 (48,4%) étaient positifs alors que les neuf échantillons d'eau de puits étaient négatifs, un seul (7,1%) des 14 échantillons d'eau de canaux d'irrigation était positif, et les trois échantillons témoins d'eau usée étaient positifs. Ainsi, même lorsque l'examen virologique ne porte que sur un nombre relativement faible d'échantillons d'eau, la concordance entre les isolements d'entérovirus et de coliphages était bonne, lorsque l'on comparait des eaux dont les niveaux de contamination étaient connus pour être différents.

Les fluctuations saisonnières des concentrations de l'eau fluviale tant en coliphages qu'en entérovirus (essentiellement des poliovirus) ont été mises en évidence par analyse statistique des données recueillies. Les concentrations s'élevaient pendant l'hiver et fléchissaient nettement pendant les mois d'été, à mesure que la température des eaux fluviales s'élevait. Dans les cours d'eau observés, la pollution chimique ne semblait pas influer sur la survie des coliphages et des entérovirus.

Ces résultats confirment la survie de longue durée des coliphages et des entérophages dans l'eau des deux rivières, ce qui indique que les coliphages peuvent servir d'indicateurs à long terme de la contamination par les entérovirus, même en présence d'une pollution chimique.

Toutefois, il faut souligner que même le meilleur indicateur de pollution virale ne peut remplacer une recherche virologique précise. Quoi qu'il en soit, les observations sur les coliphages semblent donner une bonne indication de la survie des poliovirus dans une eau donnée; mais d'autres vérifications sont nécessaires pour confirmer cette relation en ce qui concerne d'autres entérovirus.

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