Acta vet. scand. 1980, *21*, 43—54.

From the Department of Food Hygiene, College of Veterinary Medicine, Helsinki, Finland.

THE EFFECT OF CADMIUM ON INDICATOR BACTERIA IN SEWAGE

By

H. Korkeala and *J. Hirn*

KORKEALA, H. and J. HIRN: *The effect of cadmium on indicator bacteria in sewage.* Acta vet. scand. 1980, *21*, 43—54. — The effect of cadmium (Cd) on the number of different faecal indicator bacteria in sewage, and on species composition of different indicator bacteria, was studied. Different amounts of Cd were added to aliquots of a sewage sample, and after 0, 4½ and 24 h of Cd exposure at 20°C coliforms, faecal coliforms, faecal streptococci and Clostridium perfringens were enumerated by the membrane filter method.

The Cd-induced reduction in the number of coliforms and faecal coliforms during exposure was found to be greater than the decrease in the number of faecal streptococci. In the case of C. perfringens the Cd concentrations used produced no observable effect on the cell number. The addition of Cd changed the faecal coliforms and faecal streptococci density relationship. Escherichia coli seems to be more resistant to Cd than other coliforms and Streptococcus faecalis var. liquefaciens and Streptococcus durans more resistant to Cd than other faecal streptococci. No influence of Cd on gas production by faecal coliforms was observed.

Faecal streptococci and C. perfringens seem to be better indicator bacteria than coliforms and faecal coliforms in evaluating the hygienic quality of Cd polluted sewage.

coliforms; faecal coliforms; faecal streptococci; Clostridum perfringens.

The isolation of faecal indicator bacteria is commonly used to signify the potential presence of intestinal pathogens. When in water these bacteria are in an environment with possible stress factors and not favourable to maintaining the viability of most bacteria. Studies have been performed to compare the survival of faecal indicator bacteria and pathogens in different kinds of waters. The absence or numerical reduction of faecal indicator bacteria in water do not necessarily indicate the absence or similar reduction of pathogens, because of the dif-

ferent sensitivity of bacteria to varying environmental stress (*Gyllenberg et al.* 1960, *Gordon* 1972, *Cohen & Shuval* 1973, *McFeters et al.* 1974).

Toxic metals are known to have an effect on the viability of bacteria (*Winslow & Hotchkiss* 1922, *Mitra et al.* 1975). For example the sensitivity of bacteria to cadmium (Cd) varies between species (*Babich & Stotzky* 1977, *Korkeala & Pekkanen* 1978), and even within the same strain (*Novick & Roth* 1968).

The purpose of this investigation was to study the effect of Cd as a stress factor on different indicator bacteria in water and also on the species composition of different faecal indicator bacteria in water.

MATERIAL AND METHODS

The untreated sewage sample used in this study was taken at the Saarioinen-Sahalahti treatment plant, which is located about 200 km north of Helsinki. The sample represents the sewage of the rural Sahalahti community (pop. 500), which has no special industrial activity. The sample was taken in a sterilized bottle at 4 p.m. and stored at 4°C until the experiments were started on the following morning.

Effect of different Cd concentrations on the number of coliforms, faecal coliforms, faecal streptococci and Clostridium perfringens

One ml of the sewage sample was transferred to 99 ml of a modified nutrient broth developed for the preservation of bacteria (*Hirn & Pekkanen* 1977). The nutrient broth contained 0, 0.3, 3 and 30 mg of added Cd/litre broth. Ten parallel samples from each Cd concentration were taken. The samples were kept at room temperature (20°C). The numbers of the different faecal indicator bacteria were determined immediately, after 4.5 h and after 24 h. Each 1 ml sample taken from the nutrient broth was diluted with 99 ml of sterile physiological NaCl solution (pH 7.2) before membrane filtration.

The membrane filter (MF) method (*American Public Health Association* 1975) was used throughout the study for the determination of coliforms, faecal coliforms, faecal streptococci and Clostridium perfringens. Millipore HC filters (Millipore Corporation, Mass., USA) (porosity 0.70 μm) were used for faecal coliforms and Gelman GN-6 filters (Gelman Instrument Company, Mich., USA) (porosity 0.45 μm) for the other groups. The growth media used were LES Endo agar (Orion Diagnostica, Espoo, Finland) for coliforms, mFC agar for faecal coliforms and KF-Streptococcus agar for faecal streptococci. The last two media were from Difco Laboratories, Detroit, Mich., USA. Coliforms were incubated at 35°C for 24 h, faecal coliforms at

44.5°C for 24 h and faecal streptococci at 35°C for 48 h. For the enumeration of C. perfringens the tryptose-sulfite-cycloserine-egg yolk (TSCEY) agar membrane filter method was used (*Hirn & Raevuori* 1978). C. perfringens was incubated at 35°C for 24 h in GasPak jars equipped with GasPak disposable hydrogen+carbon dioxide generator envelopes (BBL, Cockeysville, Md., USA).

Identification of coliforms, faecal coliforms and faecal streptococci

The incubated LES Endo, mFC and KF-Streptococcus agar plates were obtained after membrane filtration of the sewage sample in modified nutrient broth (1:100) containing 30 mg of Cd/l. The filtration was performed after 0 and 24 h of exposure of the sewage sample to Cd at 20°C. From both sets of the incubated plates 120 colonies of coliforms, faecal coliforms and faecal streptococci were randomly isolated for further studies. The isolated strains from LES Endo agar and from mFC agar were stored at 4°C on nutrient agar (Difco), and the strains from KF-Streptococcus agar were similarly stored on brain heart infusion agar (Difco) until identification. The 240 coliform strains and 240 faecal coliform strains thus obtained were identified with the API 20E (Analytab Products Inc., La Balme les Grottes, France). For the identification of the 240 isolates of faecal streptococci, biochemical and physiological tests as described by *Facklam* (1972) were used. The final identification was carried out according to the outlines of *Facklam, Geldreich* (1976) and *Clausen et al.* (1977).

Gas production by faecal coliforms

Gas production by faecal coliforms from lactose was studied in a broth containing 3.0 g of beef extract (Oxoid, London, England), 5.0 g of peptone (Difco) and 5.0 g of lactose (BDH Chemicals, Poole, England) per 1000 ml of distilled water (pH 7.0). The tubes were incubated at 44.5°C for 48 h.

Cd analysis

Cd was determined by flameless atomic absorption spectrophotometry. The apparatus used was the Perkin-Elmer 303 atomic absorption spectrophotometer (Norwalk, Conn., USA), equipped with a graphite furnace and graphite cell power supply HGA 72 (Überlingen, German Federal Republic).

Statistical method

In all statistical calculations Student's t-test was used.

RESULTS

The effect of different Cd concentrations on the number of coliforms, faecal coliforms, faecal streptococci and Clostridium perfringens in sewage after 4½ and 24 h of incubation at 20°C

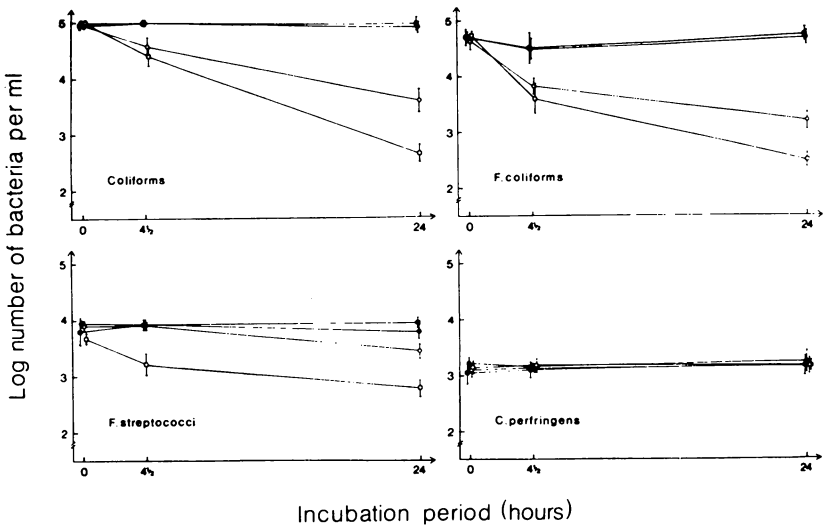

Figure 1. The effect of cadmium (Cd) on the number of coliforms, faecal coliforms, faecal streptococci and Clostridium perfringens of sewage diluted (1:100) with modified nutrient broth. The enumeration was done after 0, 4½ and 24 h of incubation with Cd at 20°C on LES Endo agar, mFC agar, KF-Streptococcus agar and TSCEY agar, respectively. The incubation times and temperatures were: LES Endo agar 24 h at 35°C, mFC agar 24 h at 44.5°C, KF-Streptococcus agar 48 h at 35°C and TSCEY agar 24 h anaerobically at 35°C. Each point represents the mean ± standard deviation of results of 10 parallel experiments, except in the case of faecal coliforms, after 4½ h. At that time the results are the mean of seven parallel experiments in broths containing 0 and 0.3 mg of Cd/l and the mean of eight parallel experiments regarding the concentration of 3 mg of Cd/l broth. Symbols: (■) no added Cd, (●) 0.3 mg of added Cd/l broth, (○) 3 mg of added Cd/l and (□) 30 mg of added Cd/l.

is presented in Fig. 1. The original Cd content of the sewage sample was 0.002 mg of Cd/l.

The means of the differences between the log numbers of bacteria after 0 and 4½ h of incubation and between the log numbers of bacteria after 0 and 24 h of incubation, for coliforms, faecal coliforms and faecal streptococci in sewage diluted with modified nutrient broth (1:100) containing 3 and 30 mg of Cd/l are presented in Table 1.

The density relationships between faecal coliforms and faecal streptococci in sewage diluted with modified nutrient broth

Table 1. Means and standard deviations of the differences between the log numbers/ml after 0 and 4½ h of incubation and after 0 and 24 h of incubation of coliforms, faecal coliforms and faecal streptococci of sewage diluted (1:100) with modified nutrient broth containing different added Cd concentrations.

Concentration	Means ± s after 0 and 4½ h of incubation at 20°C		
	coliforms	faecal coliforms	faecal streptococci
3 mg of added Cd/l	0.36 ± 0.17[1]	0.80 ± 0.18[2]	[3]
30 mg of added Cd/l	0.60 ± 0.15[1]	1.12 ± 0.24[1]	0.42 ± 0.23[1]

Table 1 (continued).

Concentration	Means ± s after 0 and 4½ h of incubation at 20°C		
	coliforms	faecal coliforms	faecal streptococci
3 mg of added Cd/l	1.38 ± 0.21[1]	1.42 ± 0.15[1]	0.43 ± 0.09[1]
30 mg of added Cd/l	2.38 ± 0.18[1]	2.23 ± 0.15[1]	0.91 ± 0.13[1]

[1] Mean of 10 parallel experiments.
[2] Mean of eight parallel experiments.
[3] Decrease in the number of faecal streptococci was not observed in any of 10 parallel experiments.

(1:100) containing different Cd concentrations after 0, 4½ and 24 h of incubation at 20°C are presented in Table 2.

The distribution of the number of bacteria as percentage of total isolated from LES Endo agar, mFC agar and KF-Strepto-

Table 2. Density relationships between faecal coliforms and faecal streptococci of sewage diluted (1:100) with modified nutrient broth containing different Cd concentrations after 0, 4½ and 24 h of incubation at 20°C.

Concentration	Incubation period		
	0 hours	4½ hours	24 hours
0 mg of added Cd/l	7.0	4.3	5.8
0.3 mg of added Cd/l	6.1	2.8	6.6
3 mg of added Cd/l	5.4	0.9	0.6
30 mg of added Cd/l	11.0	2.6	0.5

coccus agar plates after incubation of sewage 0 and 24 h in modified nutrient broth (1:100) containing 30 mg of added Cd/l broth is presented in Table 3.

Table 3. The distribution in the number of bacteria as percentage of total isolated on LES Endo agar, on mFC agar and on KF-Streptococcus agar of sewage incubated 0 and 24 h in modified nutrient broth (1:100) containing 30 mg of added Cd/l broth. The isolated strains from LES Endo agar from mFC agar were stored at 4°C on nutrient agar and the strains from KF-Streptococcus agar were similarly stored on brain heart infusion agar until identification.

Species	0 hours		24 hours	
	n	%	n	%
From LES Endo agar[1]				
Escherichia coli	66	55.0	103	88.8
Klebsiella pneumoniae	33	27.5	8	6.9
Enterobacter cloacae	11	9.2	4	3.4
Citrobacter freundii	7	5.8	1	0.8
Enterobacter aerogenes	1	0.8	0	0
Enterobacter agglomerans	1	0.8	0	0
Aeromonas hydrophila	1	0.8	0	0
	120	100	116[2]	100
From mFC agar[1]				
Escherichia coli	112	93.3	112	93.3
Klebsiella pneumoniae	7	5.8	5	4.2
Enterobacter cloacae	1	0.8	1	0.8
Citrobacter freundii	0	0	1	0.8
Enterobacter agglomerans	0	0	1	0.8
	120	100	120	100
From KF-Streptococcus agar[1]				
Streptococcus durans	67	55.8	80	66.7
Streptococcus faecium	39	32.5	26	21.7
Streptococcus faecalis var. liquefaciens	4	3.3	12	10.0
Streptococcus faecalis var. faecalis	1	0.8	1	0.8
Streptococcus faecium var. casseliflavus	1	0.8	0	0
Unclassified	8	6.7	1	0.8
	120	100	120	100

[1] LES Endo agar plates were incubated for 24 h at 35°C, mFC agar plates for 24 h at 44.5°C and KF-Streptococcus agar plates for 48 h at 35°C.

[2] Four strains died before identification.

Nine of the 240 strains isolated from KF-Streptococcus agar plates had from one to four exceptional reactions, according to the outlines used. These nine strains, however, were gram-positive cocci, failed to release O_2 from H_2O_2 and possessed the group D antigen.

The gas production from lactose at 44.5°C of bacteria isolated from mFC agar is presented in Table 4.

Table 4. Gas production from lactose at 44.5°C by bacteria isolated from mFC agar after incubation 0 and 24 h in modified nutrient broth containing 30 mg of added Cd/l broth.

Species	0 hours		24 hours	
	n	gas	n	gas
Escherichia coli	112	103	112	109
Klebsiella pneumoniae	7	0	5	0
Enterobacter cloacae	1	0	1	0
Citrobacter freundii	0	0	1	0
Enterobacter agglomerans	0	0	1	0

DISCUSSION

The results presented in Fig. 1 show that coliforms and faecal coliforms can tolerate 0.3 mg of added Cd/l medium during 24 h without a decrease in the cell number. After addition of 3 and 30 mg of Cd/l medium, a statistically significant decrease in the numbers of coliforms and faecal coliforms was observed after 4½ and 24 h ($P < 0.001$).

In the case of faecal streptococci, the addition of 30 mg of Cd/l medium gave a statistically significant decrease in cell numbers at 4½ h ($P < 0.001$), but the addition of 0.3 and 3 mg of Cd/l to the medium had no such effect. Only a slight, statistically not significant decrease in bacterial number was observed with a concentration of 0.3 mg of Cd/l at 24 h. The concentrations of 3 and 30 mg of Cd/l gave a significant decrease in the bacterial numbers after 24 h of incubation ($P < 0.001$).

In the case of Clostridium perfringens, the Cd concentrations used had no observable effect on the number of cells (Fig. 1). This is obviously due to the significant proportion of spores in populations of C. perfringens in sewage (*Bisson & Cabelli* 1979) and to the known resistance of bacterial spores to toxic chemicals.

The observed reduction in the numbers of faecal coliforms was significantly greater than the decrease in the numbers of coliforms within 4½ h after addition of 3 or 30 mg of Cd/l (Fig. 1 and Table 1) ($P < 0.001$ in both cases). After 24 h of exposure to Cd, no statistically significant differences between the mean decreases in the densities of coliforms and faecal coliforms could be observed ($P > 0.05$ in all cases). The decrease in the numbers of coliforms and faecal coliforms was significantly greater than the decrease in the numbers of faecal streptococci after addition of 3 and 30 mg of Cd/l at 4½ and 24 h ($P < 0.001$ in all cases). *Silvey et al.* (1974) reported that faecal streptococci tolerate chlorination better than coliforms and faecal coliforms, whereas *Evans et al.* (1968) did not find this effect. According to earlier studies (*Cohen & Shuval* 1973, *McFeters et al.* 1974) and the results presented in this study, it is apparent that faecal streptococci are more resistant to certain environmental factors and can thus indicate more reliably the possible presence of tolerant pathogens than coliforms and faecal coliforms. C. perfringens also seems to be a better indicator bacterium than coliforms and faecal coliforms when evaluating the hygienic quality of highly Cd-polluted sewage (Fig. 1).

According to *Geldreich & Kenner* (1969) and *Geldreich* (1976), the origin of sewage can be evaluated from the faecal coliforms and faecal streptococci density relationship (FC/FS ratio). They state that the analysis of untreated municipal sewage should reveal FC/FS ratios of 4.0 or greater. Many factors, however, have an effect on the FC/FS ratio, and the use of this ratio to ascertain whether the pollution of water is of human or animal origin has been questioned by *McFeters et al.* and *Wheater et al.* (1978). *McFeters et al.* reported different die-off rates for faecal coliforms and faecal streptococci. *Wheater et al.* found in their study that diet had a great effect on the bacterial flora of human and animal intestinal contents and that different investigation methods gave different FC/FS ratios. In our study the FC/FS ratios were over 4.0 without added Cd (Table 2). However, the addition of Cd changed the FC/FS ratio, due to the difference in die-off rates caused by Cd. In highly Cd-stressed broths this ratio was below 0.7, which according to *Geldreich & Kenner* should indicate an animal origin of the sewage. Thus the use of the FC/FS ratio is subject to limitations and should be applied with care.

When the results presented in Table 3 are examined, the coliform distribution pattern is similar to that found in other studies (*Vlassoff* 1977). Table 3 further shows that Escherichia coli seems to be the most resistant of coliforms to Cd exposure, since the decrease in numbers occurs mostly within the other coliform species. The decrease in the share of Klebsiella pneumoniae is particularly noticeable.

The proportion of the relatively Cd resistant E. coli among faecal coliforms was so great that no selection during incubation between faecal coliform species could be seen (Table 3). The share of K. pneumoniae among faecal coliforms is quite similar to that found by *Bagley & Seidler* (1977). According to the present results it is obvious that the Klebsiella population, which can grow at 35°C but not at 44.5°C, is more sensitive to Cd than the E. coli population.

Why is the decrease in density greater in faecal coliforms than in coliforms after 4½ h of Cd exposure (Fig. 1 and Table 1), although E. coli seems to be most resistant to Cd of these species (Table 3)? Evidently a part of the E. coli population was able to tolerate Cd better during 4½ h of exposure at 20°C than the others of the same species. This could be due to a better ability of the cells to repair the Cd-induced cellular damage and form colonies at 35° than at 44.5°C. After 24 h of Cd exposure the Cd-sensitive E. coli cells may have been damaged so seriously that they were unable to form colonies either at 35°C or at 44.5°C. Therefore the mean decrease in cell number after 24 h of exposure to Cd did not differ significantly between coliforms and faecal coliforms as it did after 4½ h of exposure.

The faecal streptococci were found to be more resistant to Cd than the coliforms and faecal coliforms (Fig. 1 and Table 1). This is somewhat accentuated in the present study by the absence of the relatively stress-sensitive Streptococcus bovis and Streptococcus equinus (*McFeters et al.*) from the sewage sample (Table 3). The results in Table 3 further show that in particular Streptococcus equinus (*McFeters et al.*) from the sewage sample durans seem to tolerate Cd exposure better than Streptococcus faecium. The good persistence of S. faecalis var. liquefaciens in the sample during exposure is in agreement with the results obtained by *Geldreich & Kenner*.

Gas production from lactose by faecal coliforms at 44.5°C, which is of diagnostic significance, could be influenced by Cd.

The results in Table 4 show that this is not the case regarding E. coli. The rest of the faecal coliforms did not produce gas even at 0 h.

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SAMMANFATTNING

Inverkan av kadmium på avloppsvattens indikatorbakterier.

Inverkan av kadmium (Cd) på antalet i avloppsvatten förekommande olika fekala indikatorbakterier samt på artsammansättningen av olika indikatorbakterier undersöktes. Olika Cd mängder tillsattes till delar av ett avloppsvattenprov och förekomsten av coliforma bakterier, fekala coliforma bakterier, fekala streptokocker och Clostridium perfringens undersöktes med membranfiltertekniken, sedan bakterierna hade blivit utsatta för Cd i 0, 4½ och 24 timmar vid 20°C.

Efter inkubering med Cd var minskningen av coliforma och fekala coliforma bakterier större än förminskningen av fekala streptokocker. De använda Cd koncentrationerna hade ingen betydande inverkan på antalet av C. perfringens. Tillsatsen av Cd förändrade relationerna i antalet mellan fekala coliforma bakterier och fekala streptokocker. Escherichia coli var tydligen mera resistent mot Cd än andra coliforma bakterier och Streptococcus faecalis var. liquefaciens och Streptococcus durans mera resistent mot Cd än andra fekala streptokocker. Ingen inverkan av Cd observerades på gasbildingen av fekala coliforma bakterier.

Fekala streptokocker och C. perfringens verkar vara bättre indikatorbakterier än coliforma och fekala coliforma bakterier, när den hygieniska kvaliteten av Cd förorenade avloppsvatten undersökes.

(Received September 11, 1979).

Reprints may be requested from: H. Korkeala, the Department of Food Hygiene, College of Veterinary Medicine, P.O. Box 6, 00551 Helsinki 55, Finland.