Survival of indicators of bacterial and viral contamination in wastewater subjected to low temperatures and freezing: application to cold climate waste stabilisation ponds

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Abstract The survival of bacterial and viral pollution indicators and Salmonella in urban wastewaters under freezing conditions (–14°C for up to 60 days) is reported. Presumptive, total and faecal coliforms (PC, TC, FC), salmonellae and coliphages were tested. The dynamics of somatic coliphage (E. coli C) and F-pili specific coliphage inactivation were compared at 4°C and 25°C over various run times. On freezing of the wastewater, it was found that PC, TC and FC showed a first rapid phase (days) of inactivation followed by a slower second phase (up to 4 weeks) and then stabilisation at between 1–10% of the initial population size, depending on the wastewater sample used. Salmonella spp. were detectable in 0.1 ml of raw wastewater and were still detected up to 2 days after freezing but none were detected in 100 ml samples after 4, 42 and 60 days, although microbiologically similar but antigenically different forms were found. Viral indicators of pollution showed a slow but constant decrease in viability during the first month but then stabilised at between 10–20% survivors (10% in somatic E. coli C phages, 15.8% in somatic Salmonella phages and 17.9% in F-pili specific coliphages). Using electron microscopy, no difference in susceptibility to freezing could be detected with respect to morphological phage types, which were either small icosahedral particles or complex tailed phages. The study of viral indicators at 4°C versus 25°C showed a higher survival of the various coliphages over time at 4°C. F-pili specific leviviridae were particularly susceptible to the antiviral factors at 25°C and no viable units per ml were detected after one month at that temperature, whereas somatic coliphages were detected in higher numbers after this period, especially at 4°C.

Keywords Cold waste stabilisation ponds; coliforms; coliphages; freezing; survival

Introduction

Studies on the microbiology of low temperature wastewater treatment systems are not common. Research on cold climate waste stabilization ponds (WSPs) has been carried out in Canada and Alaska (Envir. Canada, 1985 and references there; Henry, 1974; Henry and Prasad, 1986; Price et al., 1995) and recent interest in cold-adapted microbes for water treatment extends to anaerobic systems (Gatze-Lettinga and Zeeman, 2001). The biology of microorganisms at low temperatures was reviewed by Morita (1975) and Baross and Morita (1978). The microorganisms studied in our work, coliforms and salmonellae including some of their viruses, are not psychrophiles nor usually psychrotrophs, but research on their survival capacity at low temperature is needed to understand the performance of cold climate WSP systems.

WSPs in cold climates experience periods of ice cover and both the autochthonous lagoon microbiota and the allochthonous wastewater microbes go through either the freezing stress of ice crystal formation in frozen waters, or the low temperatures of the water below the ice cover. Various authors have studied the damage to microbial cells caused by freezing (Bennett et al., 1981; Calcott and Macleod, 1975; Wouters et al., 2000). The subject is of particular interest in food microbiology, where the response to freezing of a variety of pathogens and Escherichia coli has been investigated (Bollman et al., 2001; Yamamoto and Harris, 2001). In the present work, the survival dynamics of bacterial and...
viral indicators of pollution (coliphages) present in raw wastewater has been studied under freezing stress for periods of up to two months. Survival of the viral population was also investigated at 4°C and 25°C in self-depuration experiments for two months. All experiments were done in laboratory microcosms. The results add to our knowledge of the biology and fate of microbial pollution indicators in cold climate WSPs.

Materials and methods

Samples
Wastewater samples were obtained in sterile flasks from raw sewage of the city of Murcia, Spain. They were used immediately to prepare microcosms for the self-depuration experiments or subjected to freezing in two experiments of different length. Background levels of microbial pollution indicators were analysed immediately on arrival at the laboratory.

Microbiological analysis

Bacterial indicators and salmonellae. Presumptive (PC), total (TC) and faecal (FC) coliforms were analysed in duplicate using the multiple tube (MPN) fermentation method according to APHA (1995). LTB, BGB and EC culture media broths were obtained from Oxoid. The Eikjman test was performed for faecal coliforms.

Salmonella spp. presence was analysed in volumes of 0.1 ml, 1 ml, 10 ml and 100 ml of raw wastewater or in frozen wastewater after thawing, combining a pre-enrichment step in peptone water for 18 h at 37°C, enrichment in Rappaport-Vassiliadis-novobiocin (40 µg/ml, RVn) broth at 43.5°C, migration in semisolid RVn soft agar plates at 37°C, isolation on SS agar plates and identification of typical colonies in Kligler Iron agar, TSI agar and API-20E (Perales and Audicana, 1989; Emparanza-Knorr and Torrella, 1995). All isolated strains were confirmed with Difco O Salmonella antisera Poly A-I and Vi.

Coliphage counts. Somatic (E. coli C, S. typhimurium WG-45) and F-pili specific coliphages (S. typhimurium WG-49), were counted using the double layer agar method (Adams, 1959) as applied to water coliphage analysis (Grabow et al., 1998) and the already cited recommended E. coli and Salmonella strains (Havelaar and Hogeboom, 1984). Plates were incubated at 37°C for 24 h and 5 ml of a solution of 2,3,5-triphenyltetrazolium chloride (TTC) in nutrient broth was poured on the plates and after incubation for 2 hours, the supernatant was decanted and plaques counted. The bottom layer in the plates for F-pili specific coliphages contained nalidixic acid (50 mg/l) for WG-45 and nalidixic and kanamycin sulphate (20 mg/l) for WG-49. Plates for somatic E. coli C phages were prepared with enriched nutrient agar. Viral analysis of each sample was done in duplicate.

Electron microscopy
Selected lysis plaques were cut from the soft agar upper layer in plates and resuspended in 0.3 ml of distilled water with 3% formaldehyde in small glass tubes. After sedimentation of agar particles, small drops were layered on formvar coated electron microscopy grids, allowed to dry and stained with 0.5% aqueous uranyl acetate for one minute. Observations were made with a Zeiss EM-10 electron microscope at 80 Kv.

Freezing protocol
Raw wastewater samples were divided in aliquots of 150 ml and frozen in previously sterilised screw capped polyethylene bottles in a freezer at −14°C. Sample bottles (aliquots of the original sample) were thawed at room temperature after time periods of up to 60 days.
and microbiological analyses were performed immediately with appropriate dilutions of the thawed sample.

**Virus survival in self-depuration experiments**

Wastewater samples (1) in previously sterilised Pyrex bottles were kept in the dark at 4°C or 25°C for the duration of the experiment. Before each sample was taken for microbiological analysis, the bottle contents were mechanically homogenised by shaking.

**Results and discussion**

**Survival of bacterial indicators in frozen wastewater**

Two sets of independent experiments, one lasting 4 days and the other up to 60 days, were run in order to study the survival of coliforms and bacterial viruses upon freezing (–14°C). Figure 1 shows the effect of freezing a raw wastewater sample for up to 4 days on the survival dynamics of PC, TC and FC. Since a series of five parallel flasks with independent sample aliquots were used, each aliquot was frozen and thawed only once. The initial concentration of TC and FC in the raw wastewater used for this experiment was $7.90 \times 10^4$ MPN/ml. During the 4-day experiment, an initial decrease in survivors down to 6.2% of the initial concentration in TC and 4.2% in FC was observed. For the rest of the time, cell inactivation proceeded slowly and after 96 h, 1.6% of TC and 1.2% of FC survived. Data of PC have also been shown since the group includes a variety of microorganisms besides those of direct interest in sanitary water control and it was considered of interest to follow their fate. The data show a similar effect of freezing on this group compared with TC and FC, with a 0.7% proportion of survivors at the end of four days.

Figure 2 shows the results of a freezing experiment run for up to 60 days using a different wastewater sample. In this case the survival to freezing after 4 days was higher than before accounting for 68% of the PC, TC and FC detected initially in the raw wastewater. The data show a gradual decrease and some stabilisation of survivors of PC and TC during the first month (roughly 20% survivors at days 15 and 28), but during the same period the survival capacity of FC was much lower going down to 0.6%. The FC that had survived during the first month, retained their viability and were detected after 60 days: 0.01% of FC surviving after that time. The levels of viable cells of PC and TC decreased also after 60 days but their 4.4% survival was higher than for FC. Altogether the results show that the faecal bacterial indicators of pollution, and to a lesser extent, the related bacterial groups present in raw wastewater, are inactivated by freezing, particularly during an initial freezing stress during the first days to first weeks; afterwards a population of survivors remains in the frozen wastewater and is detected at least up to 60 days.

Table 1 summarises the percentage survival of the various groups of coliforms in both

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**Table 1**

<table>
<thead>
<tr>
<th>Time (days) before sample thaw</th>
<th>Presumptive coliforms</th>
<th>Total coliforms</th>
<th>Faecal coliforms</th>
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<tr>
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<tr>
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<td>$4 \times 10^3$</td>
<td>$8 \times 10^2$</td>
</tr>
<tr>
<td>28</td>
<td>$1 \times 10^3$</td>
<td>$2 \times 10^2$</td>
<td>$4 \times 10^1$</td>
</tr>
</tbody>
</table>

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**Figure 1** Effect of freezing (–14°C) of raw wastewater on presumptive, total and faecal coliforms during a 4 days experiment

**Figure 2** Effect of freezing (–14°C) of raw wastewater on presumptive, total and faecal coliforms during a 60 days experiment
experiments after various periods of freezing. Differences in the susceptibility of the coliforms was observed between the two samples, clearly indicating that a variety of uncontrolled parameters in raw wastewater may influence the survival capacity of bacteria to freezing (particles, fat droplets, different kinds of dissolved wastes, etc.). Generalisations based on short time experiments are risky. Various authors have shown however that certain microorganisms are active below ice cover and in permafrost (Rivkina et al., 2000; Skidmore et al., 2000). As the microbial indicators in our study could be trapped in water pockets with other organisms able to metabolise at low temperatures, adverse interactions of various microbial groups in these water pockets cannot be ruled out.

**Survival of viral indicators in frozen wastewater**

Figures 3 and 4 show the survival of different bacteriophages, used or proposed as viral indicators of water pollution, on freezing of the raw wastewater. During the 4-day freezing experiment, a substantial amount of viral particles, either somatic or pilispecific, remained viable (Figure 3). Throughout the two months of the long run experiment (Figure 4), viruses were slowly but constantly being inactivated. Nevertheless the concentration of viable viral units stabilised during the second month at between 10 and 20% survivors of the original population. These results suggest the possibility that a proportion of viral particles in the wastewater are trapped in “microenvironments” where the inactivating factors are active; whereas in other parts of the frozen wastewater, the remaining viruses may have escaped the action of these antiviral factors. As discussed above in the case of survival of bacterial indicators, different kinds of wastewater will probably affect viability of the viral particles differently. The morphology of viral plaques on the bacterial host lawns in Figure 5 shows that the reduction in accompanying microbiota after freezing facilitates observation and counting of plaques that develop in “cleaner lawns”. In the case of F-pili viruses, plaques formed on the lawn of strain WG-49 appear not only clearer but also larger, because the absence of contaminating bacteria in the inoculated sample allows a prolonged growth period for the viral host.

Table 2 summarizes the percentage survival of somatic and F-pili specific coliphages in frozen wastewater during the short (I) and long run (II) experiments after various time periods. In the case of the pili specific phages in Exp. II, the concentration of viable viral particles at day 4th was 175% of that detected at time zero. Either sample variation or the

| Table 1 % survival of microbial indicators of pollution in 2 raw wastewater samples subjected to freezing. Short run (4 days) and long run (60 days) freezing experiments are shown |
|----------------------------------|----------------------------------|----------------------------------|----------------------------------|
|                                  | After 4 days                     | After 28 days                     | After 60 days                     |
|                                  | PC  | TC  | FC  | PC  | TC  | FC  | PC  | TC  | FC  |
| Exp I (up to 4 days)             | 0.70| 1.60| 1.20| 0.70| 1.60| 1.20| 0.70| 1.60| 1.20|
| Exp II (up to 60 days)           | 68.0| 68.0| 68.0| 20.2| 20.2| 0.60| 4.40| 4.40| 0.01|

| Table 2 % survival of viral indicators of pollution (coliphages) in 2 raw wastewater samples subjected to freezing. Short run (4 days) and long run (60 days) experiments are shown (d = days) |
|----------------------------------|----------------------------------|----------------------------------|----------------------------------|
|                                  | % Survival of viral indicators (coliphages) |                                 |                                 |
|                                  | Somatic E. coli C, after            | F-specific (WG-49), after        |                                 |
|                                  | 4 d   | 28 d  | 60 d | 4 d   | 28 d  | 60 d |
| Exp I (up to 4 days)             | 19.4  | -     | -    | 57.8  | -     | -    |
| Exp II (up to 60 days)           | 40.7  | 25.7  | 10.2 | 175   | 79.8  | 17.9 |
dispersion or liberation of this kind of virus from unknown microhabitats due to the freezing process could explain this high count. 10.2% somatic and 17.9% pili specific coliphages were still viable after two months of freezing.

**Electron microscopy of surviving coliphages**

No special bias towards a specific bacteriophage morphology was found among the coliphages which survived a 60 days freezing period and were able to form plaques on the specific bacterial host. Examples of these are shown in Figure 6 where electron micrographs of microviridae (somatic), leviviridae (F-specific) and head and tail phages are presented.

**Salmonellae in frozen wastewater**

The susceptibility of *Salmonella* spp (mostly *S. enteritidis* agglutinating the O Antiserum Poly A-I and Vi) was studied in the 60-day experiment. Typical *Salmonella* strains according to biochemical tests and antiserum agglutination were detected in 0.1 ml raw wastewater samples and also in the same volume after 1 and 2 days in frozen samples. *Salmonella* was not detected in frozen wastewater samples of up to 100 ml analysed at day 4, 42 and 60. On the other hand, a variety of strains biochemically close to *Salmonella*, lactose negative, hydrogen sulphide positive and either saccharose positive or negative were detected in frozen samples at day 42 and 60, but none of these strains agglutinated the antiserum and accordingly were not considered to be *Salmonella*. The pathogenicity and exact taxonomic affiliation of these unknown “*Salmonella*-like” freeze resistant enterobacteria is not known at present.
Figure 6  Electron micrographs of negatively stained somatic and F-specific coliphages which survived freezing of raw wastewater for up to 60 days. (A) Somatic bacteriophages of *E. coli* C (microviridae).  
(B) F-pili specific coliphages. (C) Somatic *Salmonella* WG-45 phage and (D) somatic short tailed *E. coli* C phage

Figure 7  Inactivation of viral pollution indicators at 4°C and 25°C in laboratory microcosms
**Viral self-depuration of wastewater at 4°C and 25°C**

Inactivation of viral pollution indicators was studied in laboratory microcosms where raw wastewater was left for up to 70 days at 4°C and 25°C. The dynamics of the disappearance of various viral types are shown in Figure 7. With the exception of somatic *Salmonella* WG-45 phages whose small concentration induced erratic and difficult to interpret results in the counts, it was very clear that the ensemble of antiviral factors operating in wastewater is significantly more active at 25°C than at 4°C. At 25°C, F-pili specific phages showed a sharp decrease of two orders in 5 days, from $2.43 \times 10^5$ pfu/ml to $8.80 \times 10^3$ pfu/ml. The sample taken on day 35 contained $2.90 \times 10^1$ pfu/ml but these viruses were not detected at days 56 and 68. Inactivation of these viruses was slower at 4°C, and at day 68, $1.40 \times 10^1$ pfu/ml were still detected (0.006% of initial concentration). The temperature effect was also very clear with somatic coliphages where at 25°C a sharp decrease was observed during the first two weeks. Nevertheless, after 60 days $7.20 \times 10^1$ pfu/ml of somatic phages were detected (0.05% survival) in contrast to F-pili phages which could not be detected after 40 days. At 4°C the survival of somatic coliphages was higher than at 25°C and after 60 days, 3.67% of survivors could still be detected. It is clear that viral self-depuration processes are more active at 25°C than at 4°C. Pili-specific coliphages were particularly sensitive to the viral inactivation factors present in the raw wastewater used in this work.

**Conclusions**

Experiments using raw wastewater samples subjected to freezing showed that TC, FC and coliphages used as viral pollution indicators are subject to relatively rapid inactivation processes during the first days and weeks after freezing. A residual population of indicator bacterial survivors (0.01–10%) remained viable for periods of at least two months. The smaller concentration of *Salmonella* spp in raw wastewater meant that these pathogens could not be detected in volumes of up to 100 ml of raw wastewater after a few days of freezing. Viruses, both somatic and F-pili specific coliphages also maintained a residual (10–20%) population of survivors at the end of the two-month period. As suggested in Smith and Gerard (1981) for a cold river, survival of bacteria and viruses in frozen wastewater could take place in microscopy water pockets. Photographs of these pockets can be found in Junge et al. (2001). The inactivation of viral particles was significantly faster at 25°C than at 4°C in non frozen raw wastewater. F-pili specific coliphages were highly susceptible to antiviral factors at 25°C and concentrations decreased from $10^5$ pfu/ml to undetectable levels after one month, whereas at 4°C a few units ($1.40 \times 10^1$ pfu/ml) could still be detected after 70 days. The somatic coliphage population lasted longer than the F-pili specific one and $5.00 \times 10^3$ pfu/ml could still be detected in the 4°C experiment. Among the surviving coliphages, a variety of morphological types were found by electron microscopy: icosahedrical microviridae and leviridae and also various tailed phages. According to these results, the simplicity or complexity of the coliphage virion ultrastructure does not seem to be affected differently by the freezing stress.

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