Comparative survival of Cryptosporidium, coxsackievirus A9 and Escherichia coli in stream, brackish and sea waters

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Abstract Discharge of inadequately treated wastewater into streams may result in the dissemination of pathogens and the contamination of surface water sources. Determining the die-off rate of pathogenic microorganisms in stream and sea waters may serve as the basis for evaluating the health risks posed by the presence of pathogens in seawater. This study was conducted to determine the effect of microbial load, temperature, salinity and turbidity on the die-off of oocysts of Cryptosporidium as compared to that of coxsackie A9 virus (Cox A9) and E. coli. The test microorganisms were seeded into stream, outfall or sea waters and incubated at either 30°C (summer) or 15°C (winter). At 30ºC, the fastest die-off was observed for Cox A9 where <5-log was reduced regardless of the water quality. At 15°C Cox A9 persistence was similar to that of Cryptosporidium where no change was detected in the concentration of either throughout the study period. E coli die-off reached 5 orders of magnitude within 10d then its concentration remained unchanged. The die-off of E coli was faster than observed for Cox A9 at 15ºC regardless of the water quality. No decrease was observed in the viability of Cryptosporidium under all tested conditions throughout the study period indicating the unsuitability of E coli to serve as an indicator for the presence of parasites and viruses in stream and marine waters. The prolonged persistence of pathogenic microorganisms in marine waters suggested that discharge of contamination into streams may present a serious environmental health risk.

Keywords Cryptosporidium; die-off; health; marine water; virus

Introduction Discharge of wastewater effluents in a stream basin may result in severe contamination of receiving water bodies (lakes, sea and ocean). Rivers and streams worldwide have been found to be contaminated with pathogenic protozoan parasites and viruses, some of which originate from animal wastes (Ho and Tam, 1998). Contamination of water sources by wastewater can pose a health risk due to the presence of pathogenic protozoan and viral agents in water used for recreation, drinking and fishing. As Cryptosporidium parvum can be excreted at high concentrations from humans and farm animals, uncontrolled discharge of animal wastes can also be an important source of stream contamination in an unprotected watershed (Hansen and Ongerth, 1991). Contamination of drinking water and recreational waters has resulted in numerous foodborne and waterborne outbreaks (Tang et al., 1991; Mackenzie et al., 1994).

Little information is available about the prevalence and persistence of protozoan parasites in marine waters. Johnson et al. (1995), although using low efficiency detection methods, reported on the detection of Cryptosporidium and Giardia parasites in marine water influenced by primary sewage outfall. Studies have shown that the (oo)cyst stages of both parasites can persist for long periods under various environmental conditions. Oocysts of Cryptosporidium remained viable after one month incubation at 4°C in seawater (Robertson et al., 1992) while they retained viability after eight weeks in artificial seawater (salinity 20‰ and 30‰) at 20°C (Fayer et al., 1998). The results of these studies, combined with the fact that only a few oocysts are sufficient to cause infection in healthy adults,
indicate that serious health risk is introduced by the contamination of marine water by domestic and animal wastes (Dupont et al., 1995). This study was conducted to determine the effect of salinity, temperature and the microbial load on the die-off of Cryptosporidium oocysts, Cox A9 virus and E. coli. The suitability of faecal coliforms as an indicator for the viral and protozoan contamination of marine water was also evaluated.

Materials and methods

Water samples and analysis

Water samples (10 L) were collected from Alexander stream and from the stream outfall in the Mediterranean Sea. Marine water samples (10 L) were collected from a site with minimal direct contamination. Samples were transported to the laboratory within 2 h in a cooled container and analysed for turbidity, salinity, pH, and total bacterial count (TPC) (APHA, 1998) (Table 1). Cox A9 virus (Bozek) (ATCC 7797; from R. Hindsher, Central Virology Laboratory, Ministry of Health) was grown and enumerated using BGM cells (Nasser et al., 1993). E. coli was enumerated according to APHA (1998).

Purified, live Cryptosporidium oocyst stock suspension was obtained from Waterbone Inc. (New Orleans, LA, USA). FA staining and microscopic enumeration was accomplished as described in Method 1623 (EPA, 1999). Briefly, a 50 µL sample was applied onto a well slide and allowed to air dry. The sample was fixed with methanol and stained with FITC-conjugated monoclonal anti-Cryptosporidium (Waterbone, Inc.). After 30 min at 37ºC samples were washed, stained with 4′, 6-diamidino-2-phenylindole (DAPI) and mounted with a cover slide. Cryptosporidium was enumerated at 1,000× magnification using a Zeiss Exioscop II microscope. Samples were screened for spherical fluorescing objects 4–6 µm in diameter, for DAPI permeability and for typical Cryptosporidium morphology by differential inter-phase contrast (DIC).

Experimental design

Sea, stream and outfall water samples were seeded with Cox A9 virus, E. coli and Cryptosporidium oocysts to a final concentration of 10^5 PFU/mL, 10^6 CFU/mL and 10^5 oocysts/mL respectively. Samples were incubated either at 30ºC or 15ºC and analysed at various time points up to one month. Samples were analysed for E. coli on the same day as sampling and the remaining volume was divided and stored at 4ºC for later Cryptosporidium analysis and at -20ºC for later virus analysis. The results presented are the average of three repeated experiments. Data analysis was performed with Excel software.

Results and discussion

Die-off in stream waters

Analysis of Alexander stream water showed that human and/or animal wastes were discharged into the stream. The faecal contamination was reflected by high concentrations of faecal coliform (<10^4 CFU/mL) and high turbidity values (39NTU). Cryptosporidium

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Stream</th>
<th>Brackish</th>
<th>Sea</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>7.8</td>
<td>8.5</td>
<td>8.4</td>
</tr>
<tr>
<td>Turbidity (NTU)</td>
<td>39.0</td>
<td>13.7</td>
<td>1.7</td>
</tr>
<tr>
<td>Conductivity (ms)</td>
<td>7.0</td>
<td>13.9</td>
<td>56.6</td>
</tr>
<tr>
<td>TPC (CFU/mL)</td>
<td>1.5 × 10^5</td>
<td>5.1 × 10^4</td>
<td>48</td>
</tr>
<tr>
<td>Faecal coliform (CFU/100 mL)</td>
<td>2.3 × 10^3</td>
<td>68</td>
<td>20</td>
</tr>
</tbody>
</table>
oocysts were detected in 5/10 samples and Giardia cysts in 4/10 samples. Die-off results of Cryptosporidium, Cox A9 and E. coli in stream waters incubated at 15°C and 30°C are presented in Figure 1 and in Table 2.

DAPI inclusion/exclusion was considered an indicator for Cryptosporidium presence in addition to FA staining. Throughout the present study, high correlation was observed between the ability of the oocysts to react with FITC-conjugated monoclonal antibody and their persistence as determined by DAPI staining. Therefore, it was decided to present the results of oocysts enumerated by fluorescence staining. The die-off rate was calculated by converting the concentration of the test microorganisms at time zero and at the various studied times to $\log_{10}$ and then subtracting the former from the latter. The persistence of Cryptosporidium in stream water was not influenced by temperature and no change was observed in the concentration of the oocysts over 30d incubation at 15°C and 30°C (Figure 1). On the other hand, E. coli die-off reached four orders of magnitude within 6d in stream water regardless of the incubation temperature (Figure 1). In comparison, the die-off of Cox A9 virus was greatly influenced by the test temperature with a die-off of two orders of magnitude recorded at 15°C after 30d as opposed to five orders of magnitude within 15d at 30°C (Figure 1, Table 2). Cox A9 and E. coli survival was found to be temperature dependent, whereas the persistence of Cryptosporidium, as measured by DAPI permeability and IF, was not influenced by temperatures <30°C.

Table 2 $\log_{10}$ die-off of Cryptosporidium, Cox A9 and E. coli in waters after 30d incubation at two temperatures

<table>
<thead>
<tr>
<th>Water source</th>
<th>Cryptosporidium</th>
<th>E coli</th>
<th>Cox A9</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15°C 30°C</td>
<td>15°C 30°C</td>
<td>15°C 30°C</td>
</tr>
<tr>
<td>Stream</td>
<td>−0.4 −0.6</td>
<td>−4.4 −4.6</td>
<td>−1.9 −4.0</td>
</tr>
<tr>
<td>Brackish</td>
<td>−0.2 −0.9</td>
<td>−6.2 −4.4</td>
<td>−0.06 −4.6</td>
</tr>
<tr>
<td>Sea</td>
<td>−0.07 −0.5</td>
<td>−4.5 −3.6</td>
<td>−2.2 −5.3</td>
</tr>
</tbody>
</table>

Figure 1 Die-off of Cryptosporidium, Cox A9 and E. coli in stream water at 15°C and 30°C. A. Stream water at 15°C, B. Stream water at 30°C
Die-off of Cryptosporidium, Cox A9 and E. coli in brackish water

The persistence of *Cryptosporidium* in brackish water was not influenced by temperature and no change was recorded in its concentration at either 15°C or 30°C after 30d.

Similar results were recorded for Cox A9 at 15°C but at 30°C virus die-off reached 99.99% within 10d. The die-off pattern of *E. coli* in brackish water was similar to that observed in stream water where under both incubation temperatures the die-off reached four orders of magnitude within 7d (30°C) and 10d (15°C) incubation. The die-off pattern of *E. coli* was different to those observed for Cox A9 and *Cryptosporidium* (Figure 2, Table 2).

Die-off of Cryptosporidium, Cox A9 and E. coli in seawater

The microbial load and turbidity level of seawater was lower than those recorded for brackish and stream waters (Table 1). The persistence pattern of *Cryptosporidium* in seawater was similar to that observed for stream and brackish water samples where negligible decay was observed. *E. coli* die-off was also similar and there was no difference observed between its die-off at 15°C and 30°C (four orders of magnitude die-off within 7d at 15°C and 30°C). It is worth noting that the *E. coli* concentration decreased by four orders of magnitude within the first 7d and then remained unchanged. It appeared that the *E. coli* culture consisted of two populations – a sensitive one and a more resistant one that persisted for a longer time. Although the die-off of Cox A9 in seawater at 15°C was greater than that observed in brackish and stream water, die-off was greater at 30°C than 15°C (Figure 3, Table 2).

It seemed that the antiviral activity of marine water affected the survival of Cox A9 also at 15°C. The higher salinity level of seawater did not enhance the decay of *Cryptosporidium* oocysts at both incubation temperatures. These results were in agreement with data reported by Fayer *et al.* (1998) concerning the prolonged persistence of *Cryptosporidium* oocysts in artificial seawater. The fact that oocysts of *Cryptosporidium* could remain suspended for a long time in the water column and persist for prolonged periods has lead to suggestions that waste contamination of marine waters introduced a serious public health problem through recreational activities and shell-fishing (Medema *et al.*, 1998). Further studies are needed to evaluate the relationship between viability and infectivity of *Cryptosporidium* oocysts since *E. coli* and Cox A9 survival were determined on the basis of cultivation. This information will also be necessary to evaluate the suitabili-

![Figure 2](image-url)
ty of the detection methods developed for routine monitoring of Cryptosporidium (EPA, 1999).

Conclusions
Cryptosporidium oocyst persistence was not influenced by either salinity or temperature of up to 30°C. Regardless of the water quality and temperature, Cryptosporidium persisted longer than E. coli indicating the unsuitability of bacterial indicators for parasite contamination of estuarine and marine waters. The prolonged persistence of Cryptosporidium in marine waters may pose a serious public health problem for recreation and shell-fishing.

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References