MAMALA BAY STUDY

VIABILITY OF *CRYPTOSPORIDIUM PARVUM* IN MARINE WATERS

PROJECT MB-7

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August 30, 1995

1 EXECUTIVE SUMMARY

Mamala Bay is located on the southern shore of the island of Oahu. It encompasses the city of Honolulu, it's harbor, Pearl Harbor and many recreational beaches which are used to support tourism (Waikiki, Hanauma Bay, Ala Moana Park). Freshwater flows, stormwater and wastewater effluent inputs (Honolulu, Sand island and Pearl Harbor outfalls) all enter the Bay and may potentially impact water quality and public health. *Cryptosporidium* is a coccidian protozoan parasite which causes a diarrheal illness in humans and animals. The oocyst from this protozoan which is environmentally stable and is the infectious unit can be found in wastewater and many surface waters. There have been five recreational waterborne outbreaks since 1988 caused by *Cryptosporidium*. Key to understanding this risk is a better assessment of the survival during transport of oocysts from their sources to recreational sites.

The objective of this study was to determine the survival of *Cryptosporidium* oocysts in waters of Mamala Bay, Hawaii.

Four sets of experiments on survival of oocysts in marine waters (Pearl Harbor, Black Point and Ala Wai Canal) were undertaken at the University of South Florida and at the University of Hawaii. Oocysts suspended in the marine waters were monitored over several days using an excystation procedure (the ability of the protozoan oocyst to open up producing sporozoites, the internal stage). The percentage of oocysts within a population, excysting is related to the numbers which can open up and possible cause disease. The impact of exposure to sunlight on excystation was also examined. Temperatures between 24 and 27°C were maintained for the seeded waters set up outdoors with exposure to sunlight and without sunlight in the laboratory, the temperature was maintained at 23° C.

Cryptosporidium parvum oocysts appear to be highly resistant to inactivation in marine waters. Only a 5% to 6% decrease in excystation per day may be estimated in marine waters off the coast of Hawaii. Given the concentrations found in wastewater and the fact that these oocysts are young, it is highly probable that viable oocysts are reaching the beaches from the outfall. Sunlight does impact the inactivation rate and at least a decrease of 8.2% in excystation may be seen per day, perhaps reaching 29% decrease in excystation per day, depending on the age of the oocysts.

Cryptosporidium oocyst levels should be decreased in the wastewater effluents discharged through the marine outfall, as reliance on inactivation as a mechanism to protect the public health can not be met. Better assessment of animal sources of *Cryptosporidium* oocysts in the watershed affecting the Ala Wai canal and the transport time should be made.

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2 INTRODUCTION

Mamala Bay is located on the southern shore of the island of Oahu. It encompasses the city of Honolulu, it's harbor, Pearl Harbor and many recreational beaches which are used to support tourism (Waikiki, Hanauma Bay, Ala Moana Park). Freshwater flows, stormwater and wastewater effluent inputs (Honolulu, Sand island and Pearl Harbor outfalls) all enter the Bay and may potentially impact water quality and public health. The Mamala Bay Study Commission has brought together a team of investigators to study the public health aspects of this system. This portion of the project was aimed at assessing the viability/survivability of *Cryptosporidium parvum* oocysts in marine waters to determine the public health risk to users of recreational waters in Mamala Bay.

Cryptosporidium is a coccidian protozoan parasite which causes a diarrheal illness in humans and animals (Dubey et al., 1990. Species in this genus invade and replicate intracellularly in the epithelial cells of the digestive tract of vertebrates. As a result of this infection, the parasite produces oocysts which are shed in feces. These oocysts are infectious upon excretion and remain stable in the environment. The life cycle of *Cryptosporidium* has six stages after ingestion of an oocyst, first excystation occurs (the release of infectious sporozoites), then merogony (multiplication within the host cell), gametogony (formation of micro and macro gametes), fertilization, (formation of oocyst wall), and sporogony (sporozoite formation) (Current, 1986). Large numbers of oocysts can be shed in the feces as a result.

Cryptosporidium parvum infects most mammals including humans, making it zoonotic as well as pathogenic. Other species of *Cryptosporidium* are found in mice, fish, reptiles, and birds (Dubey et al., 1990; Fayer and Ungar, 1986). The ability of oocysts from humans and other mammals to cause infection through cross-transmission (i.e., animal to human and vice versa), makes wild and domestic animals possible reservoirs. Therefore both animal and human wastes discharged to sensitive water bodies could be associated with waterborne transmission.

Waterborne *Cryptosporidium* has recently emerged as an important public health problem (Addiss et al., 1995). The first large waterborne outbreak of Cryptosporidiosis occurred in 1985 in Carrollton, Georgia with over 13,000 people infected. The source of the contamination was thought to have been from a sewage overflow and infected cattle feces in the watershed. An epidemiologic study concluded that current methods for treatment of the public water supply may have been inadequate (Hayes et al., 1989).

The largest waterborne outbreak documented in the U.S. was caused by *Cryptosporidium* oocyst contamination of a potable water supply in Milwaukee, Wisconsin in April, 1993. Of the approximate 800,000 residents over 450,000 became ill. There were over 100 immunocompromised individuals who died from the disease. The city used conventional water treatment practices at the time of the outbreak and met all drinking water quality standards. Oocysts were able to survive the filtration and disinfection process. It is believed that the organism entered the water supply through runoff of farms upstream or possible sewage overflow events. (MacKenzie et al., 1994).

There have been 9 documented waterborne outbreaks of *Cryptosporidium* in drinking water in the U.S. (Lisle and Rose, 1995). However, this protozoan has also been associated with five recreational outbreaks since 1988. The first recorded outbreak occurred in Los Angeles between July 13 and August 14 1988 (CDC, 1990). The attack rate for varying groups of swimmers ranged from 47-100%. Four other outbreaks occurred in London, British Columbia, Lane County, Oregon and Madison, Wisconsin, (Bell et al, 1993; CDC, 1994; Joce et al., 1991; Rose et al., 1995). The source of contamination in three of the outbreaks was fecal accidents, and in at least one case there was a cross contamination from a sewage system. Attack rates from the various outbreaks ranged from 14-78% with the majority of the infected individuals being children. Because of the large number of oocysts shed by a symptomatic person, even limited fecal contamination could result in sufficient oocyst concentrations in localized areas of a pool to cause waterborne transmission (Jokippi and Jokippi, 1986).

There has only been one study to date that has looked at the effects of environmental pressures on the viability of *Cryptosporidium* oocysts. A study done by Robertson et al.(1992) reported that oocysts in marine waters were inactivated by 32-40% after 35 days. These studies were done at 4°C and are not sufficient to predict the potential for *Cryptosporidium* oocyst survival in the warmer waters of Hawaii.

Many coastal port cities around the world dump sewage effluent into marine waters where recreational beaches may be in close proximity. *Cryptosporidium* oocysts may be found in sewage at concentrations of 10^3 to 10^4 /100 L (Rose and Carnahan, 1992). These data and the reported recreational outbreaks support the hypothesis that sewage discharges impacting recreational water bodies can contribute to endemic or possible epidemic disease for the users of those waters. Key to understanding this risk is a better assessment of the survival during transport of oocysts from their sources to recreational sites.

2.1 OBJECTIVE

The objective of this study was to determine the survival of *Cryptosporidium* oocysts in waters of Mamala Bay, Hawaii, based on two methods to determine viability, vital staining techniques and excystation.

3 MATERIALS AND METHODS

3.1 Experiment 1

Cryptosporidium parvum oocysts (2 weeks of age) for this first study were obtained from Dr. Hal Stibbs at Tulane University, New Orleans, Louisiana. They were purified using percoll-sucrose density gradients and resuspended in 1% phosphate buffered saline.

Two ten gallon aquariums were utilized. The control tank contained deionized water (DI) with no additions while Instant Ocean was used to prepare the sea water tank at 30 parts per thousand. Slide-A Lyzer semipermeable dialysis chambers were used to house the oocysts with styrofoam buoys which allowed them to float within the tanks (Robertson et al., 1993). The membranes allowed for the salt water to pass through but kept the oocysts contained which were easily sampled using a syringe. Both aquariums had air stones placed in the bottom of the tanks to allow for circulation. The aquariums were placed in an environmental chamber which kept the temperature of the ambient air and the water at 20° C. Fluorescent lights within the environmental chamber were on for 12 hours and off for 12 hours per day. The dialysis chambers were inoculated with 1mL of oocysts at a concentration $1x10^6$ total organisms. Samples were extracted from the dialysis chambers at 0, 24, 48, 72 hrs, and after 7 and 14 days.

Assessment of oocyst viability was based on the viability assay described by Campbell et al. (1992) which is dependent on the inclusion and/or exclusion of two fluorogenic vital dyes, DAPI (4',6-diamidino-2-phenylindole) and PI (propidium iodide). Working solutions of DAPI (2mg/mL in absolute methanol) and PI (1mg/mL in phosphate buffered saline 0.1M, pH 7.2 (PBS)) were prepared and stored at 4^0C in the dark. Oocysts were washed with Hanks Balanced Salt Solution (HBSS) and then incubated in acidified HBSS (pH 2.75) for one hour at 37°C. (A microcentrifuge 15,000xg for 1 min. was used for all washes). The oocysts were then washed with HBSS and incubated again after the addition of 10 ul of DAPI and 10 ul of the PI working solutions for two hours, at 37°C. Oocyst suspension were viewed under both DIC (Differential Interference Contrast) optics and epifluorescence using an Olympus BH2 microscope, equipped with a UV filter block (350 nm excitation, 450 nm emission) for DAPI and a green filter block (500 nm excitation, 630 nm emission) for PI. Proportions of empty (ghost), PI positive (PI(+)), DAPI positive-PI negative (DAPI(+)/PI(-)), DAPI negative-PI negative (DAPI(-)/PI(-)) oocysts were quantified. Ten ul was placed on a glass slide with a cover slip and examined under oil emersion at 100x magnification. Oocysts fluoresced sky blue with the DAPI and DAPI+/PI-

oocysts were considered viable. Those oocysts that fluoresced red, DAPI-/PI+ or those that were DAPI+/PI+, were considered nonviable. Nonstaining oocysts containing no internal features were counted as ghosts. Oocysts containing internal features which did not take up either dye and were considered DAPI-/PI-. These non-staining populations have been considered by some to be potentially viable. In this study only the DAPI+/PI- oocysts were counted as viable.

3.2 Experiment 2

Oocysts used in this study were obtained from Dr. John Vetterling from Parasitology Services in Fort Collins, Colorado. The oocysts were secondarily purified using cesium chloride gradients and were resuspended in 2.5% potassium dichromate and stored at 4°C. These were four weeks of age. This preparation had large numbers of clean oocysts and was used through the remaining experiments.

This experiment was conducted in two 500mL polypropylene beakers. The beakers contained 100mL of seawater from Boca Ciega Bay, (St. Petersburg, Florida) with a salinity of 29 parts per thousand. One beaker, was placed on the roof in direct sunlight in a 10 gallon aquarium filled with water. This was done to compensate for large temperature fluctuations that might occur in the beaker itself and would not be indicative of daily temperature fluctuations in a large body of water such at the Boca Ciega Bay. A plastic cover was placed over the tank to keep insects and rain out of the sample. The second beaker was incubated under laboratory conditions. Each beaker was inoculated with $1x10^7$ oocysts giving a final concentration of 10^5/mL.

Aliquots were collected every 24 hours from both beakers and analyzed for viability using the previously described DAPI/PI protocol. Temperature and salinity were monitored daily and naturally-occurring marine bacteria were enumerated using marine agar and spread plate procedures.

3.3 Experiment 3

Cryptosporidium parvum oocysts were obtained from Dr. John Vetterling, Fort Collins, Colorado. These oocysts were cleaned and stored as previously described.

One liter of water from Black Point on the island of Oahu and one liter of water from Pearl Harbor was shipped express on ice to the University of South Florida. The salinity was 35 parts per thousand for both of the Hawaiian water samples.

Forty-five mls of each water sample were placed in 50mL polypropylene tubes and placed in an environmental chamber. The air and water temperature were maintained at 26°C. An inoculum of 1x 10^6 oocysts was added to each tube. Aliquots (200uL) were collected every 24 hours for a period of 14 days. Viability was assessed using two methods, fluorogenic dyes DAPI/PI described previously and excystation (Korick et al. 1990).

The excystation procedure was carried out as follows. Oocysts were washed with HBSS and suspended in 500ul of 1X PBS. The suspension was mixed with an equal volume of excystation medium (0.5% trypsin, 1.5% taurocholic acid in 1X PBS) and incubated for one hour at 37°C. Ten microliters was placed on a glass slide and examined under Differential Interference Contrast (DIC) microscopy. Full oocysts (with four sporozoites), partial oocysts (less than three sporozoites), ghost oocysts (empty shells) and free sporozoites were counted under 100X oil emersion. Percent excystation was calculated as: EO + PEO/Total oocysts x100, where EO is the number of empty oocysts (or ghosts) which have excysted, and PEO are partially excysted oocysts (containing 2 or less sporozoites). Sporozoite ratio was calculated using the following formula: number of free sporozoites / number of ghosts + number of partial oocysts (Robertson et al., 1993a). The sporozoite ratio was used to adjust the percent excystation, as empty oocysts may be indicative of inactivated organisms, rather than those which have excysted (sporozoite yield/4 x Excystation).

3.4 Experiment 4

This experiment was conducted at the University of Hawaii on the island of Oahu. *C. parvum* oocysts were obtained from Dr. John Vetterling, cleaned and stored as previously described. The two marine waters were used for this experiment were from Black Point and Ala Wai canal. The salinity of both waters were 35 parts per thousand. The control water used was 1X PBS with a salinity of 0 parts per thousand. Three (1000mL polypropylene) beakers were filled with 500 mls of the test waters and placed in the laboratory in the dark at room temperature (23°C). A duplicate of each type of water in another three beakers was set up in a similar fashion and was placed on the roof in a water bath with constant stirring. Approximately 4.5 x 10^8 *C. parvum* oocysts were inoculated in each of the 500mL of water in all 6 beakers. For those beakers stored in the sunlight, ice was added to the water bath to keep the water temperature in the beakers between 23-27° C. Samples (200uL) were collected every 24 hours. Viability was assessed by the excystation method described previously.

3.5 Statistics

Viable oocysts based on excystation were converted to a ratio of Nt/No and regressed against time (days) where Nt was the percentage of viable oocysts at any given sample time and No was the percentage of viable oocysts at time zero. Values for r, r^2, y-intercept and slope were calculated for experiments 3 and 4. Minitab version 8.2 (Minitab Inc., State College, PA) was used for the linear regression and the development of the best fit curves.

4 RESULTS

4.1 Experiment 1

This experiment demonstrated the use of environmental chambers which could possibly be u sed in-situ and the impact that salinity had on decreased survival of *Cryptosporidium* oocysts. The viability of oocysts stored in DI water decreased by 17% after 7 days while those stored in artificial sea water with a salinity of 30 parts per thousand decreased by 42% (Tables 4.1 and 4.2). The oocysts appeared to very stable in the artificial seawater and 53% were found to be viable using the DAPI procedure even after 14 days.

These experiments were run in the laboratory at temperatures of 20°C. The water temperatures in Hawaii and in the coastal waters surrounding the islands are 25 to 28°C. In addition, it was thought that sunlight may play a role in the inactivation rate of the oocysts.

4.2 Experiment 2

The second set of experiments was undertaken to examine a protocol using beakers rather t han environmental chambers for exposure of the oocysts. The semi-permeable membranes would not have a llowed for naturally-occurring bacteria to move freely and interact with the oocysts. In this case, marine waters were collected from Boca Ciega Bay in St. Petersburg, FL. The naturally occurring marine bacteria were at levels of 3×10^4 CFU/ml. The salinity was at 30 °/oo. Replicate beakers were stored in the laboratory at 22°C and in the sunlight at 36°C.

In the laboratory, oocyst viability decreased by 30 to 40% by day 4 in DI water and a similar stability was n oted in the marine water. The population of oocysts was stable thereafter up to 11 days (Tables 4.3 and 4.4). Greater inactivation was seen with the oocysts freely suspended in the waters than when the environmental chambers were used. In t he sunlight viable levels decreased by 52% by day 4, 58% by day 5 and no viable oocysts were detected by day 11 (Table 4.5). It appeared that sunlight and/or the increased temperature played a role in enhanced oocyst inactivation. However, there was a concern regarding the assessment of viability using the DAPI method as this may overestimate viable populations of oocysts. It w as therefore necessary to examine the use of excystation as a measurement of viability.

4.3 Experiment 3

The DAPI and excystation methods were compared for assessing oocyst viability in marine waters shipped from Hawaii. The oocysts were stored in 50mL test tubes, rather than beakers to minimize the surface area, therefore minimizing adsorption of the oocysts. The seeded marine waters were stored in the laboratory at 26° C, which was the average temperature of the waters in Hawaii.

Water from Pearl Harbor and Black Point both had salinities of 35 ppt. Marine bacteria levels were 9×10^4 CFU/mL in Pearl Harbor water and were 6×10^4 CFU/mL in Black Point water.

The results are shown in Tables 4.6, 4.7 and 4.8. Excystation is a measure of the numbers of oocysts which have fully or partially opened up and released sporozoites. There are 4 sporozoites per oocyst and theoretically if 100 oocysts fully excysted one should be able to count 400 sporozoites. However, during inactivation processes empty oocysts may begin to appear and therefore the sporozoite yield will drop dramatically. This was taken into account in these experiments.

The viability of the oocysts dropped from 80% excystation to 28% in DI water after 14 days while the DAPI+/PI- population went from 88% to 37%. The sporozoite yield remained fairly constant up to day 3 and than dropped in half (Table 4.6). In Black Point water the excystation began low and went from 60% to 50% over the 14 days, while the sporozoite yield went from 2.56 to 0.74 and 0.55 at days 11 and 14, respectively. The DAPI+/PI- population went from 78% to 40% (Table 4.7). A similar trend was seen with oocysts stored in Pearl Harbor waters (Table 4.8). The sporozoite yield decreased by 3 to 10 fold in the marine waters as compared to DI water.

The decrease in oocyst viability is graphically displayed in Figures 4.1, 4.2 and 4.3 for DAPI+/PI- and PI+ populations and percent excystation (adjusted based on sporozoite yield). Empty oocyst populations did not dramatically change, and the decrease in viability was noted by the increase in PI+ populations, poorer excystation and few intact sporozoites for those oocysts which did open up.

In general, DAPI/PI procedure resulted in a higher percentage of viable oocysts than did the excystation measurement. Excystation has been more readily used and accepted for viability studies on disinfectants ability to inactivate protozoa (Korick et al., 1990; Sobsey, 1989), therefore excystation was used to further evaluate survival.

The percentage of change in excystation was regressed against time (Figure 4.4) and the best-fit statistics were developed (Table 4.9). For oocysts stored at 26°C under laboratory conditions in DI water the inactivation rate (or decrease in excystation) was found to be 5.5% per day, while for oocysts stored in Black Point and Pearl Harbor waters the decrease was 5.8% and 6.6% per day.

Table 4.1. Cryptosporidium Oocyst Survival in Deionized Water in Environmental Chambers at 20 o C using DAPI/PI.

Storage Time	DAPI +/PI- *	DAPI+/PI+	DAPI-/PI+	DAPI-/PI-	Ghost
Time 0	100%	0%	0%	0%	0%
24 hrs	86%	0%	0%	12%	2%
48 hrs	85%	0%	1.5%	12%	1.5%
72 hrs	90%	1%	0%	5%	4%
7 days	83%	0%	4%	4%	9%

* considered viable

Table 4.2 Cryptosporidium Oocyst Survival in Artificial Seawater in Environmental Chambers at 20 o C using DAPI/PI.

Storage Time	DAPI +/PI- *	DAPI +/ PI +	DAPI-/PI+	DAPI-/PI-	Ghost
Time 0	100%	0%	0%	0%	0%
24 hrs	67%	0%	2%	31%	0%
48 hrs	76%	0%	1%	21%	2%
72 hrs	76%	1%	0%	23%	3%
7 days	58%	0%	2%	39%	3%
14 days	53%	0%	3%	37%	7%

* considered viable

Table 4.3 Cryptosporidium Oocyst Survival in Deionized Water Stored in Beakers Under Laboratory Conditions at 22 o C

Storage Time	DAPI +/PI- *	DAPI+/PI+	DAPI-/PI+	DAPI-/PI-	Ghost
Time 0	83%	2%	11%	2%	2%
Day 4	45%	3%	34%	18%	0%
Day 5	50%	0%	11%	38%	0%
Day 11	63%	0%	18%	17%	2%

* considered viable

Table 4.4 Cryptosporidium Oocyst Survival in Boca Ciega Bay Water in Beakers Under Laboratory Conditions at 22 o C

Storage Time	DAPI +/PI-	DAPI+/PI+	DAPI-/PI+	DAPI-/PI-	Ghost
Time 0	83%	2%	11%	2%	2%
Day 4	57%	0%	9%	39%	0%
Day 5	36%	0%	20%	45%	1%
Day 11	45%	6%	45%	0%	4%

Table 4.5 Cryptosporidium Oocyst Survival in Boca Ciega Bay Water Stored in Beakers Under Outdoor Conditions

Time / Temp.	DAPI +/PI- *	DAPI+/PI+	DAPI-/PI+	DAPI-/PI-	Ghost
Time 0/24$_0$C	83%	2%	11%	2%	2%
Day 3/ 32$_0$C	51%	2%	45%	2%	0%
Day 4/ 36$_0$C	30%	0%	70%	0%	0%
Day 5/ 35$_0$C	25%	0%	75%	7%	0%

* considered viable

Table 4.6 Comparison of *C. parvum* oocyst viability using DAPI and excystation (DI water under laboratory conditions at 26 °C.)

Time (days)	0	1	2	3	4	7	8	9	11	14
Ghost	12%	ND	13%	14%	5%	4%	2%	4%	4%	4%
Partial	58%	ND	58%	46.5%	37.5%	60%	53%	50%	38%	24%
Full	30%	ND	28%	39.5%	57.5%	36%	45%	45%	57%	72%
DAPI +	88%	82% *	67%	43%	47%	56%	50%	51%	55%	37%
PI +	9%	17% *	33%	57%	53%	44%	50%	44%	45%	63%
Spore Yield	2.27	ND	2.34	3.24	1.89	1.56	2.03	2.36	2.30	3.60
Spore x Excy.	0.40	ND	0.42	0.49	0.2	0.23	0.28	0.32	0.25	0.25

* Read 24 hours later

Table 4.7 Comparison of *C. parvum* oocyst viability using DAPI and Excystation (Black Point waters in the laboratory at 26°C)

Time (days)	0	1	2	3	4	7	8	9	11	14
Ghost	21%	12%	23%	8%	3%	6%	2%	10%	8%	8%
Partial	39%	64%	46%	36%	22%	29%	27%	22%	31%	42%
Full	40%	24%	31%	56%	75%	64.5%	71%	68%	61%	50%
DAPI +	78%	77% *	73%	60.5%	60%	56%	55%	59%	49%	40%
PI +	12%	19% *	26%	44%	38%	44%	45%	41%	51%	59%
Spore Yield	2.56	2.84	3.94	2.41	3.16	1.84	2.62	3.92	0.74	0.55
Spore x Exy	0.38	0.54	0.68	0.26	0.20	0.16	0.19	0.31	0.07	0.06

* Read 24 hours later
** Salinity was 35 ppt

Table 4.8 Comparison of *C. parvum* oocyst viability using DAPI and Excystation (Pearl Harbor waters under laboratory conditions at 26°C)

Time (days)	0	1	2	3	4	7	8	9	11	14
Ghost	12%	ND	13%	14%	5%	4%	2%	4%	4%	4%
Partial	58%	ND	58%	46.5%	37.5%	60%	53%	50%	38%	24%
Full	30%	ND	28%	39.5%	57.5%	36%	45%	45%	57%	72%
DAPI +	88%	82% *	67%	43%	47%	56%	50%	51%	55%	37%
PI +	9%	17% *	33%	57%	53%	44%	50%	44%	45%	63%
Spore Yield	2.27	ND	2.34	3.24	1.89	1.56	2.03	2.36	2.30	3.60
Spore x Excy.	0.40	ND	0.42	0.49	0.2	0.23	0.28	0.32	0.25	0.25

* Read 24 hours later
** Salinity was 35 ppt.

Figure 4.1 Percentage of DAPI positive *C. parvum* oocysts stored in DI and marine waters at 26°C under laboratory conditions

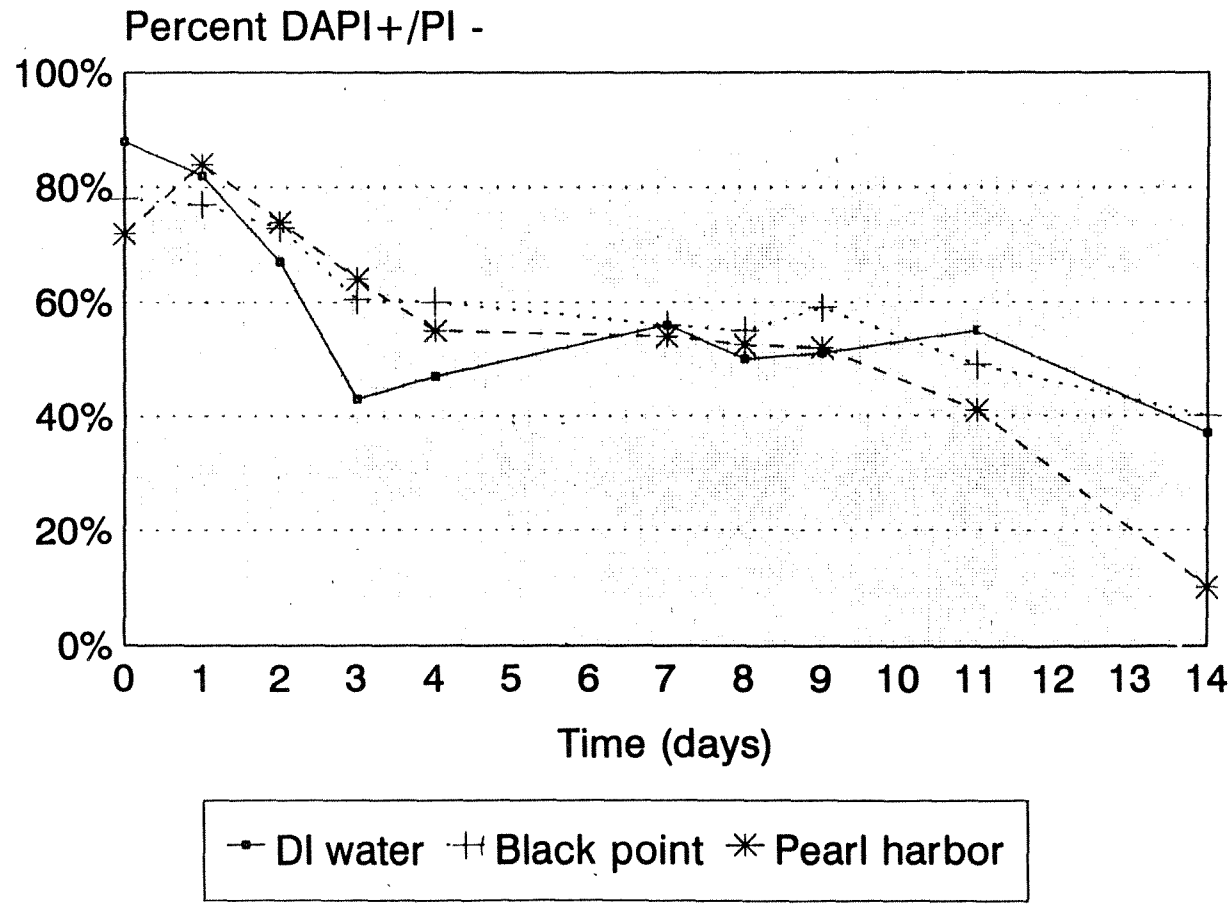


Figure 4.2 Percentage of PI positive *C. parvum* oocysts stored in DI and marine waters under laboratory conditions at 26°C

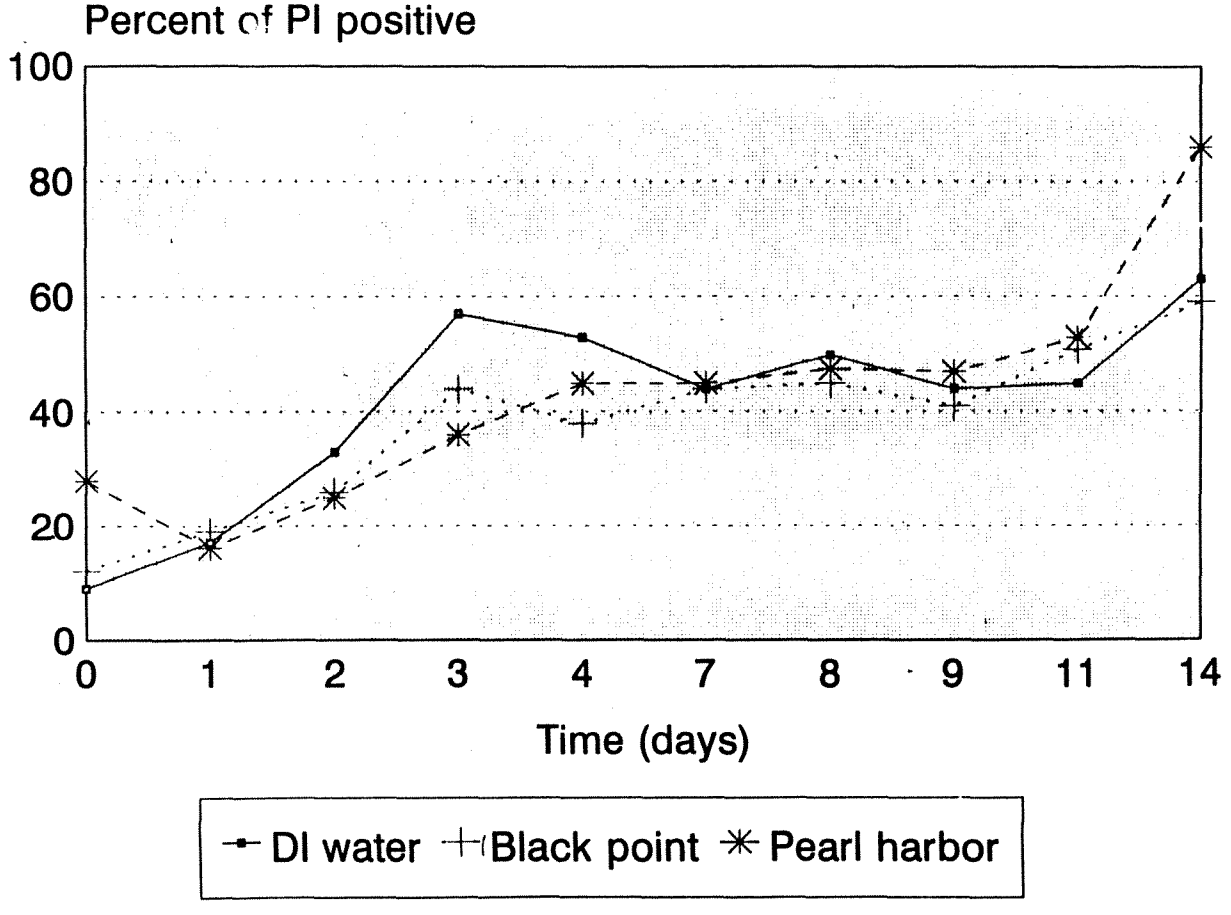
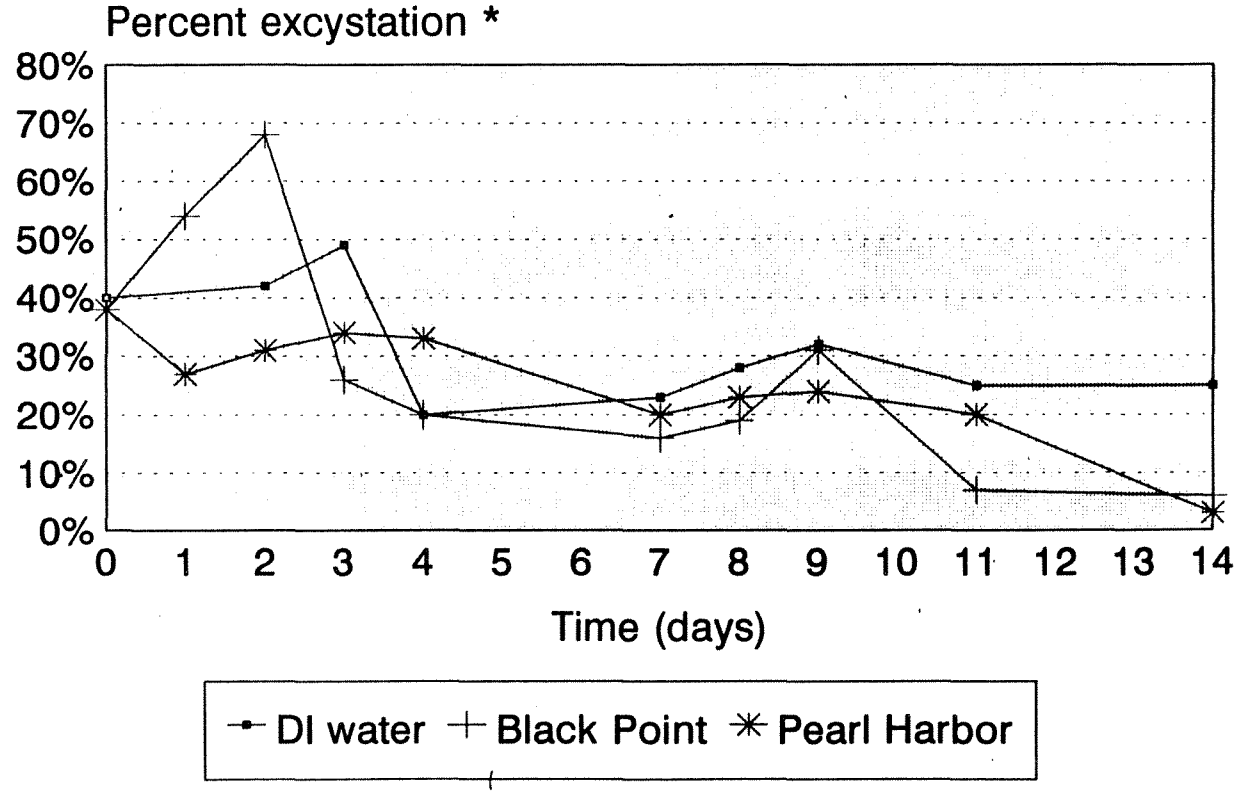
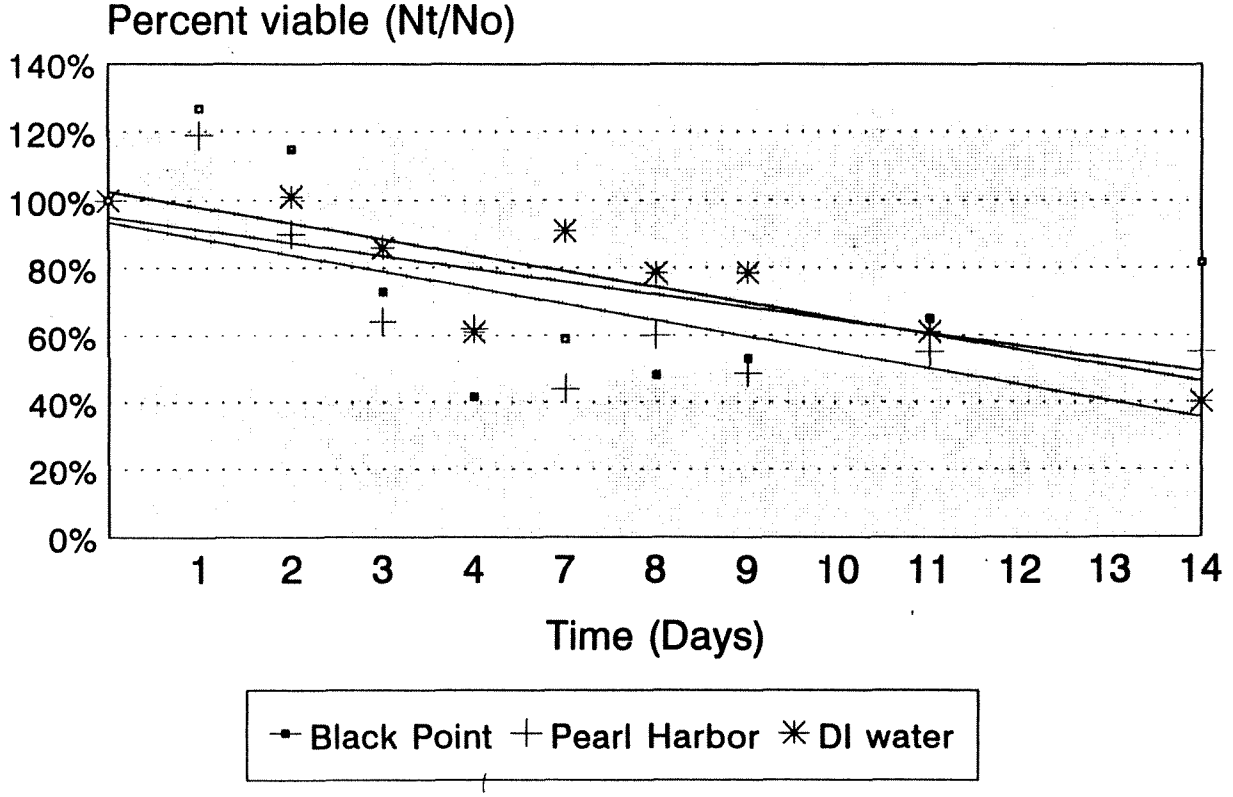


Figure 4.3 Excystation of *C. parvum* oocysts in DI water and marine waters from Hawaii
(at 26°C under laboratory conditions)



* Adjusted based on sporozoite yield

Figure 4.4 Percent Excystation of *C. parvum* oocysts in marine waters from Hawaii (Experiment 3.)



Laboratory condition was at 26°C.

Table 4.9 Statistical analysis of *C. parvum* oocysts excystation rate over time in Hawaii waters (Experiment 3.)

Statistic	Black Point	Pearl Harbor	DI
r	-0.6088	-0.8102	-0.8005
r^2	0.3707	0.6564	0.6408
y-intercept	1.0846	1.061	1.099
slope	-0.0584	-0.066	-0.055

4.4 Experiment 4

In the previous experiments the water had to be shipped from Hawaii to the USF laboratory. The final set of experiments on survival of oocysts in marine waters was set up at the University of Hawaii. In this case, one of the main goals was to examine oocyst survival in marine water and the impact of exposure to sunlight. Based on the previous results, the beaker procedure followed by excystation was used as the testing protocol. Temperatures between 24 and 27°C were maintained for the seeded waters set up outdoors with exposure to sunlight. In the laboratory, the temperature was maintained at 23° C without exposure to sunlight.

Oocysts stored in direct sunlight had a much larger and faster decrease in viability than oocysts stored under laboratory conditions at a constant room temperature using the excystation assay. Oocysts stored in 1 X PBS in the dark remained the most stable with a decrease from 62% to 13% in excystation after three days (Table 4.10). The viability of oocysts stored in 1 X PBS in the sunlight decreased from 62% excystation to 7%. Oocysts stored in Black Point waters in the dark decreased in viability from 62% excystation to 24% in the laboratory and to 6% in the sunlight (Table 4.11). The viability of oocysts stored in Ala Wai Canal water under laboratory conditions decreased from 62% excystation to 20%, while in the sunlight the excystation decreased to 6% (Table 4.12).

Comparison of oocyst excystation after exposure to the two marine waters and PBS is shown in Table 4.13. PBS seemed to be a better control solution than DI water, however, sunlight had an effect on the PBS stored oocysts as well. Temperature fluctuated slightly for the experiments conducted outdoors by 4°C. In this case the beakers were left out both during the night and during the day for the three days of the experiment.

The percentage of change in excystation over time compared to time zero is shown is Table 4-13 and this was regressed against time (Figure 4.5) and the best-fit curve and statistics were developed (Figure 4.6 and Table 4-14). For oocysts stored at 26°C under laboratory conditions in PBS the inactivation rate was found to be 8.2% per day, while for oocysts exposed to Black Point and Ala Wai Canal waters the decrease was 20.7% and 24.3% per day. In the sunlight the decrease was 27 to 29% per day.

A similar trend was seen for the sporozoite yield-excystation rate (Table 4.15). By day 3 the sporozoite yield-excystation was 0.01, 0.008 and 0.017 for the PBS, Black Point

and Ala Wai Canal, respectively exposed to sunlight. Under laboratory conditions the rates were 0.29, 0.1 and 0.11, respectively.

Table 4.10 Percent Excystation of C. parvum Oocysts (1 X PBS)

Excystation Category	Time 0	Time 0 Replicate	Time 24 Dark	Time 24 Sun	Time 48 Dark	Time 48 Sun	Time 72 Dark	Time 72 Sun
Ghost	5	11	13	0	4	1	6	1
Partial	53	56	48	31	47	16	43	6
Full	41	33	39	69	49	83	51	93
Sporozoite Yield	4.73	2.81	**	0.91	2.80	1.25	2.34	0.53
Spore x Excystation*	0.68	0.47	***	0.07	0.36	0.05	0.29	0.01

* Adjusted accordingly with sporozoite yield.
** Not determined.
*** No sporozoite yields.

Table 4.11 Percent Excystation of C. parvum oocysts in Black Point Waters

Excystation Category	Time 0	Time 0 Replicate	Time 24 Dark	Time 24 Sun	Time 48 Dark	Time 48 Sun	Time 72 Dark	Time 72 Sun
Ghost	5	11	6	1	5	0.006	3	0
Partial	53	56	31	12	23	12	21	6
Full	41	33	63	87	72	88	76	94
Sporozoite Yield	4.73	2.81	1.16	0.33	1.58	0.42	1.59	0.53
Spore x Excystation*	0.68	0.47	0.11	0.01	0.11	0.01	0.10	0.008

* Adjusted accordingly with sporozoite yield

Table 4.12 Percent Excystation of C. parvum oocysts in Ala Wai Canal waters

Excystation Category	Time 0	Time 0 Replicate	Time 24 Dark	Time 24 Sun	Time 48 Dark	Time 48 Sun	Time 72 Dark	Time 72 Sun
Ghost	5	11	6	1	6	3	3	0.004
Partial	53	56	49	17	24	14	17	6
Full	41	33	45	82	70	83	80	94
Sporozoite Yield	4.73	2.81	1.67	0.76	1.45	0.74	2.16	1.14
Spore x Excystation*	0.68	0.47	0.23	0.03	0.11	0.03	0.11	0.24

* Adjusted accordingly with sporozoite yield.

Figure 4.5 Percent Excystation of C. parvum oocysts in Hawaii waters (Experiment 4.)

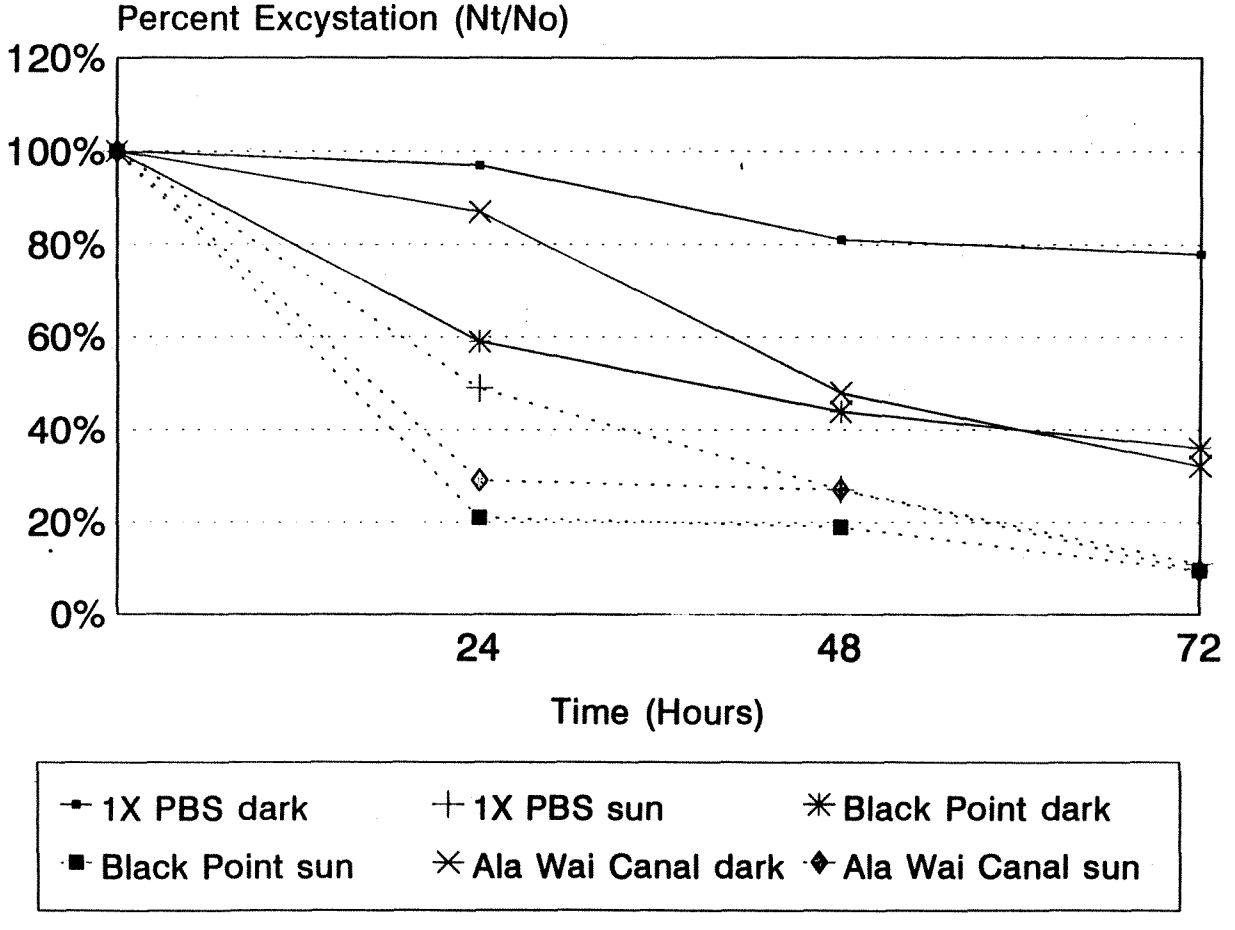


Figure 4.6 Best fit curve for C. parvum oocyst survival in Hawaii waters using excystation (Experiment 4.)

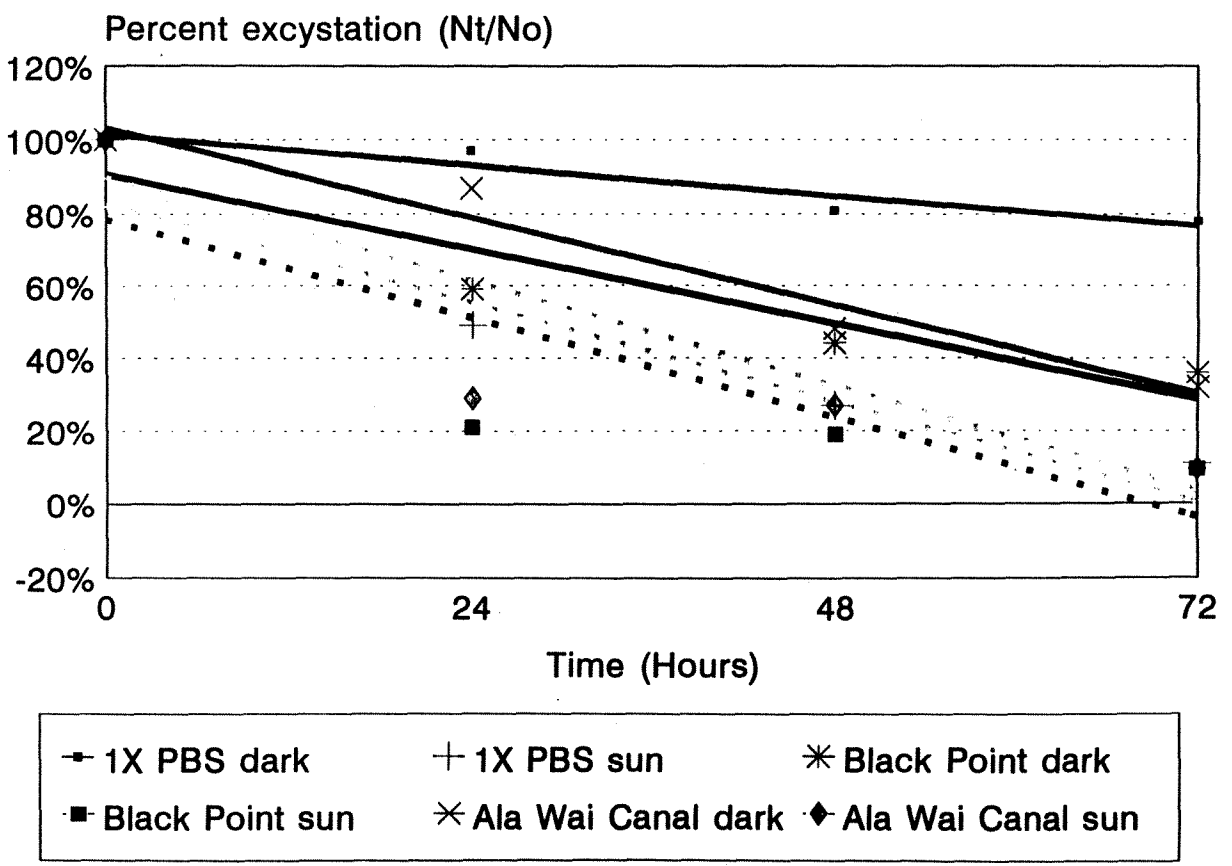


Table 4.13 Percent Excystation of C. parvum oocysts in waters from Hawaii (Experiment 4.)

Sample	Time 0 (Nt/No)	Time 24 (Nt/No)	Time 48 (Nt/No)	Time 72 (Nt/No)
1xPBS dark	63% (100%)	61% (97%)	51% (81%)	49% (78%)
1x PBS sun	63% (100%)	31% (49%)	17% (27%)	7% (11%)
Black point dark	63% (100%)	37% (59%)	28% (44%)	23% (37%)
Black point sun	63% (100%)	13% (21%)	12% (19%)	6% (10%)
Ala Wai dark	63% (100%)	55% (87%)	30% (48%)	20% (32%)
Alai Wai sun	63% (100%)	18% (29%)	17% (27%)	6% (10%)

* Oocysts used were collected from cattle April 18-28 1995.
** Roof temperature ranged from 24-27° C
*** Temperature in laboratory was 23° C

Table 4.14 Statistical analysis of *C. parvum* oocyst excystation rate over time in Hawaii waters (Experiment 4.)

Statistic	1X PBS Dark	1X PBS Sun	Black Point Dark	Black Point Sun	Ala Wai Canal Dark
r	-0.962	-0.962	-0.938	-0.840	-0.98
r^2	0.908	0.926	0.881	0.705	0.96
y-intercept	1.095	1.19	1.115	1.058	1.275
slope	-0.082	-0.289	-0.207	-0.273	-0.243

Table 4.15 *C. parvum* oocyst excystation x sporozoite yield in waters from Hawaii (Experiment 4.)

Time (hours)	0 (Nt/No)	24 (Nt/No)	48 (Nt/No)	72 (Nt/No)
1 X PBS-dark***	0.58 (100%)	0.48 (83%)	0.36 (62%)	0.29 (50%)
1 X PBS-sun**	0.58 (100%)	0.07 (12%)	0.07 (12%)	0.01 (1.7%)
Black Point-dark***	0.58 (100%)	0.1 (19%)	0.1 (19%)	0.1 (17%)
Black Point-sun**	0.58 (100%)	0.01 (1.7%)	0.01 (1.7%)	0.008(1.4%)
Ala Wai Canal-dark***	0.58 (100%)	0.23 (40%)	0.23 (40%)	0.11 (19%)
Ala Wai Canal-sun**	0.58 (100%)	0.03 (5.2%)	0.03 (5.2%)	0.017(2.9%)

* Oocysts used were collected from cattle in April 18-28, 1995.
** Roof temperature ranged from 24-27°C.
*** Temperature in laboratory was 23°C.

5 DISCUSSION

Recent outbreaks of *Cryptosporidium* in the United States have emphasized the dearth of current knowledge on the viability and survival of oocysts in fresh and potable water supplies. There is even less information pertaining to this protozoan's hardiness in marine waters. Robertson et al.,(1992) found that after 35 days in seawater, stored in the laboratory at 4°C in the dark, approximately 55% of the original inoculum (10^6 oocysts/mL) remained viable. This current study extended these author's controlled studies to reflect more in-situ conditions and assessed the viability and survival of *Cryptosporidium parvum* oocysts in marine waters off the shores of Hawaii.

Several factors may effect oocyst survival in waters including temperature, salinity, sunlight as well as specific undefined characteristics of a given water body. Table 5-1 compares the results of the excystation rate after 72 hours exposure under the various conditions tested (change in excystation compared to time zero N_t/N_o). Neither temperature at the range tested (20 to 36°C), or salinity or the type of water had any noticeable effect on the excystation rate, while it was clear that sunlight had a major impact (Figures 5-1 to 5-3).

It was found that age had an impact on the inactivation rate (Figure 5-4 and 5-5). Oocysts younger than two months as compared to 4 months were able to survive much longer in marine waters and in sunlight. When data from experiment 3 were compared to experiment 4, under laboratory conditions the decrease in excystation rate was -6.6% and -5.5% per day in marine waters for 2 month-old oocysts (Black Point and Pearl Harbor) and was -21% and -24% per day for 4 month-old oocysts (Black Point and Ala Wai Canal). This suggests that oocysts originating from fresh sources (such as wastewater discharges) would need 13 to 16 days to achieve a 90% reduction in the viable oocyst population. While oocysts originating from animals, which could remain in the soil several months until a rain washes the material into a water body would need 3.7 to 4.3 days to achieve a 90% reduction in the population.

There is currently no methodology to examine the age of oocysts found in the environment. In the future it will be important to define the sources of fecal inputs and determine the amount of time that the oocysts may remain in the environment. Up to 2 months of age the oocyst population appears to be fairly resistant to environmental pressures and this collaborates the limited studies done previously.

The methodological issues are many when attempting to address viability of the protozoa. This study would support the use of excystation as a measurement of viability. In addition the age of the oocysts must be taken into account and oocysts less than 2 months of

age should be used for all studies. A better evaluation of age as a factor influencing survival should be examined in the future.

Research has demonstrated that enteric microorganisms will survive for a longer time in fresh waters than in waters of greater salinity such as estuarine waters and seawaters (Table 5.2). Generally, there is an inverse relationship between increasing salinities and survival. Coliforms survive poorly in marine waters and this is one of the major reasons that this group of bacteria are inadequate predictors of the presence of pathogens (Feachem et al., 1983). *E. coli* survival rates are a little more reflective of the pathogens than are coliforms. At warmer temperatures with a greater reduction of *E. coli* (to low numbers) the difference between the indicator and the pathogens becomes more apparent, as the die-off rates for many of the pathogens appear to be slower than *E. coli.*

Enteric viruses may survive for months in marine waters at low temperatures. Also studies have demonstrated that virus levels may be twice as great in the sediments as in the water column and the T-90 reduction rates in sediments were reportedly 6 days at 18-21°C and 14 days at 4-15°C.

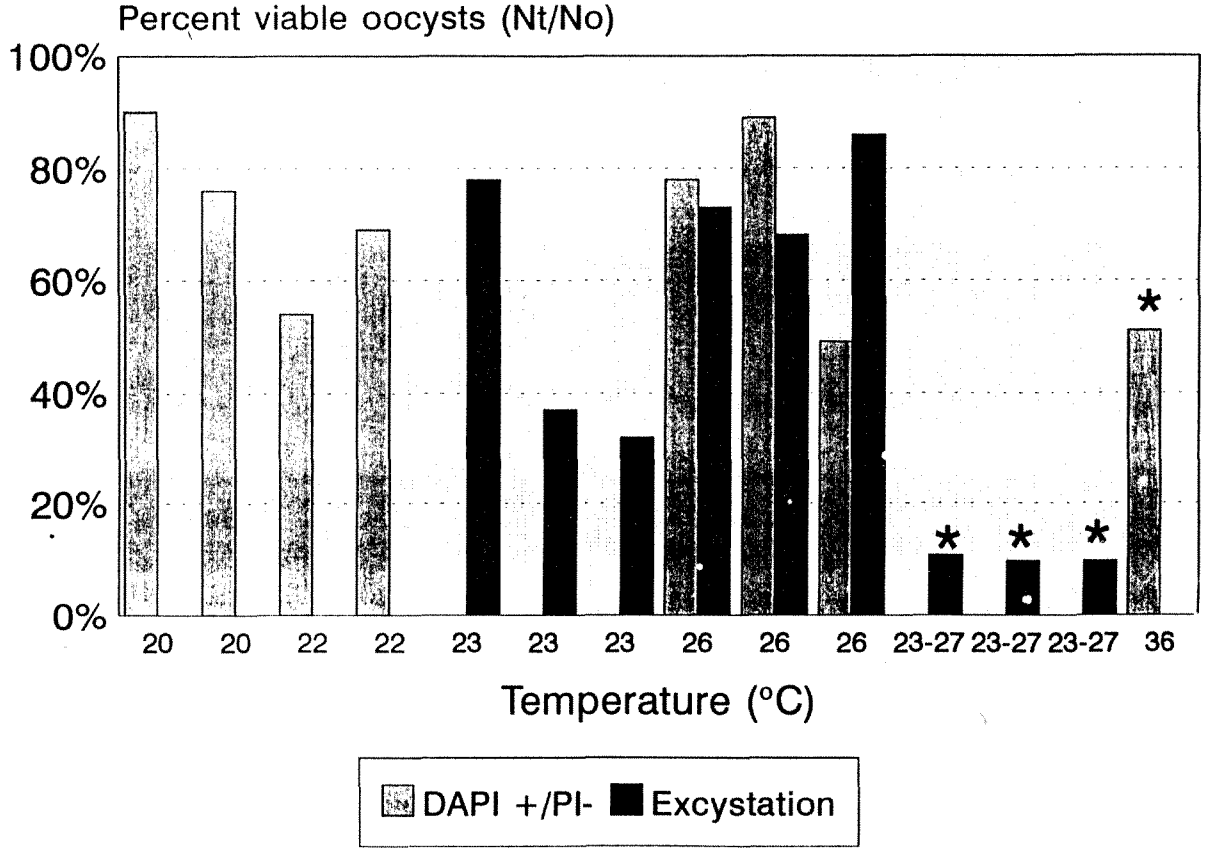
Very little information is available on the survival of the protozoa in marine waters. Investigators in the 1920s and 1930s reported that *Entamoeba* survival was unaffected by salt concentrations found in seawater. *Giardia* cysts maintained the ability to excyst at the same rate up to 12 days in seawater apparently surviving for 26 days at 10 to 20^{0}C and up to 28 days in fresh waters (DeRegnier et al., 1989). The Mamala Bay study and the work of Robertson et al. (1992) demonstrate that the oocysts of *Cryptosporidium parvum* are more resistant to inactivation than any other enteric pathogen studied to date under various environmental conditions.

Table 5.1 Summary of Percent viable C. parvum oocysts in marine waters after 72 hours

Experiment #	Temperature oC	Sunlight Y/N	Water (Salinity) (thousands per part)	% Viable (Nt/No) Excy.	% Viable (Nt/No) DAPI+/PI-
1	20	N	DI(0 ppt)	ND	90
1	20	N	Artificial seawater (35 ppt)	ND	76
2	22	N	DI (0 ppt)	ND	54**
2	36	Y	Boca Ciega (30ppt)	ND	51
2	22	N	Boca Ciega (30 ppt)	ND	69**
3	26	N	Black Point (35 ppt)	73	78
3	26	N	Pearl Harbor (35 ppt)	68	89
3	26	N	DI (0 ppt)	86	49
4	23-27	Y	PBS (0 ppt)	11	ND
4	23-27	Y	Black Point (35 ppt)	10	ND
4	23-27	Y	Ala Wai Canal (35 ppt)	10	ND
4	23	N	PBS (0 ppt)	78	ND
4	23	N	Black Point (35 ppt)	37	ND
4	23	N	Ala Wai Canal (35 ppt)	32	ND

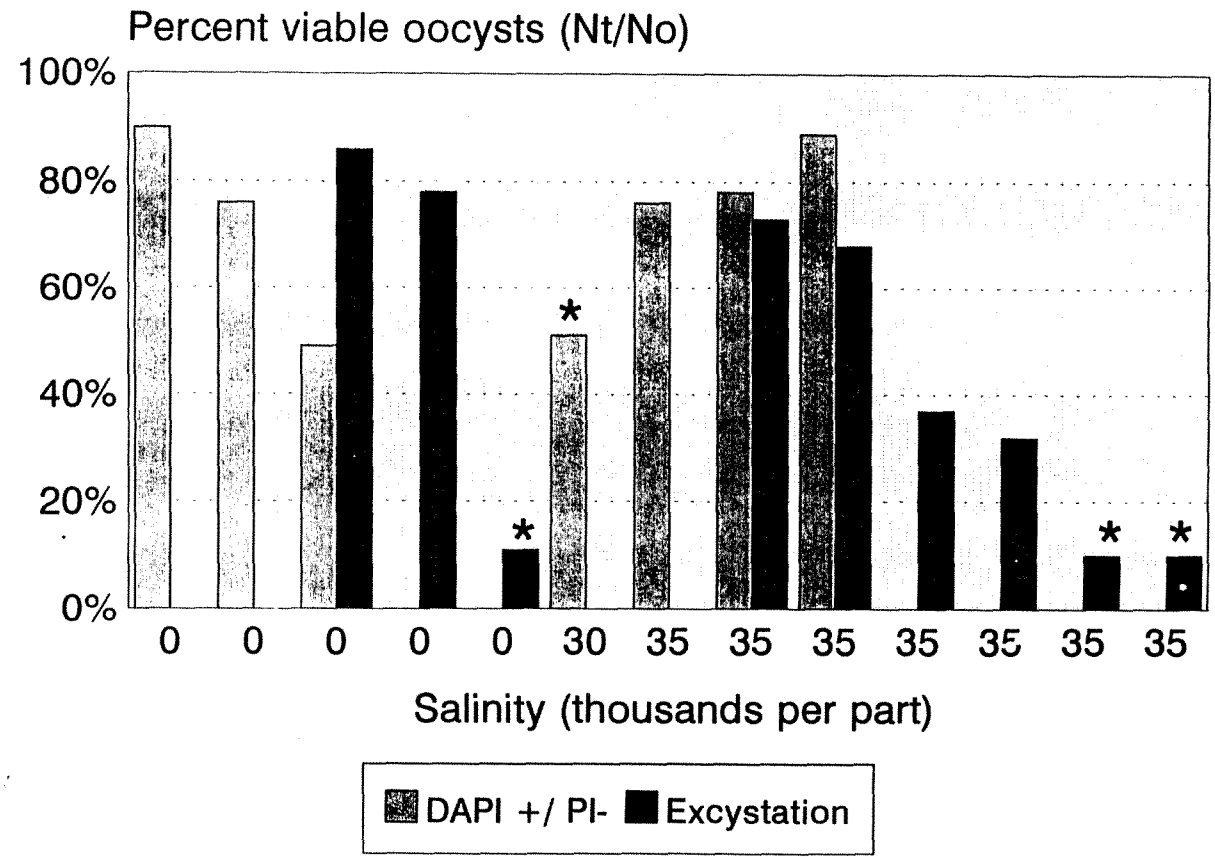
** Time at 96 hours

Figure 5.1 Percent viable *C. parvum* oocysts relative to temperature at 72 hours after exposure in marine waters



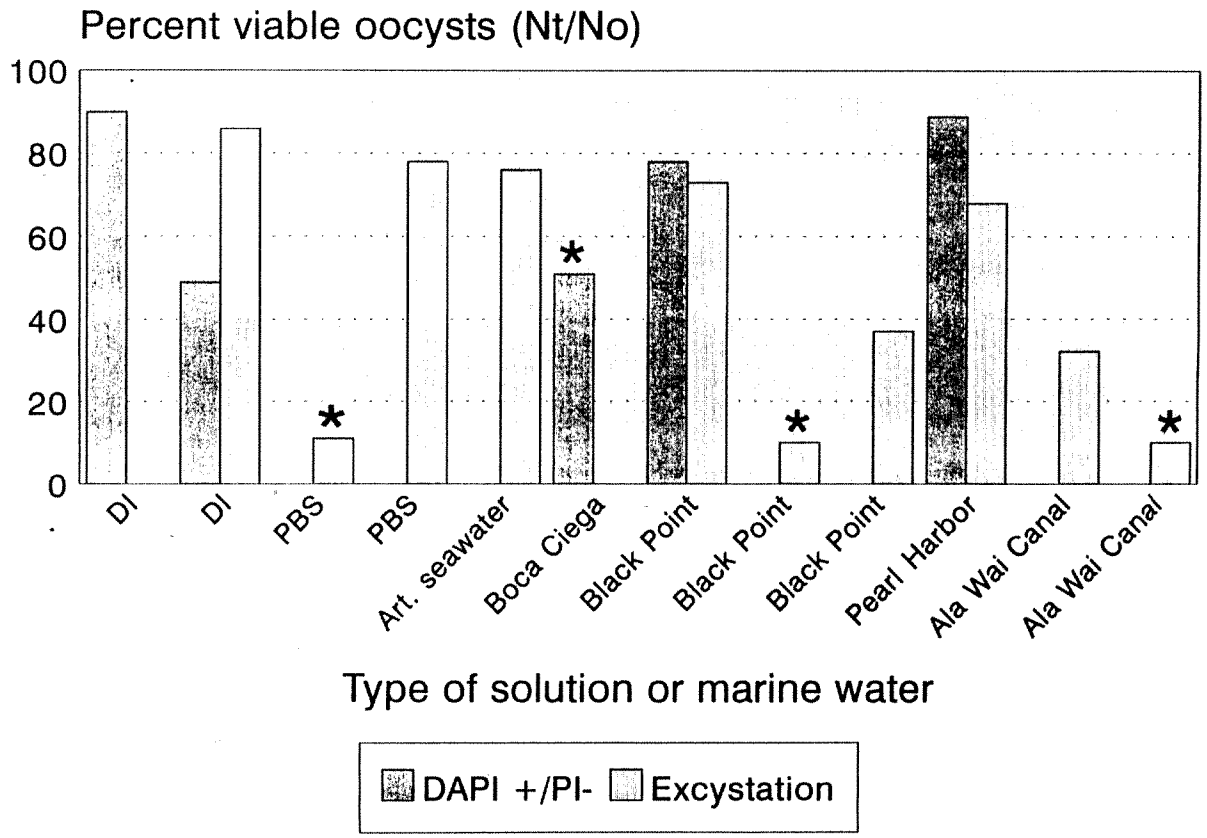
* Time at 96 hours and stored in sunlight

Figure 5.2 Percent viable C. parvum oocysts after 72 hours in different salinity waters



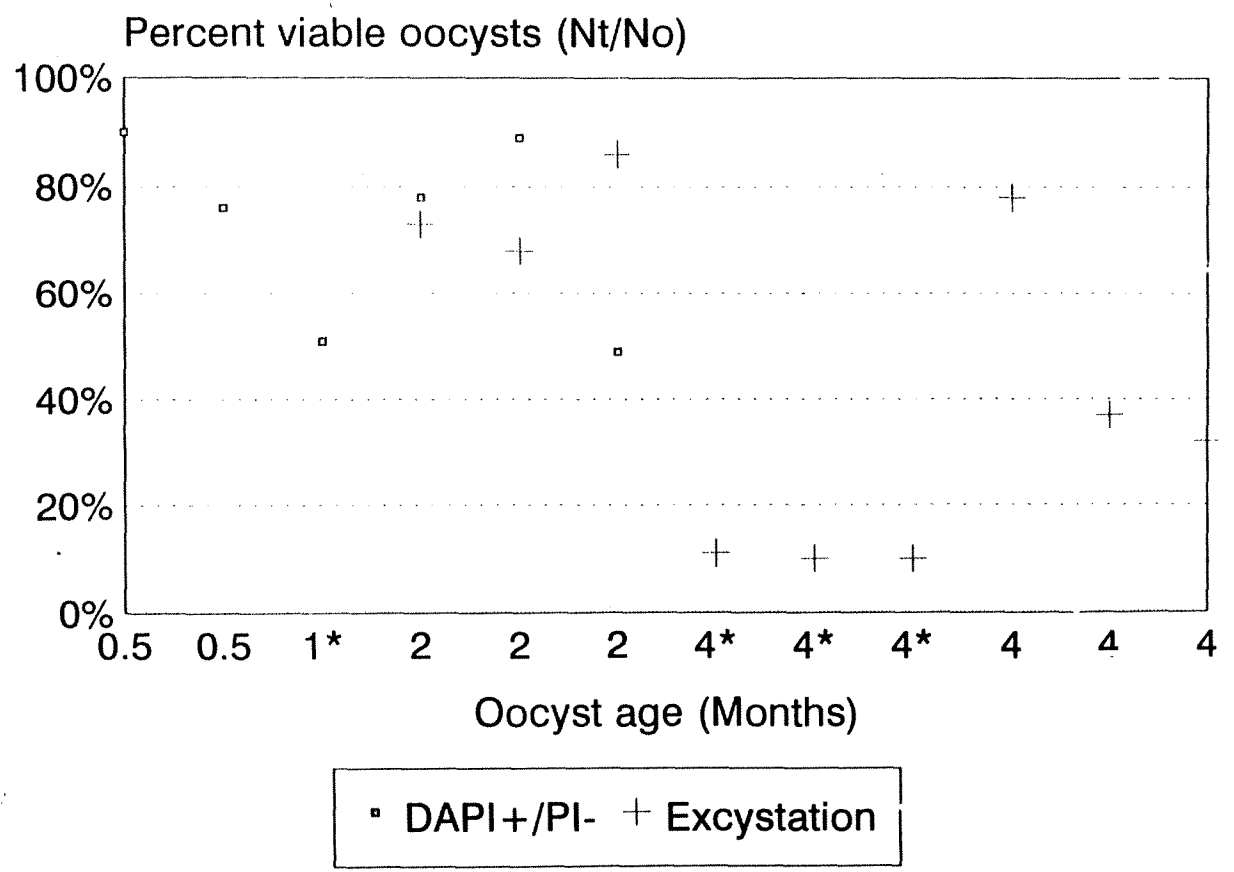
* Stored in sunlight

Figure 5.3 Percent viable *C. parvum* oocysts after 72 hours in different marine water and solutions



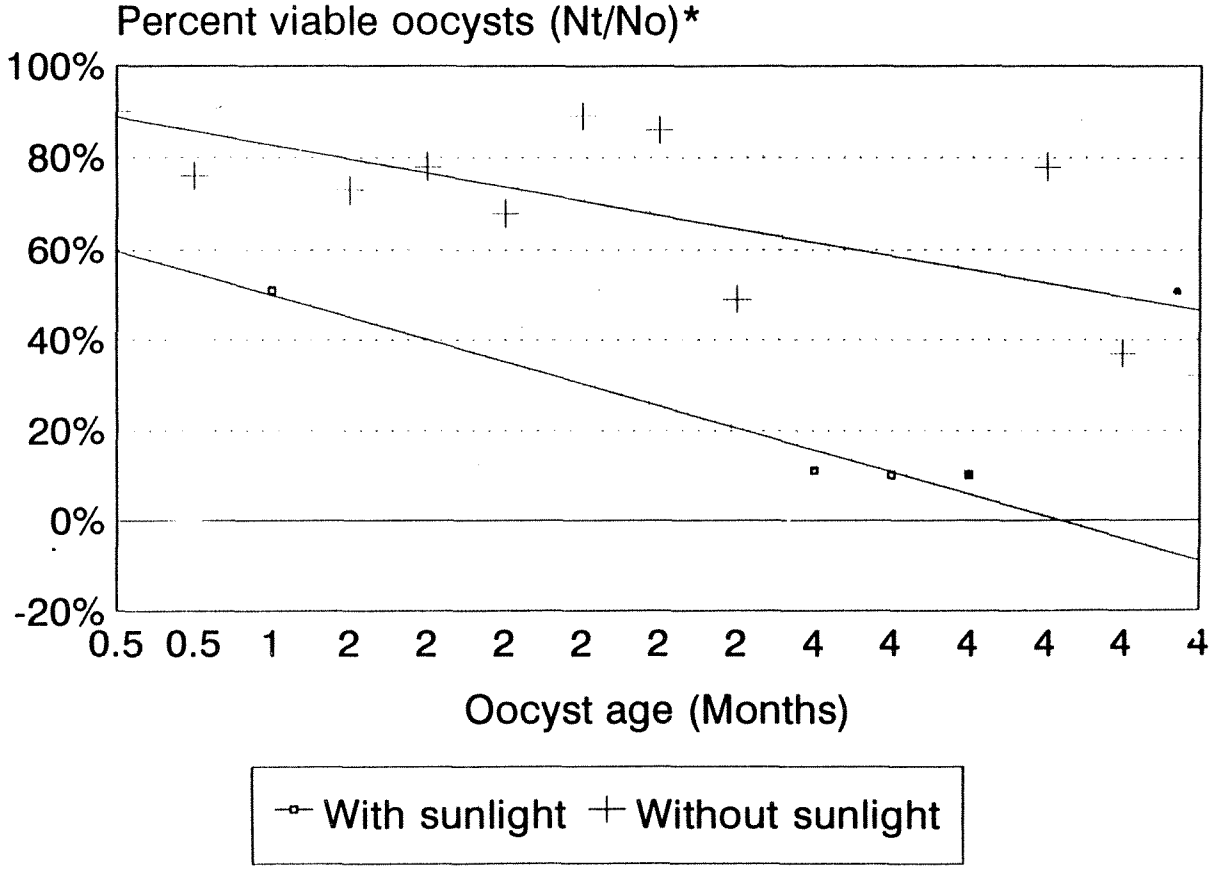
* Stored in sunlight

Figure 5.4 Percent viable C. parvum oocysts relative to the age of the preparation after 72 hours exposure to marine waters



* Stored in sunlight.

Figure 5.5 Best fit curve for the survival of *C. parvum* oocysts of various ages with and without sunlight after 72 hours exposure to marine waters



* Percent viable oocysts detected by either excystation or DAPI+/PI-.

Table 5.2 Survival of enteric pathogens and indicator bacteria in fresh and marine waters

	Time for 90% reductions (days)			
	Marine waters		Fresh waters	
Microorganism	Temp. oC	T-90	Temp. oC	T-90
Coliforms	10-20	0.025-0.33 (Avg.=0.083)	10-20	0.83-4.8 (Avg.=2.5)
E.coli	0	1.6	15	3.7
	30	0.58		
Salmonella	4	0.96	10-20	0.83-8.3
	37	0.7		
Yersinia	4-37	0.6	5-8.5	7
Giardia			2-5	14-143
			12-20	3.4-7.7
enteric viruses	20	0.67-1.0	4-30	1.7-5.8
	18-20	6.0*		
	4-15	14.0*		
Cryptosporidium	20-27	13-16**	4	60
		4***		

* In sediments.
** For oocysts under 2 months old.
*** For oocysts 4 months old.

6 CONCLUSIONS

Cryptosporidium parvum oocysts appear to be highly resistant to inactivation in marine waters. Only 5% to 6% decrease in excystation may be estimated per day in marine waters off the coast of Hawaii. To achieve 90% reductions in viable oocyst populations, 13 to 16 days would be needed in the water environment. Given the concentrations found in wastewater and the fact that these oocysts are young, it is highly probable that viable oocysts are reaching the beaches from the outfall. Sunlight does impact the inactivation rate and at least a decrease of 8.2% in excystation may be seen per day, perhaps reaching 29% decrease in excystation per day, depending on the age of the oocysts.

7 RECOMMENDATIONS

1. *Cryptosporidium* oocyst levels should be decreased in the wastewater effluents discharged through the marine outfall, as reliance on inactivation as a mechanism to protect the public health can not be met.

2. Better assessment of animal sources of *Cryptosporidium* oocysts in the watershed affecting the Ala Wai canal and the transport time to recreational sites should be made.

8 REFERENCES

Addiss, D.G., Arrowood, M.J., Bartlett, M.E., Colley, D.G., Juranek, D.D., and Kaplan, J.E., 1995. Assessing the public health threat associated with waterborne cryptosporidiosis: Report of a workshop. In: Morbidity and Mortality Weekly Report. Atlanta, GA: Centers for Disease Control and Prevention, 44/No RR-6, 1.

Bell, A., G uasparini, R., Meeds, D., Mathias, R.G., and Farley, J.D. Farley. 1993. A Swimming p ool-associated outbreak of *Cryptosporidiosis* in British C olumbia. Revue Canadienne De Sante Publique, 84 (5):334-337.

Campbell, A.T., Robertson, L.J., and H.V. Smith. Viability of *Cryptosporidium parvum* oocysts: correlation of in vitro excystation with inclusion or exclusion of fluorogenic dyes. Appl. Environ. Microbiol. 1992 58:3488-3493.

CDC, 1 990. Swimming Associated Cryptosporidiosis- Los Angeles County. MMWR: 39(20) 343-345.

CDC, 1 994. Cryptosporidium Infections Associated with swimming pools-Dane County, Wisconsin, 1993. MMWR:43 561-563.

Current, W., 1986. *Cryptosporidium:* its biology and potential for environmental transmission. Crit. Rev. Environ. Control: 17:21-51.

DeRegnier, D.P., Cole, L., Schupp, D.G., and Erlandsen, S. L. 1989. Viability of *Giardia* Cysts in Lake, River, and Tap Water. Appl. and Env. Microbiology. Vol. 55 No. 5. pp. 1123-1129.

Dubey, J.P., Speer, C.A., and Fayer, R. (eds) 1990. *Cryptosporidiosis of Man and Animals*. CRC Press, Boca, Raton, FL.

Fayer R. and Ungar B. L. P., 1986. *Cryptosporidium* spp. and cryptosporidiosis. Microbiol. Rev. 50:(4) 458-483.

Feachem, R.G., Bradley, D.H., Garelick, H., and Mara, D.D. 1983. *Sanitation and Disease Health Aspects of Excreta and Wastewater Management.* John Wiley and Sons, New York, NY.

Hayes, E.B., Matte, T.D., O'Brien, T.R., McKinley, T.W., Logsdon, G.S., Rose, J.B., Ungar, B.L.P., Word, D.M., Pinsky, P.F., Cummings, M.L., Wilson, M.A., Long, E.G., Hurwitz,

E.S. and Juranek, D.D., 1989. Large community outbreak of cryptosporidiosis due to contamination of filtered public water supply, New Engl. J. of Med., 320, 1372.

Joce, R.E., Bruce J., Kiely, D., Noah, N. D., Dempster, W.B., Stalker, R., Fumsley, P., Chapman, P.A., Norman, P., Watkins, J., Smith, H.V., Price, T.J., and D. Watts. 1991. An Outbreak of cryptosporidiosis associated with a swimming pool. Epidemiol. Infect. 107:497-508.

Jokippi, L. and Jokipii, A.M.M., 1986. Timing of symptoms and oocyst excretion in human cryptosporidiosis. New Eng. J. of Med. 315:1643-1647.

Korich,D.G., Mead, J.R., Madore, M.S., Sinclair, N.A., and C.R. Sterling. Effects of Ozone, Chlorine Dioxide, Chlorine, and Monochloramine on *Cryptosporidium parvum* oocyst viability. Appl. and Environ. Microbiol. (1990) 56(5) 1423-1428.

Lisle, J. T. and Rose, J.B., 1995. *Cryptosporidium* contamination of water in the USA and UK: a mini review. J water SRT-Aqua 44(3) 103.

MacKenzie, W., Neil, M., Hoxie, N., Proctor, M., Gradus, M., Blair, K., Peterson, D., Kazmierczak, J., Addiss, D., Fox, K., Rose, J. and Davis, J., 1994. A massive outbreak in Milwaukee of *Cryptosporidium* infection transmitted through the public water supply. New England Journal Med., 331, 161.

Robertson, L.J., Campbell, A.T., and H.V. Smith. 1992. Survival of *Cryptosporidium parvum* oocysts under various environmental pressures. Appl. Envir. Microbiol. 58:3494-3500.

Robertson, L.J., Campbell, A.T., and H.V. Smith. 1993a. In vitro excystation of *Cryptosporidium parvum*. Parasitol 106, 13-19.

Robertson, L.J., Campbell, A.T., and H.V. Smith, 1993. A low cost, low technology container for studying the survival of transmission stages of parasites and other pathogens in water related environments. Wat. Res.: 27(4) 723-725.

Rose, J., Lisle, J. and LeChevallier,M. (1995). Waterborne cryptosporidosis: incidence, outbreaks and treatment strategies. In: *Cryptosporidium and Cryptosporidosis*, R. Fayer (ed.) CRC Press, Boca Raton. (in press)

Rose, J.B. and Carnahan, R.P., 1992. Pathogen removal by full-scale wastewater treatment. Final Report SP249 (Florida Department of Protection), Tallahassee, FL.

Sobey, M.D., 1989. Inactivation of health-related microorganisms in water by disinfection processes. Wat. Sci and Tech. 21:(3)179-195.