BENCH-SCALE TECHNICAL REPORT

THE EFFECT OF ELECTRON BEAM TREATMENT ON DELAYED MORTALITY AND EPHIPPIA HATCH RATES

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ABSTRACT

This technical report presents findings from bench-scale tests evaluating the ability of electron beam treatment to kill aquatic algae and zooplankton, specifically examining delayed mortality within 48 hours following treatment. This evaluation was the first attempt to assess the effect of electron beam treatment on hatch rates of *Daphnia magna* ephippia.

Researchers from the Lake Superior Research Institute (LSRI) of the University of Wisconsin-Superior (UWS) in Superior, Wisconsin, USA traveled to the Fermi National Accelerator Laboratory, hereafter Fermilab, to conduct the bench-scale evaluation in May 2022. The test apparatus is a stationary copper electron beam accelerator that supplies a radiation dose expected to cause mortality in standard test organisms. Testing conducted in this evaluation was developed based on findings from earlier tests at Fermilab that demonstrated electron beam treatment was effective at causing complete mortality in *E. coli* at the lowest dose employed (1 kGy) and was effective at causing immediate mortality in *D. magna* and *Eucyclops* spp. at doses between 5 and 10 kGy (Polkinghorne et al., 2022). *S. capricornutum* was more resistant to immediate effects of electron beam treatment in initial tests (Polkinghorne et al., 2022).

All experimental exposures were conducted at Fermilab. The irradiation process was conducted by Fermilab staff in the Illinois Accelerator Research Center's (IARC's) Accelerator Applications Development and Demonstration (A2D2) machine. Doses ranged from 1 to 50 kGy.

Dose effectiveness testing was completed in lab water only, based on initial tests demonstrating minimal differences in treatment effect between high and low challenge water (i.e., low vs. high organic carbon content, suspended solids, and UV-transmittance). In samples examined 48 hours post-treatment, *S. capricornutum* had >99% mortality and *Eucyclops* spp. had 90% mortality at the 2 kGy dose. *D. magna* had 100% mortality at the lowest dose. No *D. magna* emerged from ephippia treated at any dose with the electron beam.

Key Words: Bench-scale, Ballast Water, Delayed Mortality, Electron Beam, Fermilab, Ephippia

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1 INTRODUCTION

A major focus area of the LSRI-Great Waters Research Collaborative (GWRC) is providing unbiased, independent data in support of the accelerated development of technologies having the potential for preventing the introduction and/or controlling the spread of non-indigenous organisms within the Laurentian Great Lakes. This report details the bench-scale evaluation of A2D2, a 9 MeV electron beam accelerator that was used to apply a radiation dose to lab water samples containing standard test organisms to further explore the utility of electron beam in ballast water applications. Initial research with the electron beam showed high levels of effectiveness in causing mortality in three different size classes of organisms at economically feasible electron beam dosages (Polkinghorne et al., 2022). The electron beam was provided by the Department of Energy's Fermilab managed by the Fermilab Research Alliance. Research was conducted at Fermilab (Batavia, IL, USA).

This technical report presents qualitative findings from bench-scale tests of the electron beam, which took place in May 2022. The research questions answered were:

1. What is the relationship between electron beam treatment dose and response, in terms of delayed mortality, for freshwater organisms (protists and zooplankton)?
2. Does electron beam treatment have an impact on hatch rates of *Daphnia magna* from ephippia?

2 TEST METHODS

2.1 TEST PLAN AND SOPS

A Test Quality Assurance Plan (TQAP) (Polkinghorne et al., 2022) and standard operating procedures (SOPs) were used to implement all test activities. These procedures facilitated consistent conformance to technical and quality system requirements and increased data quality in addition to providing unbiased, independent data. The TQAP details: collection, analysis, handling, quality objectives, and quality assurance/quality control (QAQC) requirements for samples and data. It was approved by both LSRI-GWRC and Fermilab prior to the start of bench-scale test activities. The procedures followed throughout testing are described in the *Test Methods* section and listed in the *References* section of this report.

2.2 BALLAST WATER MANAGEMENT SYSTEM AND EXPERIMENTAL APPARATUS

The electron beam accelerator used by LSRI-GWRC was stationary, however, compact and portable units are being developed at Fermilab that may be capable of functioning as an in-line treatment on a vessel. The technology would treat a vessel's ballast water as it is taken onboard and/or discharged to the receiving body of water.

All test exposures were conducted at Fermilab. The irradiation process was conducted by Fermilab staff in the Illinois Accelerator Research Center's (IARC's) Accelerator Applications Development and Demonstration (A2D2) machine. Located in the pit floor (Figure 1) of the Heavy Assembly Building, A2D2 (Figure 2) was the source of 9 MeV electrons from a repurposed teletherapy linear accelerator, or linac.

Its foundation is a normal conducting multi-cell 2.85 GHz accelerator structure known as a cavity. Electrons are generated by a thermionic electron gun and the radiofrequency power is supplied by a classic Klystron amplifier. Once the electrons are accelerated, they are collimated by thin slits and a 270-degree bending magnet. When combined, these electrons have a narrow momentum spread and are well focused leaving the vacuum window. From a maximum of 1.2 kW of beam power, A2D2 is capable of varying dose rates from 0.2 to 1.2 kGy/sec from its internal settings. For this application, A2D2 was run at 1 kGy/sec and total dose was manipulated by adjusting the irradiation time. Ozone is generated as the electron beam passes through the air above the sample. If the system was designed to treat ballast water, it could be configured in a way that would produce minimal to no ozone. To prevent toxicity caused by ozone, samples were covered during the exposure process. Safety procedures included a roped off area for operation personnel only, an alarm sounding to indicate that irradiation was about to occur, and appropriate personal protective equipment.

Figure 1. IARC building pit and electron cave where A2D2 is located.

Figure 2. The electron cave of A2D2 with a sample in the exposure location. Note that GWRC samples were covered during exposure.

2.3 TRAVELING TO CONDUCT BWMS EVALUATION

LSRI staff members Jen Maki, Michael Nagel, and Christine Polkinghorne traveled 440 miles to Batavia, IL on May 23, 2022 with test water, organisms, equipment, and supplies. Bench-scale evaluations were conducted at Fermilab from May 24-25, 2022. The team worked closely with Charles Cooper, Yichen Ji, and Slavica Grdanovska of Fermilab to set up equipment, prepare samples, and expose samples to the electron beam. LSRI staff were informed of the recommended operating conditions and safety measures required during operation and when navigating from the lab to the pit. Analysis of the algae samples and zooplankton samples, after the 0-hour analysis, were conducted in a mobile laboratory set up in a hotel room in Naperville, IL.

2.4 EXPERIMENTAL WATER PREPARATION

Dechlorinated laboratory water was used to conduct the dose effectiveness testing described in the following sections. Dechlorinated laboratory water represents a minimally challenging water quality for ballast water treatment technologies in terms of organic carbon concentration, total suspended solids, and water clarity. Previous testing of the electron beam as a ballast water treatment examined effectiveness in both the minimally challenging laboratory water as well as in water amended with test dust, Micromate™, and humic acid sodium salt to provide a higher challenge to the treatment. Minimal difference was noted in the treatment effect of the two water types, so testing was conducted with only laboratory water. Table 1 outlines the target ranges for each parameter in laboratory water for acceptance. Laboratory water was prepared as follows:

> *Laboratory Water (LW):* One day prior to the start of testing at Fermilab, two 19 L carboys were filled with LW to the 17 L mark via the hatchery headbox, a temperature controlled and aerated water source, located in the LSRI Toxicity Testing Lab. LW is municipal water from the City of Superior, Wisconsin that is passed through an activated carbon column to remove the majority of the chlorine present. The remaining residual chlorine is removed through injection of sodium sulfite and the resulting total residual chlorine concentration is below the limit of detection (i.e., < 5.91 µg/L Cl_2). LW has a low concentration of organic carbon and suspended solids, and a high UV transmittance.

Table 1. Water quality acceptable limits for laboratory water (LW) prepared for GWRC bench-scale evaluations.

Parameter	Acceptable Range for Initiating Bench-Scale Testing
Temperature	22–28 °C
pH	6.5–9.0
Specific Conductivity	120–170 µS/cm
Salinity	<1ppt
Dissolved Oxygen (DO)	4–12 mg/L
Total Suspended Solids (TSS)	<1.25 mg/L
Particulate Organic Matter (POM)	<1.25 mg/L
Dissolved Organic Carbon (DOC)	<0.77–2 mg/L
Non-Purgeable Organic Carbon (NPOC)	<0.77–2 mg/L
Percent Transmittance at 254 nm (%T)	93.0–100 % (filtered and unfiltered)
Total Alkalinity	40–60 mg/L
Total Hardness	40–60 mg/L

2.5 WATER QUALITY METHODS

Water quality samples were collected and measured from stock water one day prior to beginning testing at Fermilab. Water quality measurements included determination of total suspended solids (TSS), percent transmittance (%T), particulate organic matter (POM), non-purgeable organic carbon (NPOC), dissolved organic carbon (DOC), total alkalinity, total hardness, dissolved oxygen (DO), temperature, specific conductivity, and pH.

Temperature, DO, specific conductivity, and pH measurements were made on the stock LW containers prior to addition of organisms at the Fermilab. After exposures were completed, temperature, DO, specific conductivity, and pH measurements were made on the chemistry replicates for each exposure.

2.5.1 TOTAL SUSPENDED SOLIDS, PARTICULATE ORGANIC MATTER, AND MINERAL MATTER

Total Suspended Solids (TSS) analysis was conducted according to *LSRI/SOP/SA/66 – Analyzing Total Suspended Solids (TSS), Particulate Organic Matter (POM), and Mineral Matter (MM)*. Sample volumes (± 1%) were vacuum filtered through ashed, pre-washed, dried, and pre-weighed glass fiber filters (i.e., Whatman 934-AH, 1.5 µm particle retention). After each sample was filtered it was dried at 103-105°C in an oven and brought to a constant weight. TSS values were determined by calculating the weight of particulates collected on the filter. To determine POM, the residue on the filter from TSS analysis was ignited to a constant weight at 550°C in a muffle furnace. The concentration of POM was determined by calculating the difference of the dry weight of the particulates on the filter before and after ignition (the mass lost due to combustion). Mineral matter was defined and calculated as the difference between TSS and POM (determined by calculating the mass left on the filter after combustion).

2.5.2 PERCENT TRANSMITTANCE AT 254 NM

Percent Transmittance (%T) analysis was conducted according to LSRI/SOP/SA/69 – *Laboratory Determination of Percent Transmittance (%T) of Light in Water at 254 nm* on both unfiltered and filtered aliquots of each TSS sample collected using a Perkin Elmer Lambda 35 UV-Vis spectrophotometer. For analysis of the filtered aliquots, an appropriate volume of sample was filtered through a glass fiber filter (i.e., Whatman 934-AH, 1.5 µm particle retention). Deionized water was used as a reference to adjust the spectrophotometer to 100%T, and then each unfiltered and filtered sample aliquot was measured in a pre-rinsed sample cuvette with a 1 cm path length at 254 nm.

2.5.3 ORGANIC CARBON ANALYSIS

Dissolved organic carbon (DOC) and non-purgeable organic carbon (NPOC) analysis was conducted following *LSRI/SOP/SA/47 – Measuring Organic Carbon in Aqueous Samples*. Organic carbon analysis was performed using a Shimadzu Total Organic Carbon Analyzer, Model TOC-L, using a 1,000 mg/L organic carbon standard to prepare a working standard of 50 mg/L C. This standard was acidified to a pH < 2 with concentrated hydrochloric acid (HCl; ~ 0.2% v/v) and was used to generate a calibration curve. Before analysis, the NPOC samples were acidified to a pH < 2 with concentrated HCl and DOC samples were filtered through a glass fiber filter (i.e., Whatman GF/F, 0.7 µm nominal particle retention) and acidified in the same manner as NPOC samples. During analysis, samples were purged with ultra-pure air to remove the inorganic carbon and purgeable organic carbon and injected into the analyzer.

2.5.4 HARDNESS

Total hardness was analyzed using the ethylenediaminetetraacetic acid (EDTA) titrimetric method through manual titration according to the method described in *LSRI/SOP/GLM/02 – Procedure for Measuring Total Hardness*. Samples were analyzed immediately after collection and total hardness was reported as mg/L $CaCO_3$.

2.5.5 ALKALINITY

Analysis of total alkalinity was conducted using the sulfuric acid titrimetric method through manual titration and following the method described in *LSRI/SOP/GLM/01 - Procedure for Measuring Alkalinity*. Samples were analyzed immediately after collection and total alkalinity was reported as mg/L $CaCO_3$.

2.5.6 DISSOLVED OXYGEN

The DO concentration of the test water was measured using an YSI ProSolo Handheld Meter and Optical Dissolved Oxygen/Temperature Probe (YSI, Incorporated, Yellow Springs, OH) according to *LSRI/SOP/GLM/34 - Calibrating, Maintaining, and Using the YSI ProSolo Handheld Meter and Optical Dissolved Oxygen/Temperature Probe to Measure Dissolved Oxygen in Water Samples*. The DO meter was calibrated daily.

2.5.7 PH

The pH of the test water was measured using an Orion 3 Star meter with a pH combination electrode and automatic-temperature-compensation (ATC) probe, following *LSRI/SOP/GLM/05 – Procedure for pH Meter Calibration and pH Measurement for Ballast Treatment Systems Utilizing pH as the Active Substance*. The pH meter was calibrated daily with a three-point calibration and verified for accuracy using a pH 8.00 check buffer. The pH 8.00 buffer was measured at regular timepoints throughout analysis to verify that the meter did not drift.

2.5.8 SPECIFIC CONDUCTIVITY

The specific conductivity of the test water was measured using an Oakton Model CON 150 Conductivity/TDS/Temperature Meter (Oakton Instruments; Vernon Hills, IL), hereafter referred to as the CON 150 Meter. The CON 150 Meter was calibrated on a monthly basis and was verified for accuracy each day prior to sample analysis using a Daily Check Standard (i.e., 0.0100 M potassium chloride) according to *LSRI/SOP/GLM/28 - Procedures for Calibrating and Using the Oakton CON150 Conductivity/TDS/Temperature Meter.*

2.5.9 TEMPERATURE

Temperature was measured using a Fisher digital thermometer that was calibrated quarterly against a certified National Bureau of Standards mercury thermometer according to *LSRI/SOP/GLM/17 – Procedures for Thermometer Verification and Calibration*.

2.6 DOSE EFFECTIVENESS TESTING METHODS

Dose effectiveness tests measured treatment effects, in terms of mortality or hatching rate, of the electron beam treatment on organisms traditionally used for laboratory toxicity testing. The organisms used in dose effectiveness tests were cultured by LSRI with the exception of the *D. magna* ephippia which were purchased from Environmental Bio-detection Products, Inc., (EBPI, Burlington, Ontario, Canada). The overall experimental design was outlined in the test plan (Polkinghorne et al., 2022). The nominal dose, in units of kilograys (kGy), applied to each organism is shown in Table 2.

For each control and exposure dose for *D. magna*, *Eucyclops*, and *S. capricornutum*, there were three replicates and an additional chemistry replicate for water quality measurements. For the *D. magna* ephippia testing there were five replicates and an additional chemistry replicate for each control and exposure dose. This was due to the variability in hatch rates that have been historically observed. Once control and treatment sample vessels were filled with the appropriate water and organisms, they were taken to the electron cave. A labeled dosimetry film was placed on the cap of the sample vessel before exposure. After the film completely developed (approximately 24 hours), it was used to determine the actual dose applied to each sample. For each dose (1, 2, 5, 10, 20, and 50 kGy) sample replicates were placed individually into the A2D2 and exposed to the electron beam (Figure 2). Table 2 outlines the dates when tests were conducted and the doses applied to samples. Throughout the report the doses displayed in tables and figures are nominal doses, while actual doses applied are shown in Appendix 1.

Table 2. Dates, water type, and organisms tested during electron beam treatment testing.

Date	Organisms	Dose (kGy)
24 May 2022	*D. magna/Eucyclops*	0, 1, 2, 5, 10
	S. capricornutum	0, 1, 2, 5, 10, 20, 50
25 May 2022	*D. magna* Ephippia	0, 1, 2, 5, 10, 20, 50

2.6.1 ALGAE DELAYED MORTALITY

For *S. capricornutum* testing, a 10-L carboy containing 7-L of LW was spiked with 12.44 mL of algae from 4- to 8-day old cultures (1.125×10^8 cells/mL), to obtain approximately 200,000 cells/mL, following *LSRI/SOP/GWRC/11 – Assessing Bench-Scale Dose Effectiveness of Potential Ballast Water Treatment Processes on* Selenastrum capricornutum. Following addition of *S. capricornutum*, the water was manually mixed by inverting the carboy. The spiked water was then portioned into 8-ounce polypropylene exposure vessels to a level of 3 cm (~160 mL) for exposure and control use.

After all exposures were completed, post-treatment water quality measurements were recorded and samples were held in a portable incubator set to maintain a temperature of 25°C in the dark for the 48-hour holding period. Samples were transported to the laboratory set up in a hotel room and at approximately 6, 24 and 48 hours post treatment, a 1 mL subsample of the *S. capricornutum* was collected from each replicate for algae enumeration and analyzed by staining the cells with the mortal stain SYTOX® Green. *LSRI/SOP/GWRC/11* was followed for staining and counting. Counting was conducted by enumerating the number of live and dead cells within a known area on a hemocytometer using a compound microscope equipped with epifluorescence able to excite samples at 450-490 nm under 400x magnification.

2.6.2 ZOOPLANKTON DELAYED MORTALITY

Approximately 500 *D. magna* neonates were collected from brood boards that had been renewed three days prior. Approximately 500 mixed age *Eucyclops* spp. adults were collected from mixed *Lumbriculus variegatus/Eucyclops* spp. culture tanks. The *D. magna* and *Eucyclops* were transported in two separate 1-L plastic bottles in the dark in a portable incubator set to maintain a temperature of 25°C.

D. magna and *Eucyclops* spp. sample vessels were filled with a small volume of the laboratory water (temperature adjusted to 25°C ± 3°C) and then both organisms were transferred into the vessels via a wide-mouth Pasteur pipette. The vessels were then filled to 3 cm after a quality control count was conducted by a second analyst.

Following treatment exposures, post treatment water quality measurements were recorded and initial live/dead counts were recorded. The samples were placed in the dark in a portable incubator set to maintain a temperature of 25°C for the 48-hour holding period. Survival counts and water quality measurements were made at approximately 4, 24 and 48 hours post-treatment. Mortality in the zooplankton was determined by visual observation of samples on a light table for any movement of the organisms following *LSRI/ SOP/GWRC/9– Assessing Bench-Scale Dose-Effectiveness of Potential Ballast Water Treatment Processes on* Eucyclops *spp*. and *LSRI/SOP/GWRC/10– Assessing Bench-Scale Dose Effectiveness of Potential Ballast Water Treatment Processes on* Daphnia magna. Nonmotile organisms were gently prodded with a metal probe to induce a response before declaring the organism deceased.

2.6.3 EPHIPPIA HATCH RATE

Vials of *D. magna* ephippia were composited into a glass bowl containing laboratory water. Groups of 10 ephippia were transferred to 1 oz. plastic cups, the cups were verified by a second individual to contain 10 ephippia, and the contents of five cups were added to an exposure vessel. The exposure vessel was then filled to the 3 cm mark with laboratory water. Following exposures, the vessels were placed in the dark in a portable incubator set to maintain a temperature of 25°C. The samples remained in the incubator until return to the LSRI lab on May 26th. Upon arrival to campus, water quality was measured in the chemistry replicate of the exposed vessels. The lab water and ephippia in each exposure container was poured into a sieve with 63 µm Nitex mesh to remove the water. The ephippia were rinsed with moderately hard reconstituted water and then 10 ephippia were transferred into wells of a hatching tray containing 10 mL of moderately hard reconstituted water following *LSRI/SOP/GWRC/15– Assessing Bench-Scale Dose Effectiveness of Potential Ballast Water Treatment Processes on* Daphnia magna *ephippia*. Parafilm was used to cover the junction between the cover and the trays. The trays were placed into an incubator set at 21°C with constant lighting at 6000 lux (±600 lux) for 72 hours prior to counting each well for young hatched from ephippia.

Figure 3. Michael Nagel transferring ephippia from sieve to hatching tray.

2.7 DEVIATIONS

There were no deviations from the test plan and SOPs that occurred during testing at Fermilab.

3 RESULTS

Findings from the electron beam dose effectiveness tests are presented in the following subsections.

3.1 WATER QUALITY PRIOR TO EXPOSURE

Water quality was measured in stock solutions of LW immediately after they were prepared in LSRI's lab, one day prior to testing at Fermilab (Table 3). Water quality measurements of the stocks met the water quality acceptance requirements listed in the test plan and in Table 1 (Polkinghorne et al., 2022). Additionally, water quality measurements of the stocks were measured daily prior to addition of organisms to ensure there was no significant change in water quality (Table 4).

Table 3. Water quality values measured on stock solutions prior to use in dose effectiveness tests of the electron beam.

Parameter	LW Stock -1	LW Stock -2
Temperature (°C)	23.6	23.5
pH	7.28	7.27

Parameter	LW Stock -1	LW Stock -2
DO (mg/L)	7.1	6.5
Conductivity (µS/cm)	156.7	156.4
Hardness (mg/L $CaCO_3$)	50.7	52.3
Alkalinity (mg/L $CaCO_3$)	53.1	52.7
TSS (mg/L)	<1.25	<1.25
%T Filtered/Unfiltered	96.8/96.8	96.8/96.9
NPOC (mg/L)	1.4[J]	1.2[J]
DOC (mg/L)	1.4[J]	1.3[J]
POM (mg/L)	<1.25	<1.25
MM (mg/L)	<1.25	<1.25

[J] = Value between limit of detection (0.77 mg/L) and limit of quantitation (2.6 mg/L).

Table 4. Water quality measurements of stock water in each carboy taken prior to preparing sample vessels for exposure on the day of testing at Fermilab.

Parameter	LW Carboy 1 (*Selenastrum capricornutum*)	LW Carboy 1 (*Eucyclops* and *D. magna*)	LW Carboy 2 (*D. magna* Ephippia)
Temperature (°C)	24.9	25.1	26.8
pH	7.44	7.48	7.54
DO (mg/L)	8.2	8.4	7.9
Conductivity (µS/cm)	156.8	161.7	155.5

3.2 ALGAE

The results of water quality measurements taken immediately following *S. capricornutum* exposures are shown in Table 5. In general, temperature increased and pH and DO concentrations decreased as the applied dose increased. In the 50 kGy LW samples, water temperature increased 14.1°C, pH decreased minimally, and DO decreased by 4.4 mg/L compared to the controls. The conductivity was not impacted by treatment with the electron beam.

Table 5. Temperature, pH, DO, and conductivity results of exposure solutions measured after treatment with electron beam involving *S. capricornutum* in LW.

Dose (kGy)	SC-LW			
	Temp. (°C)	pH	DO (mg/L)	Conductivity (µS/cm)
0 - Control	22.0	7.44	8.5	160.0
1	22.0	7.48	7.2	157.2
2	22.4	7.43	6.7	156.9
5	23.1	7.35	6.4	157.0
10	23.9	7.30	6.1	157.4
20	26.0	7.28	5.5	157.8
50	36.1	7.27	4.1	158.7

Water quality measurements were taken in the chemistry replicate of the samples at 6, 24, and 48 hours of incubation (Table 6). After 100% mortality was achieved in a dose, the samples for that exposure were no longer held. The temperature in the incubated samples ranged from 23.9°C to 25.5°C. The pH of the samples increased from 6 hours to 24 hours after incubation. Dissolved oxygen increased in the treated samples, likely because air was dissolving back into the water as the containers were not air tight.

Table 6. Temperature, pH, and DO of exposure solutions during the *S. capricornutum* incubation period.

Dose (kGy)	SC-LW											
	6 Hours				24 Hours				48 Hours			
	Temp. (°C)	pH	DO (mg/L)	Conductivity (µS/cm)	Temp. (°C)	pH	DO (mg/L)	Conductivity (µS/cm)	Temp. (°C)	pH	DO (mg/L)	Conductivity (µS/cm)
0 - Control	24.3	7.31	8.4	150.2	24.5	7.53	8.0	159.2	24.9	7.41	8.0	173.6
1	23.9	7.23	8.2	149.6	24.8	7.59	8.0	158.5	25.4	7.55	7.9	158.3
2	24.9	7.22	7.9	149.5	25.4	7.56	8.0	157.6	25.5	7.56	8.0	159.7
5	24.3	7.22	7.7	149.1	24.8	7.56	7.9	160.8	-	-	-	-
10	24.3	7.35	7.5	149.7	23.9	7.51	7.9	160.7	-	-	-	-
20	24.4	7.44	7.2	149.6	-	-	-	-	-	-	-	-
50	24.3	7.45	7.0	149.5	-	-	-	-	-	-	-	-

Initial counts of *S. capricornutum* in the 10 L carboy were targeted to be approximately 200,000 cells/mL. The average concentration of *S. capricornutum* was 178,549 cell/mL ± 31,428 cell/mL. Results of *S. capricornutum* exposures to electron beam, presented as percent mortality, are shown in Table 7. Initial post-treatment counts began approximately 3 hours post treatment and continued for 3 hours. For initial post-treatment counts the first replicate of each dose was counted with the second and third replicates completed sequentially to capture any mortality that was increasing within a dose over time post-treatment. The mortality within the control and lowest doses (1 and 2 kGy) were consistent over the duration of enumeration. The mortality of replicates 2 and 3 of the 5 and 10 kGy doses was higher than replicate 1, suggesting death of the cells increasing as time passed following treatment. There was 100% mortality in the two highest doses in the initial post-treatment count. Mortality continued to increase with time following treatment. There was 100% mortality in the 5 and 10 kGy treated samples counted 24 hours post-treatment and 99.6% mortality in the 2 kGy treatment at 48 hours post-treatment. At 48 hours post-treatment the lowest electron beam dose had 68.9% mortality indicating high impact of electron beam treatment even at low doses.

Table 7. Percent mortality (± standard deviation) of *S. capricornutum* during dose effectiveness testing with the electron beam.

Dose (kGy)	Percent Mortality (Std Dev)		
	6 hours	24 hours	48 hours
0 - Control	0.7 (0.7)	0.4 (0.7)	0.9 (0.9)
1	0.7 (0.6)	39.4 (6.8)	68.9 (11.1)
2	6.2 (5.0)	82.9 (8.7)	99.6 (0.6)
5	28.8 (18.2)	100 (0)	-
10	82.0 (5.5)	100 (0)	-
20	100 (0)	-	-
50	100 (0)	-	-

3.3 ZOOPLANKTON

Results of water quality measurements taken immediately following zooplankton exposure are shown in Table 8. As was seen in the algae exposure solutions, the electron beam had no effect on conductivity values, but did cause an increase in the temperature and a decrease in both pH and DO values.

Table 8. Temperature, pH, DO, and conductivity results of exposure solutions measured after treatment with the electron beam involving *Eucyclops* spp. (EU) and *D. magna* (DM).

Dose (kGy)	DM/EU LW			
	Temp. (°C)	pH	DO (mg/L)	Conductivity (µS/cm)
0 - Control	22.7	7.61	8.5	160.3
1	22.6	7.59	7.6	160.1
2	22.8	7.49	6.7	158.9
5	23.8	7.39	6.6	158.3
10	24.1	7.35	6.5	159.8

Water quality measurements were taken in the chemistry replicate of the samples at 4, 24, and 48 hours of incubation (Table 9). After 100% mortality was achieved in a dose, the samples for that exposure were no longer held. Temperatures throughout the holding period ranged from 23.2°C to 25.6°C. Dissolved oxygen levels were similar in all samples and ranged from 7.8 to 8.4 mg/L oxygen.

Table 9. Temperature, pH, and DO of exposure solutions during the zooplankton incubation period.

Dose (kGy)	DM/EU LW											
	4 Hours				24 Hours				48 Hours			
	Temp. (°C)	pH	DO (mg/L)	Conductivity (µS/cm)	Temp. (°C)	pH	DO (mg/L)	Conductivity (µS/cm)	Temp. (°C)	pH	DO (mg/L)	Conductivity (µS/cm)
0 - Control	23.2	7.24	8.4	166.2	23.7	7.69	8.0	172.8	24.9	7.65	8.0	172.2
1	23.2	7.28	8.1	164.2	24.1	7.67	8.0	165.2	25.6	7.75	8.1	161.7
2	23.3	7.20	7.8	162.1	24.6	7.61	7.9	166.0	25.2	7.62	8.1	161.7
5	-	-	-	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-	-	-	-

Based on previous work at the Fermilab which indicated total mortality in *D. magna* and *Eucyclops* spp. at electron beam doses greater than 10 kGy, zooplankton were only exposed to doses up to 10 kGy to examine delayed mortality effects (Polkinghorne et al., 2022). Counts made immediately post-exposure of mortality in *D. magna* determined that that was 100% mortality in the 5 and 10 kGy doses and 13.3%

mortality in the 2 kGy dose (Table 10). By 48 hours post-treatment *D. magna* exposed to even the lowest electron beam dose experienced 100% mortality.

Table 10. Treatment effect of the electron beam on *D. magna*.

Dose (kGy)	*D. magna* Percent Mortality (Std Dev)			
	0 Hour	4 Hour	24 Hour	48 Hour
0 - Control	0 (0)	0 (0)	15.0 (7.1)	15.0 (7.1)
1	0 (0)	0 (0)	80.0 (17.3)	100.0 (0)
2	13.3 (11.5)	93.3 (5.8)	100.0 (0)	-
5	100.0 (0)	-	-	-
10	100.0 (0)	-	-	-

Mortality in *Eucyclops* spp. exposed simultaneously to electron beam treatment with *D. magna* showed similar results (Table 11). *Eucyclops* were slightly less impacted by electron beam treatment with some organisms surviving at both the 1 and 2 kGy doses at 48 hours post-treatment.

Table 11. Treatment effect of electron beam on *Eucyclops* spp.

Dose (kGy)	*Eucyclops* spp. Percent Mortality (Std Dev)			
	0 Hour	4 Hour	24 Hour	48 Hour
0 - Control	0 (0)	0 (0)	5.0 (7.0)	10.0 (0)
1	0 (0)	0 (0)	0 (0)	20.0 (17.3)
2	6.7 (5.8)	93.3 (5.8)*	73.3 (15.3)	90.0 (10.0)
5	100.0 (0)	-	-	-
10	100.0 (0)	-	-	-

* Counts were conducted without prodding organisms

3.4 EPHIPPIA

Results of water quality measurements taken following electron beam exposures of the *D. magna* ephippia samples are shown in Table 12. As with the algae and zooplankton exposures, temperature increased as the dose increased. In the case of ephippia testing, however, the 50 kGy exposure was treated first rather than last, and the temperature of the sample cooled while the other exposures occurred. The trends of decreasing pH and DO with increased dose were seen with ephippia. As with the algae and zooplankton treatments, there was no effect of treatment on conductivity. Following an approximately 31-hour incubation period, the temperature of the samples ranged from 24.4°C to 25.2°C. pH increased during the holding time, but the pH remained highest in the control and lowest in the 50 kGy dose.

Table 12. Temperature, pH, DO, and conductivity measured in solutions at each dose after exposure in *Ephippia* dose effectiveness tests with the electron beam.

Dose (kGy)	EDM-LW							
	0 Hour				31 Hour			
	Temp. (°C)	pH	DO (mg/L)	Conductivity (µS/cm)	Temp. (°C)	pH	DO (mg/L)	Conductivity (µS/cm)
0 - Control	24.3	7.41	8.0	159.3	25.1	7.65	7.9	157.7
1	23.4	7.43	7.2	171.7	25.1	7.65	7.9	172.5
2	23.5	7.37	6.4	159.9	24.7	7.65	8.0	157.9
5	23.6	7.28	6.4	156.7	24.9	7.59	7.9	159.4
10	25.1	7.29	7.0	157.1	25.2	7.53	7.7	158.0
20	26.6	7.26	5.7	157.7	24.4	7.50	7.8	160.8
50	24.3	7.28	4.4	156.7	24.5	7.53	7.6	157.4

Following the 72-hour incubation of ephippia at the optimal hatching conditions, the five control replicates had an average of 12 young with a standard deviation of 4.1. No young were present in any of the exposures that had been treated with the electron beam.

4 QUALITY ASSURANCE/QUALITY CONTROL – DATA QUALITY OBJECTIVES

4.1 WATER QUALITY DATA QUALITY OBJECTIVES

The data quality objectives (DQO) for water quality analyses conducted during the evaluation of the electron beam are summarized in Table 13.

Table 13. Data quality objectives, criteria, and performance measurement results from water quality analyses conducted during the electron beam dose effectiveness testing.

Data Quality Indicator	Evaluation Process/Performance Measurement	Data Quality Objective	Performance Measurement Result	
Precision	Samples (10%) were collected and analyzed in duplicate with performance measured by average relative percent difference (RPD).	< 20% average RPD	Percentage of Samples Collected and Analyzed in Duplicate:	Duplicate Relative Percent Difference
			%TF: 25.0%	%TF: 0.1%
			%TU: 25.0%	%TU: 0.0%
			NPOC: 50.0%	%NPOC: 0.8%
			DOC: 50.0%	%DOC: 1.0%
			POM: 50.0%	POM: NC
			TSS: 50.0%	TSS: NC
			Hardness: 50.0%	Hardness: 5.4%
			Alkalinity: 50.0%	Alkalinity: 5.9%
Bias, Method Blanks	%T method blanks were prepared by filtering deionized water samples (one per analysis date).	> 98% average %T	Number of %T Method Blanks Analyzed: 1	Method Blanks (%T): 99.8%
	TSS/POM method blanks were prepared by filtering deionized water samples from a 1L sample bottle (one per analysis date) and then drying, weighing, ashing and weighing the filter.	< 1.25 mg/L average TSS/POM	Number of TSS Method Blanks Analyzed: 1	Method Blanks (TSS)= 0 ± 0 mg/L
			Number of POM Method Blanks Analyzed: 1	Method Blanks (POM)= 0 ± 0 mg/L
	NPOC blanks were prepared by acidifying a volume of deionized water to 0.2% with concentrated hydrochloric acid.	< 0.77 mg/L average NPOC	Number of NPOC Blanks Analyzed: 2	Blanks (NPOC)= 0.4 ± 0.1 mg/L
	DOC method blanks were prepared by filtering deionized water samples (one per analysis date).	< 2.6 mg/L average DOC	Number of DOC Method Blanks Analyzed: 1	Method Blanks (DOC)= 0.9 ± 0.0 mg/L
Accuracy	Samples (10%) were spiked with a total organic carbon spiking solution with performance measured by average spike-recovery (SPR).	75% - 125% average SPR	Percentage of NPOC/DOC Samples Spiked: 50.0%	NPOC/DOC Spike Recovery= 102.2 ± 1.4%
	Performance was measured by average percent difference (%D) between all	One per analysis day < 20% average D	Percentage of Analysis Days Containing a Reference Standard:	Reference Standard Percent Difference
			TSS: 100%	TSS: 5.6%

Data Quality Indicator	Evaluation Process/Performance Measurement	Data Quality Objective	Performance Measurement Result	
	measured and nominal reference standard values.		POM: 100%	POM: 4.3%
			NPOC: 100%	NPOC: 1.6%
			Hardness: 100%	Hardness: 3.3%
			Alkalinity: 100%	Alkalinity: 8.6%
		A least one per 10 samples < 10% average D	Percentage (vs total samples) Check Standards:	NPOC 10 mg/L Standard % Difference
			NPOC/DOC: 50%	0.7 ± 0.8%
Represent-ativeness	All samples were collected, handled, and analyzed in the same manner.	Not Applicable – Qualitative.	All water chemistry/quality samples were collected, handled, transported and analyzed in the same manner using the appropriate SOPs.	
Comparability	Routine procedures were conducted according to appropriate SOPs to ensure consistency between tests.	Not Applicable – Qualitative.	The SOPs listed in the methods and references section were used for all water chemistry and water quality analyses.	
Completeness	Percentage of valid (i.e., collected, handled, analyzed correctly and meeting DQOs) water chemistry samples measured out of the total number of water chemistry samples collected. Performance is measured by percent completeness (%C).	> 90% C	TSS: 100%	
			%T Filtered: 100%	
			%T, Unfiltered: 100%	
			NPOC: 100%	
			DOC: 100%	
			Hardness: 100%	
			Alkalinity: 100%	
Sensitivity	The limit of detection (LOD) and limit of quantification (LOQ) for each analyte and analytical method utilized was determined annually unless a reporting limit was used based on the amount filtered as was the case with TSS/POM.	Not Applicable	TSS/POM RL: 1.25 mg/L based on filtering 800 mL of sample	
			NPOC/DOC LOD: 0.77mg/L	
			NPOC/DOC LOQ: 2.6 mg/L	
			Determined 19 October 2021	

NC: Not Calculable

4.2 ALGAE TESTING

During *S. capricornutum* testing, data quality was measured by analyzing a minimum of 10% of samples in duplicate and by having a second individual conduct quality assurance counts on a minimum of 10% of samples. For all testing with *S. capricornutum*, the minimum number of duplicate and quality assurance samples were met or exceeded (Table 14). The average relative percent difference of live and dead counts was less than 20% during duplicate analysis and quality assurance counts.

Table 14. Average Relative Percent Difference (RPD) for *S. capricornutum* counts conducted during the electron beam dose effectiveness testing.

Water Type	Duplicate or Quality Assurance Count	Percent of Samples with QA/Duplicate	DQO	Average Relative Percent Difference	
				Live	Dead
LW	Duplicate	13.3	RPD ≤ 20%, when greater than 10 cells of live/dead are counted	13.7	14.8
	Quality Assurance	13.3		4.2	6.6

4.3 ZOOPLANKTON TESTING

During dose effectiveness testing with *Eucyclops* spp. and *D. magna* data quality was ensured by having a second individual conduct counts on a minimum of 10% of the samples. QA counts were conducted on 40% of zooplankton samples with an RPD of 0%, both meeting the DQO.

4.4 EPHIPPIA TESTING

During dose effectiveness testing with D. magna ephippia data quality was ensured by having a second individual count counts on a minimum of 10% of the samples. QA counts were conducted on 43.75% of the samples with an RPD of 0%, both meeting the DQO.

5 CONCLUSIONS AND DISCUSSION

Over the course of several days in May 2022, LSRI-GWRC evaluated electron beam treatment at Fermilab as a potential ballast water treatment method. The electron beam accelerator used for this study functioned as planned during testing and no delays were encountered. The data discussed in the previous sections was used to evaluate the following questions.

> *Objective 1: What is the relationship between electron beam treatment dose and response, in terms of delayed mortality, for freshwater organisms (protists and zooplankton)?*
>
> Results of delayed mortality with both protists and zooplankton showed that mortality was immediate in the highest doses and increased over a 48-hour period in lower doses following exposure to electron beam. In *S. capricornutum*, the 2 kGy dose had >99% mortality by the 48-hour sample time (Table 7). With zooplankton, *D. magna* in all exposures were dead by the 48-hour sample time, while 90% of the Eucyclops spp. exposed to 2 kGy electron beam were dead by the 48-hour sample time (Table 10 and Table 11, respectively). It is beyond the scope of this project to determine the mechanism for delayed mortality versus immediate mortality, but it has been previously demonstrated that electron beam treatment can cause direct damage by creating single- or double- strand breaks in the DNA molecule beyond cell repair or recovery (Shehata, M.M.K. et al., 2011 and Lei, J. et al., 2020).

Objective 2: Does electron beam treatment have an impact on hatch rates of Daphnia magna from ephippia?

Resting eggs have been resistant to many ballast water treatment efforts due to the presence of a protective coating designed to prevent desiccation of the organisms during periods of drought (Cáceres, C.E., 1997). In testing where *D. magna* ephippia were exposed to electron beam treatment at Fermilab followed by incubation at optimal hatching conditions at the LSRI lab, no *D. magna* hatched from any of the treated samples while the control samples with the same number of ephippia had an average of 12 young ± 4.1 young emerging after exposure to optimal hatching conditions.

The results from this testing conducted at Fermilab indicate that electron beam has great potential for success as a ballast water treatment if a more compact system capable of being installed on a ship can be designed. The demonstration of the high mortality in organisms examined 48 hours post-exposure indicates that exposures to electron beam treatment at levels as low as 2 kGy cause mortality in the standard test organisms tested here and that 100% of the organisms examined were dead within 24 hours after a 5 kGy dose. Previous testing conducted at Fermilab showed even the 1 kGy dose to be 100% effective at eliminating *E. coli* in samples.

Bench scale testing previously conducted with *D. magna* ephippia exposed to a variety of ballast water treatments including UV and ozone demonstrated those treatments have no effect on hatch rates or potentially cause increased hatch rates (Anders, O. et al., 2019, Anders, O. et al., 2020, respectively). The effectiveness of the e-beam at eliminating emergence from *D. magna* ephippia at even the lowest dose indicates that e-beam could be an important control mechanism for aquatic nuisance species present in ballast water as resting eggs.

Charlie Cooper, General Manager of the Illinois Accelerator Research Center, Fermilab, has indicated that a system could be designed for ballast water application that would have less electron energy, thereby requiring less shielding than is required with the A2D2 which was designed for a wide scope of applications. The low doses required for effectiveness will allow relatively low power input allowing lower cost of operation and increased safety.

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8 APPENDIX A. MEAN MINIMUM DOSE IN KILOGRAY (±SD) DELIVERED TO SAMPLES IN LAB WATER DURING DOSE EFFECTIVENESS TESTING WITH THE ELECTRON BEAM.

Dose (kGy)	*S.capricornutum*	*D. magna/Eucyclops*	*D. magna* Ephippia
1	1.20 (0.05)	1.13 (0.12)	0.98 (0.14)
2	2.64 (0.13)	2.25 (0.23)	1.82 (0.17)
5	5.21 (0.27)	5.81 (0.33)	5.90 (0.32)
10	10.68 (0.22)	11.66 (0.44)	12.42 (0.29)
20	23.71 (0.80)	-	22.06 (0.40)
50	57.81 (0.33)	-	46.88 (1.21)

9 APPENDIX B. LIST OF SOPS USED DURING DOSE EFFECTIVENESS TESTING AT FERMILAB.

Category	Title	Code
General Lab Maintenance	Procedure for Measuring Alkalinity	LSRI/SOP/GLM/01
General Lab Maintenance	Procedure for Measuring Total Hardness	LSRI/SOP/GLM/02
General Lab Maintenance	Procedure for Calibration and Operation of pH Meters Utilizing Automatic Temperature Compensation (ATC)	LSRI/SOP/GLM/05
General Lab Maintenance	Procedures for Thermometer Verification and Calibration	LSRI/SOP/GLM/17
General Lab Maintenance	Procedures for Calibrating and Using the Oakton CON150 Conductivity/TDS/Temperature Meter	LSRI/SOP/GLM/28
General Lab Maintenance	Calibrating, Maintaining, and Using YSI ProSolo Handheld Meter and Optical Dissolved Oxygen Probe to Measure Dissolved Oxygen in Water Samples	LSRI/SOP/GLM/34
Great Waters Research Collaborative	Assessing Bench-Scale Dose Effectiveness of Potential Ballast Water Treatment Processes on *Eucyclops* spp.	LSRI/SOP/GWRC/9
Great Waters Research Collaborative	Assessing Bench-Scale Dose Effectiveness of Potential Ballast Water Treatment Processes on *Daphnia magna*	LSRI/SOP/GWRC/10
Great Waters Research Collaborative	Assessing Bench-Scale Dose Effectiveness of Potential Ballast Water Treatment Processes on *Selenastrum capricornutum*	LSRI/SOP/GWRC/11

Category	Title	Code
Great Waters Research Collaborative	Assessing Bench-Scale Dose Effectiveness of Potential Ballast Water Treatment Processes on *Daphnia magna* ephipphia	LSRI/SOP/GWRC/15
Sample Analysis	Measuring Organic Carbon in Aqueous Samples	LSRI/SOP/SA/47
Sample Analysis	Analyzing Total Suspended Solids, Particulate Organic Matter, and Mineral Matter	LSRI/SOP/SA/66
Sample Analysis	Laboratory Determination of Percent Transmittance of Light in Water at 254 nm	LSRI/SOP/SA/69