Evaluation of an *in situ* early life stage test with cutthroat trout, *Oncorhynchus clarki*, for environmental monitoring – a case study using mine effluent
B. Chalmers, J. Elphick, G. Gilron and H. Bailey

**ABSTRACT**

This study evaluated an *in situ* early life stage test using cutthroat trout for potential use in Canada’s *Metal Mines Effluent Regulations*’ Environmental Effects Monitoring (EEM) program. Current field monitoring approaches focus on either adult fish surveys or mesocosm studies, but both of these have inherent limitations that may affect their suitability on a site-specific basis. This study evaluated an alternative approach, namely an *in situ* toxicity test, as part of an EEM program for a zinc, copper and gold mine. Hatchboxes containing cutthroat trout embryos were placed in a creek that receives treated effluent from the mine, and monitored through the swim-up stage to evaluate hatching success, survival, normal development and growth. Advantages of the method include: no feeding requirement during exposure, fixed exposure locations, relevant endpoints and high statistical sensitivity. In addition, the extended exposure period integrated long-term exposure variables, including low-flow and freshet events. This approach also has application to other salmonid species and types of discharges.

**Key words** | cutthroat trout, early life stage test, Environmental Effects Monitoring (EEM), *in situ* toxicity testing, mine effluent, *Oncorhynchus clarki*

**INTRODUCTION**

The Environmental Effects Monitoring (EEM) program originated as part of the Canadian *Pulp and Paper Effluent Regulations (PPER)* under the *Fisheries Act* in 1992, and is now also part of the *Metal Mines Effluent Regulations (MMER)*. The objective of metal mining EEM is to evaluate the effects of treated mine effluent on fish, fish habitat and the use of fisheries resources. Because the Canadian mining industry is characterized by a wide variety of ore deposits, receiving environments and mining and milling processes, the use of uniform effluent concentration limits may not ensure adequate protection under all conditions. Therefore, to provide a more complete evaluation of the effects of treated mine effluent on the aquatic environment, site-specific EEM studies are required to be conducted every 5 years by all mines subject to the *MMER* (*Environment Canada* 2001).

The EEM program consists of laboratory-based chemical characterization of the effluent, sub-lethal toxicity testing, and biological monitoring studies in the receiving environment, including evaluation of fish health and benthic invertebrate community surveys. Basic requirements of the fish health survey include the monitoring of a minimum of 20 adults of each sex from each site; obtaining some of the health metrics for the studies requires sacrificing the fish.

Myra Falls Operations is primarily a zinc and copper mine, but also produces smaller quantities of gold and silver. The mine is located within Strathcona Provincial Park, on Vancouver Island, British Columbia, Canada. Myra Creek is a small creek that flows through the mine site and supports a local population of cutthroat trout, which are the only species of fish present. As historical data indicated that there is only a small population of
cutthroat trout in the creek (Hallam Knight Piesold Ltd 1999), it appeared that there would be insufficient trout present to conduct the fish health survey, and that any attempt to do so could adversely affect the entire population. Furthermore, because the fish-containing reach of the creek is relatively short (i.e., 5 km), and virtually the entire spawning and side-channel habitat is located upstream of the discharge, there would be some question as to the actual extent of separation (i.e., exposure history) between fish sampled above and below the discharge point.

Due to the limitations of applying the traditional EEM fish health survey to this site, the development of an alternative approach was desirable (Munkittrick et al. 2002). Such an approach would: directly evaluate potential impacts on the fish species of interest, not have an adverse impact on the existing population in the creek, and ensure separation between reference and exposure groups. The proposed study design was an in situ exposure in which cutthroat trout embryos obtained from a hatchery were placed in hatchboxes at different locations in the creek bed, and monitored through swim-up to identify any potential adverse effects. This general approach has been used in the past; for example, Erickson & Deniseger (1987) used eyed steelhead trout embryos in situ to evaluate the impacts of acid rock drainage. In addition, the province of British Columbia has published a manual of field methods, including a brief description of procedures for in situ tests with salmonid embryos (BCWLAP 2005). In general, the use of early life stage toxicity tests is appropriate because of their demonstrated sensitivity, sublethal endpoints, and extended exposure period that encompasses a number of developmental stages (Rand & Petrocelli 1985; Environment Canada 1998; ASTM 2002). Other considerations in evaluating the practical application of this approach for use in future EEM programs included ease of deployment and monitoring, control performance and statistical sensitivity (Dubé et al. 2002).

METHODS AND MATERIALS

Study site

The mine discharges treated effluent into Myra Creek, upstream of Buttle Lake, within Strathcona Provincial Park, on Vancouver Island. The discharge point is within a 5-km stretch of the creek that supports a limited population of cutthroat trout, but is bounded by impassable waterfalls both upstream and downstream. Creek flows range between 2 and 20 m$^3$/s, depending on the season, but average 6 m$^3$/s. Treated effluent from the mine is discharged into the creek approximately 2.5 km upstream of Buttle Lake, at an average of 0.44 m$^3$/s throughout the year. In addition to the discharge point, other suspected sources of metals include groundwater seeps located along the base of the tailings dam. The study site is illustrated in Figure 1.

The primary exposure sites consisted of three locations related to the discharge (Figure 1): a reference site located approximately 2 km upstream of the discharge point, a site located approximately 50 meters downstream of the discharge point, and a far-field site located approximately 1 km downstream of the discharge point outside of the mixing zone. Additional exposure sites were located at approximately 50-m intervals along the base of the tailings dam to evaluate impacts associated with potential seepage along the dam face.

Test organisms and deployment

Eyed cutthroat trout Oncorhynchus clarki embryos (approximately 266 degree-days post-fertilization) were obtained from the Vancouver Island Provincial Trout Hatchery (Duncan, British Columbia) on April 15, 2005. The embryos were transported in plastic bags under oxygen in coolers to the study site. At the site, the embryos were randomly distributed to Whitlock-Vibert hatchboxes (Federation of Fly Fishers, Livingston, Montana, USA), such that each box contained 25 embryos (see Figure 2). The boxes were previously modified by applying 3-mm plastic mesh over the larger openings that would otherwise have allowed the fry to escape at swim-up. Each box was then placed in a barbeque rotisserie basket, which was subsequently filled with 1.5” washed river gravel to diffuse current flow and protect the embryos from sunlight.

The baskets were placed in the creek over a 2-day period between April 15 and 16 in depressions manually excavated in the stream bed to a depth of approximately 15 cm, and covered with the excavated gravels (Figure 2). In cases

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where the substrate was too large to move, baskets were placed on the creek bed and covered with large rocks to protect them from the current and sunlight. To ensure that the baskets placed in deeper channel sections were not lost during high-flow events, they were attached to the bank with small diameter rope. Four replicate baskets were placed at each of the primary study sites; most of the sites along the tailings dam were represented by one replicate,
but multiple replicates were placed in several locations to provide a measure of variability.

One hundred embryos from the same batch were also incubated at an off-site laboratory to assess hatching success and survival under laboratory conditions. This provided a measure of general health of embryos used for the study, as well as an indication of any adverse effects associated with transportation to the site. These eggs were reared in bottled spring water in an incubator with controlled temperature adjusted weekly to match the actual temperature at the field site. By tracking the field temperature in the laboratory, we were able to identify the timing of developmental events, which helped to determine inspection periods in the field. At the end of the exposure period, survival among these controls averaged 77 ± 8%, indicating that the overall quality of the embryos used to initiate the study was good.

**Monitoring**

The embryos were reared in Myra Creek for a mean of 585 degree-days from fertilization, with a standard deviation of 21 degree-days across the sites. The primary study sites were monitored three times a week for temperature, conductivity and pH. Dissolved oxygen was monitored at the time of set-up, and twice more over the exposure period.

The hatchboxes were inspected on May 2, 9 and 26, and June 10. During these inspections, each box was opened and the contents (i.e., eggs or alevins) were visually inspected, counted, and any dead eggs or alevins were removed. The gap between inspections on May 9 and May 26 was due to high water levels in the creek making the boxes inaccessible.

Samples were also collected for the assessment of water chemistry; samples were collected every 50 m on the tailings dam side of the creek on April 15, May 12 and June 10, 2005 as part of routine operations monitoring, and analyzed on-site for zinc using a PerkinElmer AAAnalyst 200 Atomic Absorption Spectrometer (AA). These samples were also analyzed for pH, conductivity, and temperature using an Oakton Waterproof pH/CON 10 portable meter. In addition, water samples were collected periodically, and submitted to ALS Environmental Laboratories (Vancouver, BC) for metals analyses. Finally, daily 24-hr composite water samples were collected with Sigma Streamline Portable Auto-Samplers located at the far-field site and from the effluent just prior to discharge into Myra Creek. These composites were analyzed in-house for zinc using AA.

**Study termination**

The fish were left in hatchboxes in the creek until just prior to swim-up. Calculation of degree-days indicated that fish at the effluent site were developing more quickly because the water temperature averaged 1 degree warmer than the other sites. Consequently, these boxes were terminated on May 31, 10 days ahead of the other sites, which were terminated on June 10.

At study termination, the hatchboxes were removed from the creek and transferred into small plastic containers filled with creek water. These containers were then transported to an on-site laboratory, where live fish were separated from any dead fish. The dead fish were counted and discarded because they were too decomposed to measure and weigh, and the live fish were sacrificed using a 5 mg/L solution of MS222 (tricaine methanesulfonate). In some cases, there were discrepancies between the number of live fish observed, and the number of dead fish enumerated. Consequently, when calculating percent survival, missing fish were presumed to have died and decomposed between monitoring events. This approach is conservative with respect to identifying possible adverse effects, and is consistent with standard guidance for conducting toxicity tests using early life stages with salmonids (Environment Canada 1998; USEPA 2002). Thus, the survival endpoint is based on the number of live fish remaining, regardless of the number of dead fish that were actually observed.

The surviving fish were measured, weighed, and assessed visually for external deformities. Standard lengths were determined to the nearest 0.5 mm by placing the fish onto a laminated sheet of graph paper with 1-mm grids. The fish were also photographed on this paper in order to retain records of observations. Once measured, the surviving fish from each box were placed in an aluminum weigh boat, and wet weight determined to the nearest 0.001 g with a Mettler AE 260 DeltaRange balance. Average wet weight per fish was then calculated for each replicate.
Data analyses

Effluent discharge

Evaluation of potential impacts associated with the effluent discharge focused on identifying differences between the upstream reference site, and the sites located near the discharge point and downstream of the mixing zone (i.e., far field). Specifically, the analysis focused on lethal (cumulative survival) and sub-lethal (i.e., deformities, length and weight) endpoints. Kolmogorov–Smirnov and Levene’s tests were applied to the data to confirm normality and homogeneity of variance, respectively, prior to undertaking analysis of differences between sites. The assumptions of normality and homogeneity of variance were confirmed for all endpoints ($p = 0.01$); therefore, Dunnett’s test (one-tail) within the analysis of variance (ANOVA) was applied to identify differences between reference and discharge and far-field sites (Zar 1984). Differences among sites were considered significant at $p = 0.05$.

It should be noted that the far-field site originally had four replicates, but three of these were lost during the last week of the study when they were stranded during an unexpectedly rapid drop in flows. Consequently, the one remaining replicate was grouped with two hatchboxes located along the tailings dam, downstream of the discharge point and associated mixing zone (sulfate and conductivity measurements along both sides of the creek indicated that the treated effluent was fully mixed within 300 m downstream of the discharge point). Thus, these boxes were considered representative of fully-mixed conditions and, therefore, appropriate to use to characterize far-field conditions relative to the discharge. Specifically, the far-field replicates used in the data analysis were located approximately 600 m, 800 m and 1 km downstream of the treated effluent discharge point.

Tailings dam seeps

The analysis of the stations located along the base of the tailings dam was complicated by the fact that use of single replicates limited measurements of ‘within-site’ variability and precluded making quantitative statistical comparisons among sites. Consequently, responses from these sites were evaluated qualitatively by comparing the results for each site with the results obtained for the pooled reference and far-field sites. The basis for this approach was that the reference and far-field sites were not statistically different for any of the endpoints measured, and their locations bracketed the stations located along the base of the tailing dam. Thus, it is reasonable to assume that the pooled reference and far-field sites provided a robust estimate of the expected performance (e.g., growth and survival) of cutthroat trout embryos reared in the creek. On this basis, results obtained for each of the stations located along the tailings dam were compared with the range of values (i.e., ± two standard deviations) associated with each of the endpoints derived from the pooled reference and far-field data. Any value that fell outside these limits was considered potentially indicative of an impact because the probability of such an occurrence was $<0.05$.

RESULTS AND DISCUSSION

Effluent discharge

Comparisons of the different endpoints associated with the upstream reference site, the discharge site, and the far-field site are presented in Table 1. Cumulative survival of trout embryos to swim-up ranged between 82.0 and 89.3%, with no significant differences observed among sites. Normal development ranged between 78.3 and 92.7%, with the discharge site being significantly lower than the reference site, but no difference between the reference and far-field sites. Length averaged 22.3, 21.1 and 22.7 mm at the reference, discharge, and far-field sites, respectively, with no adverse effects

| Table 1 | Comparison of survival, normal development and growth at reference, effluent and far-field sites |
|-------------|---------------------------------|-----------------|---------------|
| Reference   | Effluent | Far-field |
| Survival (%) (mean ± SD) | 82.0 ± 8.1 | 89.0 ± 11.0 | 89.3 ± 8.3 |
| % Normal (mean ± SD) | 90.9 ± 4.1 | 78.3 ± 9.6 | 92.7 ± 2.0 |
| Length (mm) (mean ± SD) | 22.3 ± 0.91 | 21.1 ± 1.04 | 22.7 ± 1.04 |
| Weight (mg) (mean ± SD) | 120 ± 0.5 | 114 ± 5.5 | 129 ± 3.5 |
| No. replicates | 4 | 4 | 3 |

*aSignificantly less than at reference site.*
apparent compared with the reference site. Average wet weight ranged between 114.0 and 129.3 mg per fish across sites; none of the weights at the sites were significantly less than the reference site. Overall, these data suggest that sub-lethal effects (specifically, small decreases in normal development) were apparent just downstream of the discharge point, but were limited to the mixing zone, since there was no evidence of adverse impacts in the far-field replicates.

**Tailings dam seeps**

Four seeps were identified along the tailings dam, on the basis of the proximate in situ exposures, and the supporting water chemistry. The locations of three seeps were suspected previously on the basis of local water chemistry, and one was identified as a result of conducting this study. Survival, growth, and abnormalities at these sites are provided in Table 2, compared with the pooled data from the reference and far-field sites. SEEP 1 exhibited 6% survival, while SEEPs 2, 3 and 4 exhibited survivals of 76, 100 and 0%, respectively, compared with the pooled mean survival (i.e., 85.1%) at the reference and far-field sites. Thus, survival was not adversely effected at SEEPs 2 and 3, but survival at SEEPs 1 and 4 was well below two standard deviations of the pooled mean.

Analyses of possible sub-lethal effects were limited to SEEPs 2 and 3 due to the high level of mortality at SEEPs 1 and 4. The percentages of normally developed fry were 93 and 92%, respectively, at SEEPs 2 and 3, which were comparable with the pooled mean of 91.6% for the reference and far-field sites. Thus, these data indicate that there were no adverse effects on normal development at these sites. The pooled mean length associated with the reference and far-field sites was 22.5 mm, compared to average lengths of 21.2 mm at SEEP 2 and 22.4 mm at SEEP 3. Both of these values were within two standard deviations of the pooled mean, suggesting that there were no adverse effects on length at these two sites.

The pooled mean wet weight for the reference and far-field sites was 124 mg/fish, compared with SEEPs 2 and 3, which exhibited average weights of 105 and 127 mg, respectively (Table 2). The average weight at SEEP 2 was well below two standard deviations of the pooled mean, whereas the average weight at SEEP 3 was similar to the pooled mean. Thus, the weight data suggest that an adverse effect on growth occurred at SEEP 2, compared with the pooled reference and far-field sites. Overall, the data suggest that measurable adverse effects were present at three of the four SEEP sites.

**Potential causes of impacts of seeps from the tailings dam**

Although this study was not designed to identify cause and/or link cause and effect, the analytical results were evaluated to determine the extent to which elevated metals concentrations might be associated with toxicity. Based on measured concentrations, only two metals exceeded the BC freshwater guidelines for aquatic life: zinc and copper (BC Ministry of Environment 1987, 1999). Concentrations of both metals tended to exhibit similar patterns; i.e., the highest concentrations were associated with SEEPs 1 and 4, which were consistent with the observed patterns in mortality. Maximum concentrations of zinc observed at SEEPs 1 and 4 during seepage events reached 1.4 and 1.9 mg/L, respectively, thus exceeding the BC provincial guideline for maximum exposure (i.e., 0.033 mg/L) by at least 40-fold. Similarly, concentrations of copper ranged up to 0.046 and 0.030 mg/L at SEEPs 1 and 4, respectively, exceeding the BC provincial maximum exposure guideline of 0.005 mg/L by six- to nearly 10-fold. Concentrations of both metals were also substantially higher than background levels of 7.1 and 0.5 μg/L for zinc and copper, respectively.

In direct comparisons with actual toxicity data, zinc concentrations observed at SEEPs 1 and 4 exceeded acute LC50 s for juvenile rainbow trout exposed to zinc in water with similar hardness: e.g., 0.126 mg/L (Bailey et al. 1999); 0.170 mg/L (Bradley & Sprague 1983); and 0.370 mg/L

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Table 2 | Comparison of biological endpoints at the seep sites; values at seep sites are compared to mean ± 2SD of pooled reference and far-field sites

<table>
<thead>
<tr>
<th>Ref - Far-field</th>
<th>SEEP 1</th>
<th>SEEP 2</th>
<th>SEEP 3</th>
<th>SEEP 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Survival (%)</td>
<td>85.1 ± 16.4</td>
<td>6.0</td>
<td>75.7</td>
<td>100.0</td>
</tr>
<tr>
<td>% Normal</td>
<td>91.6 ± 6.4</td>
<td>–</td>
<td>93.3</td>
<td>92.0</td>
</tr>
<tr>
<td>Length (mm)</td>
<td>22.5 ± 1.9</td>
<td>–</td>
<td>21.2</td>
<td>22.4</td>
</tr>
<tr>
<td>Weight (mg)</td>
<td>124 ± 10.3</td>
<td>–</td>
<td>105*</td>
<td>127</td>
</tr>
</tbody>
</table>

*a* less than 2SD of mean of pooled reference and far-field sites.
(Holcombe & Andrew (1978). Similarly, copper concentrations were within the range reported to be acutely toxic to rainbow trout fry (USEPA 2007). Collectively, these data suggest that zinc and copper were potentially responsible for the toxicity observed at SEEPs 1 and 4. However, given that average concentrations of zinc and copper in the treated effluent were 0.244 and 0.035 mg/L, respectively, during the study period, and no adverse effects on survival were observed downstream of the discharge point, elevated concentrations of zinc were the most likely cause of mortalities at the seep sites. Interestingly, while intermittent elevations in conductivity suggested periodic seepage in the vicinity of SEEP 3, the overall lack of biological effects and generally low metal concentrations indicated that metals were not being mobilized at this site (Chalmers 2006).

The potential impacts of treated mine effluent and seep discharges on Myra Creek

Based on the results of the in situ exposures obtained during this study, the observed adverse effects were limited to the immediate vicinity of the effluent discharge and isolated seeps along the length of the tailings dam. Conversely, embryos exposed downstream of the mixing zone (i.e., far-field) did not exhibit any adverse effects on survival, growth, or normal development. Adverse effects noted at specific SEEP sites included both lethal and sub-lethal responses, but these effects were not observed at adjacent exposure points along the tailings dam, implying that the seeps and associated effects were highly localized. Notably, the mine has subsequently undertaken a significant effort to eliminate unintended seeps that percolate through waste rock and discharge through the banks of the engineered channel. With the construction of a perimeter drainage system, this run-off is now captured and treated prior to discharge.

Application to EEM programs

This study demonstrated that the in situ approach used is able to detect both lethal and sub-lethal effects in quantifiable terms. Moreover, the approach provided a known exposure to suspected metal sources and separation between exposure and reference groups, thus eliminating one of the major difficulties with fish surveys in waterways that do not have physical barriers between reference and exposure populations. Notably, if a survey cannot distinguish between these populations, it becomes problematic to clearly identify the presence or degree of effect related to an effluent or other impacts. In addition, this approach avoids potentially significant impacts associated with destructive sampling of large numbers of fish.

Test sensitivity (i.e., the ability to detect adverse effects) is frequently of concern in field studies due to high variability, but was not an issue in this study. Minimum significant differences calculated for each endpoint based on the critical value of Dunnett’s t-test, were less than 20% of the corresponding reference mean for all parameters. This level of sensitivity is comparable to that achieved in toxicity tests conducted under controlled laboratory conditions otherwise applied in EEM studies and similar effluent monitoring programs (Bailey & Young 1997; USEPA 2002).

Current EEM guidance also allows for the use of mesocosms in place of the fish survey (Environment Canada 2001). This method involves setting up large tanks containing various concentrations of effluent mixed with water from the receiving environment on a continuous-flow basis. However, this latter approach is equipment-intensive, can be quite costly, and is ultimately little more than a large-scale laboratory test that does not take into account all of the variability and interactions associated with discharge operations and the receiving environment (Courtenay et al. 2002). Conversely, the in situ hatchbox method requires comparatively little equipment and can also be used with other species of salmonids; with appropriately modified test chambers, other organisms can also be used. Depending on the species and spawning period, this approach also allows for monitoring over different seasons. In addition, there are strains of rainbow trout that are available as embryos throughout the year and, with minimal effort, the embryos can be produced as sterile triploids. This offers the advantages of a widely available test organism that is not capable of reproducing in the receiving environment in the event of escape.

There are challenges associated with using the in situ hatchbox approach. Loss of replicates due to high or low flows is the single largest concern, but this risk can be reduced dramatically by the careful selection of exposure
sites. Replicates placed within stream gravels are surprisingly resistant to high-flow events, but closely monitoring the replicates (and moving them if necessary) may be necessary during periods of declining flow to prevent stranding. Addition of back-up sites at different locations also serves to reduce risk. In this study, provisions to move the replicates were in place at each site in the event of high or low flows; however, the rate at which the flows decreased was not anticipated and the replicates were not observed closely enough at the critical time to relocate them before stranding.

It was initially anticipated that high freshet flows might present a problem, but this did not turn out to be the case, except for replicates placed in the engineered channel where the bottom composition prevented them from being situated within the gravels. During freshet, the protective rocks surrounding several of the hatchboxes were stripped away, leaving the boxes exposed to direct sunlight and scouring. Ultimately, these replicates were excluded from the analyses because the effect of sunlight and variable flows could not be adequately quantified. Regardless, should this technique be employed at sites with substrate that prevents burial, a more effective system for securing and protecting the hatchboxes needs to be in place.

Another potential confounding factor for the method is predation; in this study, a predatory insect larva was discovered in one of the hatchboxes, along with alevins that appeared to have been partially consumed and other alevins that were missing entirely. Since it was impossible to determine the exact impact of predation on this particular replicate, it was necessary to exclude it from the analysis. However, this situation was observed in only one of the replicates tested, suggesting that it was a relatively rare occurrence, and use of multiple replicates at a given site reduces the overall impact of such events.

While the in situ hatchbox approach has its challenges, they are readily addressed and offset by the advantages. The method requires relatively little equipment to set up, and can be employed with relevance in most habitats that support salmonid populations. Cutthroat trout were used at this site, but it would be straightforward to substitute another species of salmonid based on seasonal and local relevance. Tissue metal concentrations could also be measured at the end of the exposure period to aid in relating exposure to effects. Another advantage is that it is relatively easy to monitor the study while in progress, requiring only weekly or bi-weekly checks, depending on anticipated changes in flow levels and temperatures. In addition, no feeding is required throughout the exposure period. Temperature, as well as other parameters, can also be monitored with data loggers in the hatchboxes to provide a more accurate assessment of water quality conditions during the exposure. Indeed, subsequent deployments at this and other sites have incorporated the suggestions noted above, and have further demonstrated this method to be a robust and informative approach for monitoring water quality.

In summary, the extended duration of the exposure period, the ability to integrate ‘real-world’ variations in both discharge and environmental conditions, and clear delineation of reference and exposure groups collectively provide a powerful tool to increase our understanding of the impacts of various discharges on aquatic systems. Using this approach, management decisions can be based on empirically derived evidence of actual impacts in the receiving environment of concern. Moreover, follow-up exposures can readily be used to assess whether process or infrastructure changes have achieved the desired improvements in the receiving environment.

ACKNOWLEDGEMENTS

Initial deployment of the hatchboxes was accomplished with the capable assistance of Andy Diewald and Armando Tang.

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First received 22 August 2012; accepted in revised form 10 September 2013. Available online 14 October 2013