



Toxicological Reconnaissance of Arsenal® Use along the Rio Grande River through Big Bend National Park

Final Report

Natural Resource Report NPS/BIBE/NRR—2024/2640



ON THE COVER

Study site Treatment 2 at Big Bend National Park. Photo Rio Bravo Restoration.

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April 2024

U.S. Department of the Interior
National Park Service
Natural Resource Stewardship and Science
Fort Collins, Colorado

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Please cite this publication as:

Walker, D. and J. Renfrow. 2024. Toxicological reconnaissance of Arsenal® use along the Rio Grande River through Big Bend National Park: Final report. Natural Resource Report NPS/BIBE/NRR—2024/2640. National Park Service, Fort Collins, Colorado.
<https://doi.org/10.36967/2302769>

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Executive Summary

This study examined the biological response of aquatic organisms, collected in situ and from laboratory toxicity testing of water and soil to the herbicide Arsenal® primarily used to decrease the invasive species *Arundo donax* in the Rio Grande River through Big Bend National Park in Texas (USA). Biological, toxicological, physicochemical, and flow data were collected before, during, and after an Arsenal® treatment from 4 reference and 3 treatment sites. Significant differences existed in ecoregion 24 B-IBI scoring, and in toxicity testing of water and sediment, between treatment and reference sites. Greater toxicity, and a higher inverse relationship to B-IBI scoring, was observed in sediment samples compared to water samples. This could be due to the surfactant nonylphenol ethoxylate (NPE), added to the Arsenal® formulation rather than the active ingredient imazapyr.

Care must be implemented with the results of this research due to many confounding variables that occurred during the study period including flash flooding. Although flashy hydrology is relatively common to the region, it can influence aquatic macroinvertebrate assemblage structure, function, and species richness measures with these dropping due to scour and downstream displacement within a reach during, and for some period following, flooding. Therefore, flash flooding would be expected to have some effect on aquatic macroinvertebrate assemblages and subsequent B-IBI scoring.

Additionally, flooding will cause some displacement, and deposition, of sediment throughout the study area. This flooding could impact the sediment toxicity results as sediment-bound contaminants could be spread some distance from their origin.

Arsenal® treatment occurred during this study near sites Treatment 1 and Treatment 2 on 5/29 and 5/31 2022 respectively. This represented an extreme condition as far as concentration of potential toxicants in the water are concerned and these relatively high concentrations would be expected to have decreased within a relatively short period of time, especially in the water.

It was outside of the scope and budget of this project to obtain concentrations of imazapyr or surfactants from sediment or water. Therefore, exact doses of imazapyr or surfactant for response curves could not be obtained.

No sample site exists in isolation rather, influences from one reach can significantly impact downstream areas. This is especially true for aquatic ecosystems in arid regions that often exhibit flashy hydrology. However, we believe the findings from this study provide indication of potential toxicity of some magnitude as well as provide direction for management. Decisions of whether the toxic effects of herbicide use to aquatic macroinvertebrates outweigh the beneficial effects of *Arundo donax* control should be carefully considered.

Introduction

To restore aspects of hydroecological functioning of the Rio Grande River through Big Bend National Park (BIBE), the non-selective, systemic herbicide imazapyr is often used to treat invasive plant species, particularly giant cane (*Arundo donax*). The objective of this project was to examine the potential effects of Arsenal® on aquatic macroinvertebrate species from the Rio Grande near and through the Big Bend National Park.

Aquatic macroinvertebrates (AMs) are often used to calculate state-specific benthic indices of biotic integrity (B-IBI) and have been frequently used as bioindicators of water quality and stream structure and function for decades (US EPA, 2022). They are less mobile than fish and have a longer generational time and permanence than algae or periphyton. Also, diversity of AMs, especially in terms of pollution-tolerance and functional feeding groups (how they acquire food) make them ideal bioindicators of stream ecosystem condition. The use of AMs as bioindicators of ecosystem health is not without issue, however. Although they show greater permanence than algae, AMs are readily affected by changes in stream discharge and assemblage dynamics can change within a few hours following a flash flood or otherwise heavy flow rate. Temporal and spatial variability of AMs, while less than periphytic assemblages, can often be high (Robinson, 2011). Often, long-term databases of AM assemblage structure and function are needed to determine biotic integrity of an aquatic ecosystem. Additionally, pollution tolerance values of individual species of AMs are derived mostly for levels of dissolved oxygen (Hilsenhoff, 1988) and not for organic pollutants many of which may be toxic yet have little to no effect on dissolved oxygen levels.

Due to inherent spatial and temporal variability of AMs, it is often best to combine controlled and replicated laboratory analyses with field sampling. Laboratory experimentation foregoes some environmental relevance for quantitative accuracy while field sampling alone provides a greater degree of relevance at the cost of quantitative accuracy. Combining analyses between field and controlled laboratory work provides the best representation of aquatic ecosystem structure and function. This is especially true for determining potential toxicity of contaminants that may not affect levels of dissolved oxygen as do more conventional pollutants.

There is an absence of research examining the totality of potential effects that Arsenal® could have on aquatic ecosystems. This is especially true in terms of the effects, additive or synergistic, that nonylphenol ethoxylate (NPE), the surfactant commonly used with Arsenal®, exert when combined with imazapyr. The environmental relevance of mostly lab-based studies of imazapyr alone is low. More studies are needed examining the totality of effects Arsenal® may have on aquatic ecosystems using a combination of in situ and laboratory studies. This was the design approach chosen for this study examining the potential effects of Arsenal® on AM species from the Rio Grande near and through the Big Bend National Park.

Imazapyr is highly mobile in water with a very short half life in water (3-4 hours) with slightly longer half lives in soil of 2-5 days (Dugdale et al., 2020). It is considered low to practically non-toxic to birds (Tu et al., 2001), mammals (Hess et al., 2010), fish (US EPA, 2005a), and amphibians (Yahnke et al., 2013).

Very few studies have been performed examining toxicity of imazapyr to aquatic invertebrates. One uncited study was mentioned in a publication submitted to the state of Washington by AMEC consultants (AMEC, 2009) where *Daphnia* (species not mentioned) were exposed to Arsenal® containing an “unspecified surfactant”. This uncited study mentioned in AMEC (2009) found a 48-hour LD50 of 350 mg of Arsenal®/L. Although the surfactant was never identified, very few, if any, studies have been performed examining the combined toxicity of imazapyr plus the surfactant. Kintner and Forbis (1983), in a study using imazapyr without surfactant, found an LC50 (following 24 and 48 hours) of > 100 mg/L (the highest dose analyzed).

The only study that examined the effect(s) of Arsenal® on in situ aquatic macroinvertebrates was performed by Fowlkes et al. (2003) for cypress wetlands in Florida. The authors used in situ microcosms consisting of PVC pipes embedded in wetland soils and treated or not treated with imazapyr. Aquatic invertebrates (mostly pollution-tolerant species of chironomids) were collected and assessed for species abundance, richness, diversity, and deformity between treatment and control following a 14-day exposure period. The authors found no significant statistical difference due to treatment effects.

The surfactant used with Arsenal®, nonylphenol ethoxylate, and its primary degradation compound 4-nonylphenol (4NP), are known to cause significant endocrine disruption in vertebrate species (Gaido et al., 1997; Soto et al., 1991). The degradant, 4NP, is believed to be more toxic than the parent compound (US EPA, 2005). Besides causing endocrine disruption, NPE and 4NP are believed to interfere with cellular signaling in many organisms causing genotoxicity (Soares et al., 2008). Additionally, both NP and 4NP moderately bioaccumulate and biomagnify (Chen et al., 2010).

NP and 4NP are both hydrophobic and partition strongly to sediment (Chen et al., 2010). They have half lives in soil and sediment of years to decades; much longer than imazapyr (Soares et al., 2008).

For an in-depth literature review detailing the chemical properties and toxicological effects of imazapyr and the surfactant NPE and 4NP, please see Appendix B.

The use of Arsenal® along the Rio Grande River through Big Bend National Park is necessary to restore some semblance of hydroecological functioning due to the invasion and spread of *Arundo donax*. This study, using a field and laboratory approach, attempts to understand the potential implications of using Arsenal® on AMs along the Rio Grande through BIBE.

Park Resource Management Issues

Briggs et al. (2021, p. 1471) characterized giant cane (*Arundo donax*) as “a perennial, clump-forming hydrophyte that has transformed the structure and function of many subtropical and semiarid riparian zones worldwide.” Since the early 2000’s the rapid spread of giant cane in the Rio Grande, and throughout BIBE, has caused significant channel narrowing and hydrologic alteration that puts native species at risk. Giant cane also increases the risk of flooding to nearby communities as well as increased fire frequency and magnitude (Briggs et al., 2021). These ecosystem alterations and invasion of giant cane are exacerbated, and perhaps caused by, an unnatural hydrologic regime due to increases in upstream water withdraws for human needs.

Since 2005, attempts at decreasing giant cane through BIBE has consisted of reducing above ground biomass via burning followed 4-6 weeks with the herbicide Arsenal® (Briggs et al., 2021). Spot treatments with Arsenal® occur 1-2 years following primary treatment. This methodology has shown promise in decreasing giant cane biomass (Briggs et al., 2021) however, although herbicide is never applied directly to the water, overspray drift does occur (pers. comm. Jeff Renfrow). The effects of Arsenal® on aquatic life to this point has not been quantified. This study will provide insight into the effects of giant cane control using Arsenal® on aquatic macroinvertebrate species in the Rio Grande through BIBE.

Surveys were conducted under permit number BIBE-2022-SCI-0018.

Methods

Sample Design

All field work and sample collection occurred during May and June of 2022. Biological, physicochemical, toxicological, and flow data was obtained from all sites approximately one week before, and one month following Arsenal® treatment. Four reference and three treatment sites were sampled (Table 1, Figures 1 and 2). Sites were chosen based on the contribution of spring flow above a west-east gaining reach throughout the study area with sites farthest to the west having less spring flow contribution than those to the east. The Santa Elena (SE-Out) site is situated above this gaining reach. Arsenal® treatment history varied throughout the study area. No treatment has ever occurred at the Santa Elena (SE-Out) site. These sites, and two others (Table 1), were included as reference sites. The site labelled as “downstream treatment” (DS-Treatment) was just downstream of the treatment reach but was contaminated by Arsenal® treatment immediately upstream, therefore, this site was included as a treatment site for analysis. Arsenal® treatment only occurred during this study period near sites Treatment 1 and Treatment 2 on 5/29 and 5/31 2022 respectively. The only sites within the reference category that have ever been treated were La Clocha (April 2020) and Hot Springs (April 2019).

Table 1. Reference and treatment site naming from east to west.

Site Name	Abbreviation	Treatment/Reference	Northing/Easting ¹
Santa Elena Take Out	SE-Out	Reference	3225906/636291
La Clocha	La Clocha	Reference	3226061/693803
Hot Springs	Hot Springs	Reference	3229625/695093
Upstream Treatment	US-Treatment	Reference	3230398/696905
Treatment 1	Treatment 1	Treatment	3229947/697681
Treatment 2	Treatment 2	Treatment	3229142/699022
Downstream Treatment	DS-Treatment	Treatment	3229402/699262 ²
Downstream Treatment	DS-Treatment	Treatment	3230398/696905

¹ Datum = NAD83, Zone 13 North

² The first coordinate was used during the initial sampling for this site on 5.23.22. Due to low water flow on 6.12.22 and 6.29.22, this site was moved downstream to the second coordinate listed.

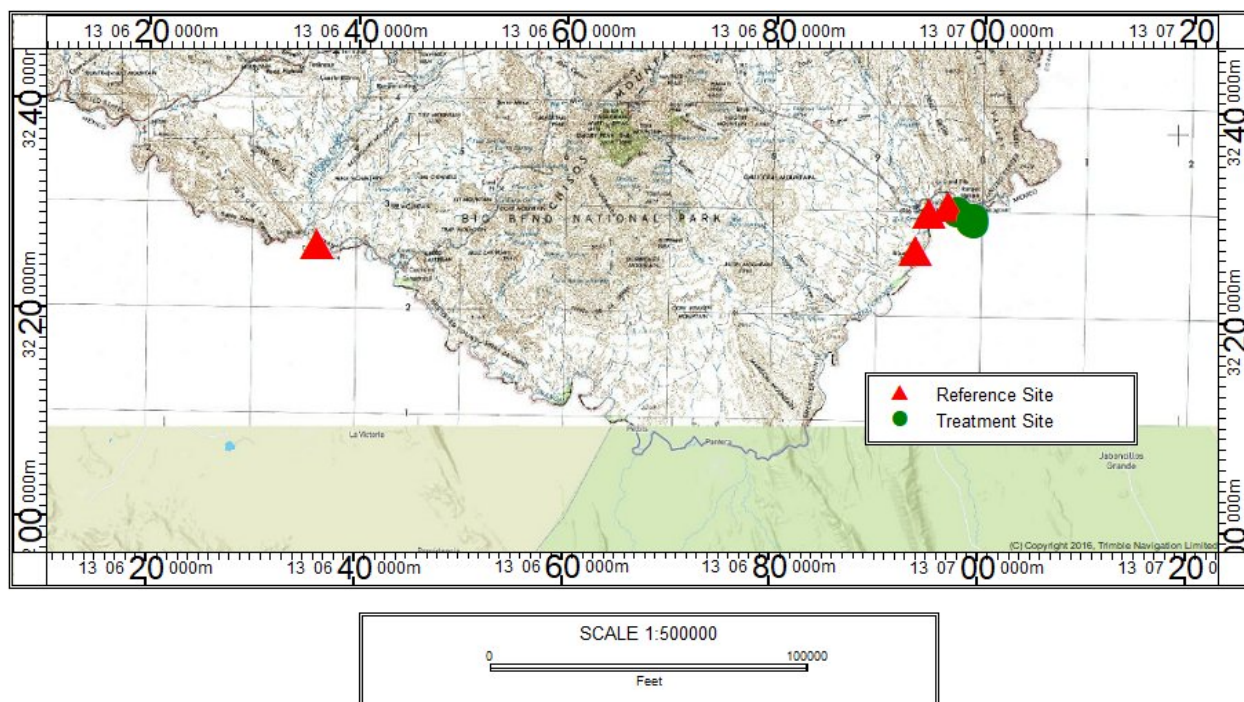


Figure 1. Overview map of sampling sites. The site furthest to the west is SE-Out.

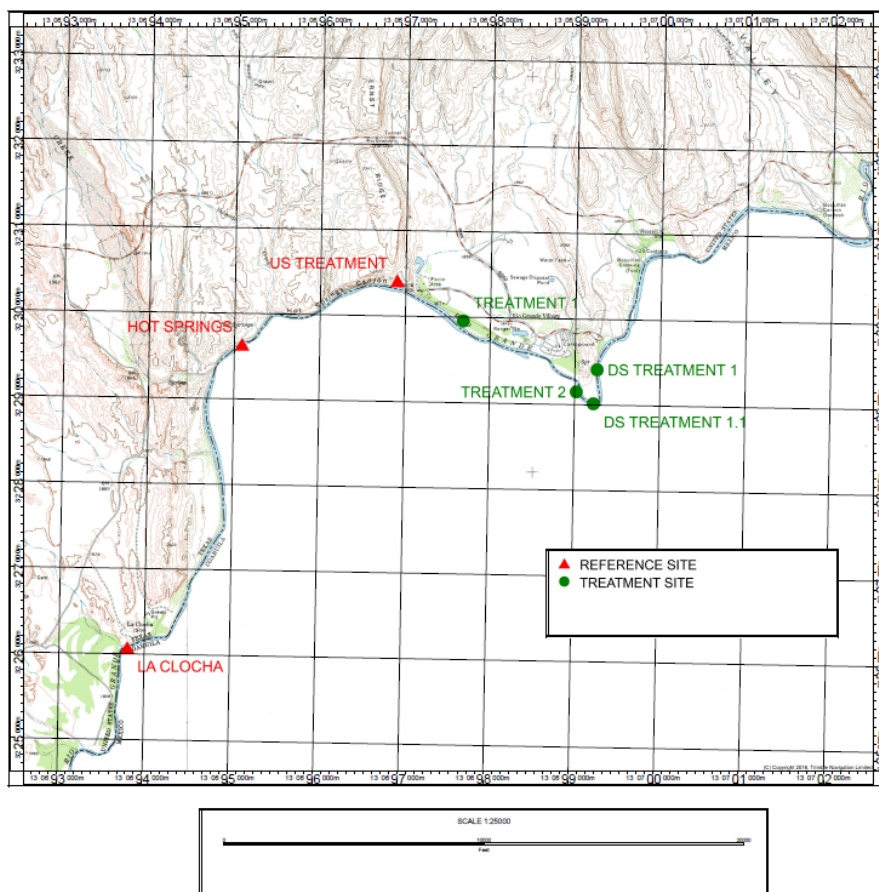


Figure 2. Map of sites downstream of SE-Out

Survey Methods

Flow

Flow was calculated for each site using a calibrated AA meter and physicochemical variables were obtained in the field using a YSI Pro Plus® data display and sonde. Specific field measurements included water temperature, pH, specific conductivity, oxidation-reduction potential, dissolved oxygen (mg/L and percent saturation), and turbidity. The SE-Out site was pooled for most of the study but did have 8 cfs of flow during the second sampling. The site DS-Treatment was moved farther downstream (~400 m) during the latter two samplings due to low streamflow at the original site (Table 1).

Biological

Biological sampling included collection, identification, enumeration, and calculation of the benthic index of biotic integrity (B-IBI) for aquatic macroinvertebrates using the Texas Commission on Environmental Quality (TCEQ) protocols for the Level 3 ecoregion 24 (TCEQ, 2014). Field collection consisted of a one five-minute sample at each site using a D-frame kick net primarily from runs, riffles, and glides. Sampling also occurred from snags and overhanging macrophytic vegetation wherever this was deemed to be a significant portion of the habitat. De-watered samples were collected in 1-liter, wide mouth Nalgene bottles filled with 91% isopropyl for storage and transport back to the laboratory for identification and enumeration.

Sorting consisted of removing large debris only after it was proven to have no aquatic macroinvertebrates clinging to it under a dissecting scope. On average, it would take one, 8-hour day to sort through an entire sample. Samples were all checked by Dr. Walker to ensure no organisms had been missed in the sorting process. Primary dichotomous keys to identify organisms to Genus included Merrit and Cummins (2019), and Pennak (1989).

Following sorting, identification, and enumeration, the ecoregion 24 B-IBI score was calculated for each site using an Excel macro provided by TCEQ (Table 2). The final B-IBI score was then used for further statistical analysis.

Table 3 summarizes sampling dates and obtained samples by site. No flow data was obtained from SE-Out on 5/22/22 (due to a pooled condition) but was obtained on 6/28/22. Treatment 1 and Treatment 2 sites were sampled during Arsenal® treatment that occurred on 5/29 and 5/31, 2022 respectively. Sampling from these sites during this period were limited to collection of soil and water for toxicological testing only and no other data was obtained.

Table 2. Example of TCEQ scoring for Ecoregion 24 (Hot Springs site). Score range: Exceptional >26; High 21-26; Intermediate 18-20; Limited <18.

Metric	Value	Score
Taxa Richness	12	2
Number of Ephemeroptera Taxa	4	3
% Trichoptera	18.97	4
% Chironomidae	6.32	3
% Diptera	6.32	3
% of Total Trichoptera as Hydropsyche	42.42	3
% Collector-Gatherers	19.35	3
Biotic Index (HBI)	3.21	3
Number of Taxa with Tolerance Value <8.5	12	4
Total Score	n/a	28*

*Exceptional

Table 3. Sampling dates by site.

Site	Samples Obtained	Dates
SE-Out	Biological/Physicochemical/Toxicological	5/22/22
SE-Out	Biological/Physicochemical/Toxicological/Flow	6/28/22
La Clocha	Biological/Physicochemical/Toxicological/Flow	5/22/22, 6/28/22
Hot Springs	Biological/Physicochemical/Toxicological/Flow	5/22/22, 6/28/22
US-Treatment	Biological/Physicochemical/Toxicological/Flow	5/23/22, 6/12/22, 6/29/22
Treatment 1	Biological/Physicochemical/Toxicological/Flow	5/23/22, 6/12/22, 6/29/22
Treatment 1	Toxicological	5/29/22
Treatment 2	Biological/Physicochemical/Toxicological/Flow	5/23/22, 6/12/22, 6/29/22
Treatment 2	Toxicological	5/31/22
DS-Treatment	Biological/Physicochemical/Toxicological/Flow	5/23/22, 6/12/22, 6/29/22

Laboratory Methods

Water samples were collected at all sites for calculation of EC50 (effect concentration for 50% of the population) values of *Daphnia magna* using testing equipment purchased from Environmental Bio-detection Products Inc. (EBPI) in Mississauga, Ontario Canada. Water samples collected during the initial sampling were placed in a cooler with wet ice and frozen within 24 hours of field collection. Subsequent samples collected were frozen before sending via overnight delivery to the lab. All samples were run in duplicate.

Serial dilutions of the field-collected water (“effluent”) from each site were performed and analyzed using 100%, 50%, 25%, 12.5%, and 6.25% of sample water. Following a 3-day incubation of *D. magna* ephippia, all neonates were pre-fed a cyanobacterium food source (*Spirulina*) 2 hours prior to placement into multiwell plates containing four wells of each of the serial dilutions including a

control row of standard freshwater consisting of de-ionized water mixed with NaHCO₃, CaCl₂, MgSO₄, and KCl salts (Appendix A). A total of five neonates were placed into each cup of the multiwell plate along with 10 mL of each serial dilution and the control row of standard freshwater.

Parafilm was placed over each multiwell plate and was then incubated at 20° C in darkness for 24 and 48 hours. At the end of each incubation period, the number of dead or immobilized *D. magna* neonates were calculated and recorded on the result sheet (Appendix A). Following 48 hours, the Hill slope equation was then used to determine the logMedian EC₅₀ for each multiwell plate (using an Excel-enabled macro provided by EBPI Inc.) (Appendix A). Average logMedian EC₅₀ values from the two multiwell plates for each site were then used for subsequent statistical analysis using JMP® Pro version 16.1.0.

Soil-sediment samples were also collected from each site to be used in laboratory toxicity testing using the ostracod *Heterocypris incongruens*. Unpreserved composite samples were collected in 500 mL glass jars from the wetted edge of each site (on average, 3-5 areas were selected for collection at each site). Samples were refrigerated at the lab.

Following a 48-hour hatching period of ostracod cysts at 25° C and 3,500 lux illumination, neonates were pre-fed the cyanobacterium *Spirulina* and allowed to feed for 4 hours before length measurements were obtained. Using the same freshwater made for *D. magna* testing, ten neonate ostracods were transferred from the hatching Petri dish into a clear, length measurement multiwell plate. A micrometer slip was placed on the stage of a dissection microscope and the multiwell placed on top of the micrometer. Lugol's preservative was used to immobilize the selected neonates and length (in µm) were used as "day zero" readings (Appendix A).

Following day zero length measurements, 2 mL of standard freshwater and 1,000 µL of sediment was added to a separate multiwell plate composed of 6 wells. Two mL of an algal suspension containing the chlorophyte *Scenedesmus* was then added to each well (Appendix A). Ten ostracod neonates were then added to each of the 6 wells from the hatching Petri dish. Each plate was covered with Parafilm® and the lid closed on top. Plates were incubated for 6 days at 25° C in darkness. Duplicates were performed for each sediment collected in the field and for control plates using reference soil provided by EBPI Inc.

Following the 6-day incubation period, all ostracods were collected from each well of the multiwell plates and transferred to the clear, multiwell plate for length measurement. The micrometer and dissection scope was again used to calculate growth between each sediment and control plate. Dead or immobilized ostracods were also determined. The end points for this test was growth inhibition and mortality compared to controls (Appendix A).

Statistical Methods

All statistical analyses were performed using JMP® Pro 16.1.0 and assumed a 95% confidence interval. Statistical significance is inferred for any *p* value < 0.05.

Sample standard deviation was calculated using the formula $\sigma_{\bar{x}} = \sigma/\sqrt{n}$ where σ = population standard deviation, n = population size, and $\bar{x}$ = each value from the population. Standard error was calculated using the formula $s/\sqrt{n}$ where s = standard deviation and n = the number of observations.

Half maximal effect concentration (EC50) values for *Daphnia magna* using collected water from the field were determined from the point of inflection using the Hill (1910) equation. The calculated inflection point is where 50% of the organisms were dead or immobilized. Following this inflection point, increases in concentration dose have less of an effect on an organism. Dose-response analyses often cover large concentration values of up to 500-fold or more. Using the logarithm of EC50 values serves two purposes; one is that it is essential to turn the Hill equation into a sigmoid logistic function suitable for dose-response analysis and the other is that comparison of very large value differences become more comprehensible. All EC50 values were reported as “logMedianEC50”. It’s important to note that higher logMedianEC50 values represent less toxicity whereas lower values represent increasing toxicity.

Principal component analysis (PCA) was performed of relevant variables upon parametric analyses. Primarily, we were interested in determining the relationship, if any, between in situ biological measures (B-IBI values) and laboratory toxicology testing of water and sediment. To achieve this, the in situ biological variables chosen were ecoregion 24 B-IBI scores, total number of aquatic macroinvertebrates, taxa richness, and pollution tolerance (HBI) values. The in situ biological variables were run separately against sediment (percent growth inhibition and percent mortality) and water (logMedian EC50 values) laboratory toxicity testing. Other variables that may be significant were included such as streamflow and dissolved oxygen concentrations. The B-IBI values were the first principal component in the model.

Results

Care should be used in the interpretation of all following results as most suffer from very low sample size and unequal distribution(s). For an overview of treatment versus reference sites, please review Table 1.

Field Survey

Discharge

Discharge was quite variable between sites, but the overall mean values were significantly greater in treatment compared to reference sites (Figure 3 and Table 4). As mentioned, the first and second samples from SE-Out were pooled during one sampling with a small amount of flow during the second. The La Clocha site contained the next lowest flow followed by the Hot Springs site. Sites Treatment 1, Treatment 2, DS-Treatment, and US-Treatment had similar flows as did the area just upstream of treatment.

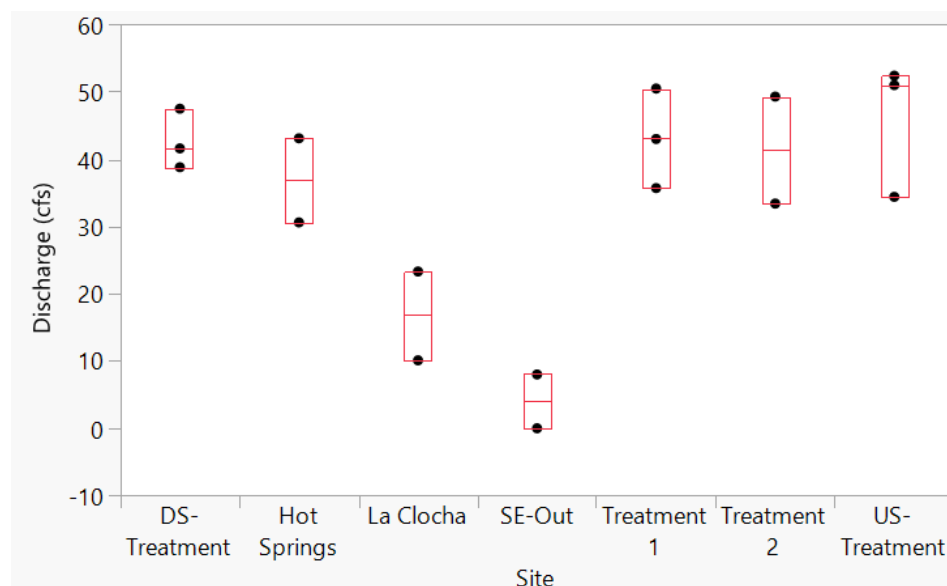


Figure 3. Discharge (cfs) by site during the study period.

Table 4. Means and standard deviations of discharge (cfs) by site during the study period.

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
Hot Springs	2	36.850	8.838	6.250	-42.563	116.263
La Clocha	2	16.700	9.333	6.600	-67.160	100.560
SE Out	2	4.000	5.656	4.000	-46.824	54.824
Treatment 1	3	43.066	7.400	4.272	24.683	61.449
Treatment 2	3	41.066	7.965	4.598	21.280	60.853
Treatment DS	3	42.633	4.441	2.564	31.601	53.665
Treatment US	3	45.933	10.012	5.7808	21.060	70.806

Typical for the region, hydrologically flashy flows did occur during the study period. Such flows did occur outside of the typical sampling regiment for each site as evidenced by the USGS streamflow gauge at Rio Grande Village in Big Bend NP (Figure 4). This gauge is located just downstream of the sampling site US-Treatment. Streamflow dynamics are important determinants when analyzing aquatic macroinvertebrate dynamics as higher discharge, especially episodic, often results in flushing many macroinvertebrates downstream. This can have a significant effect on the calculation of B-IBIs and determinations of assemblage metrics.

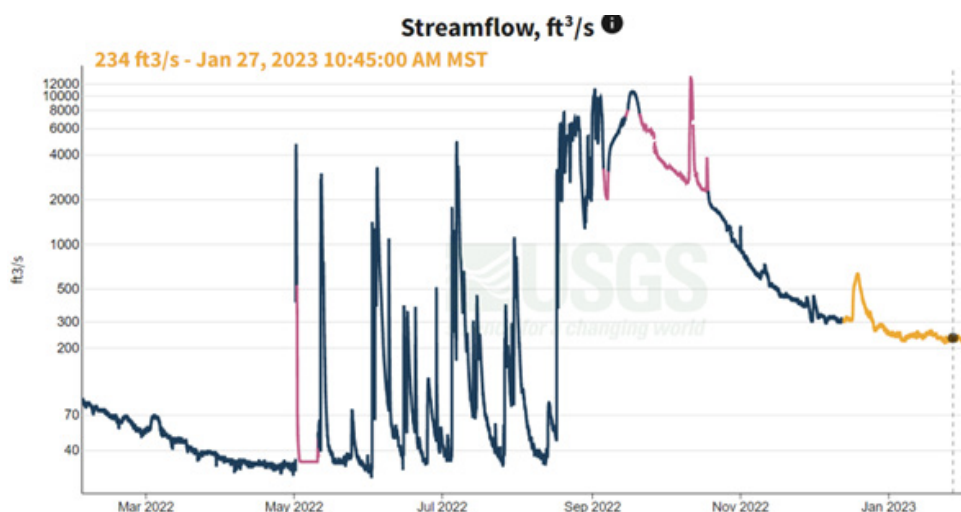


Figure 4. Streamflow as measured at the USGS gauge station near Rio Grande Village, BIBE

Physicochemical

Physicochemical variables were collected at each site on the dates shown in Table 2.

Sites within and near the treatment reach appeared to have slightly higher water temperatures than did other reference sites (Figure 5 and Table 5). SE-Out had significantly lower water temperatures than other sites during the study period. The remaining sites, however, had very warm relative water temperatures.

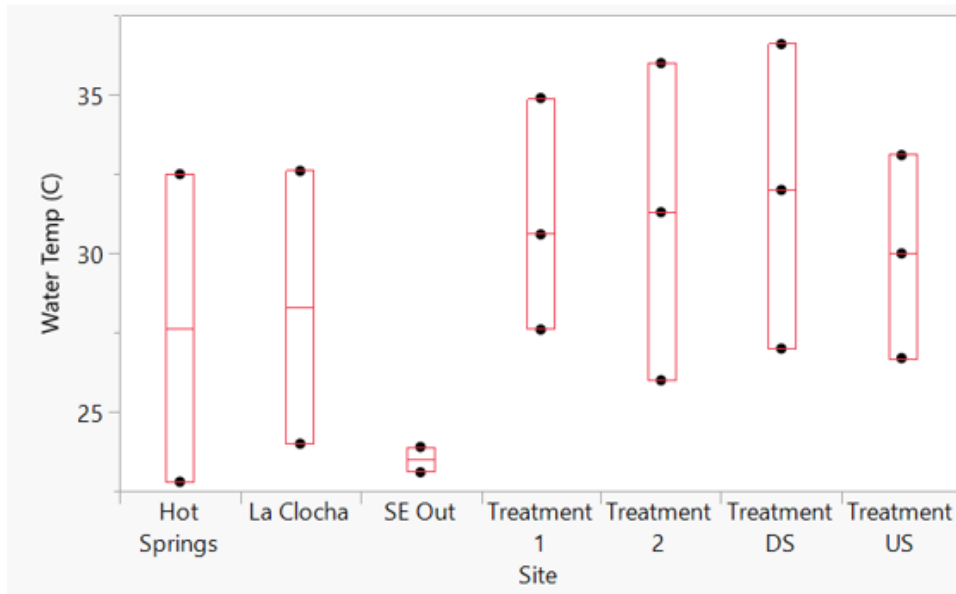


Figure 5. Water temperature (°C) by site during the study period.

Table 5. Means and standard deviations of water temperature (°C) by site during the study period.

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
DS Treatment	3	31.866	4.8013	2.7720831	19.939	43.793
Hot Springs	2	27.650	6.8589	4.850000	-33.975	89.275
La Clocha	2	28.300	6.0811	4.300000	-26.336	82.936
SE Out	2	23.500	0.5656	0.400000	18.417	28.582
Treatment 1	3	31.033	3.6692	2.118400	21.918	40.148
Treatment 2	3	31.100	5.0029	2.888400	18.671	43.528
US Treatment	3	29.933	3.2005	1.847800	21.982	37.883

Although the DO probe accurately calibrated the night before field data collection on 6/28/22, it was noticed on 6/29/22 that the probe was calibrating low and likely needed membrane replacement. DO readings for sites US-Treatment, Treatment 1, Treatment 2, and DS-Treatment were obtained from the nearby USGS gauge located at Rio Grande Village for 6/28/23. Unfortunately, this USGS gauge was deemed too far from the Hot Springs and La Clocha sites to be representative and DO concentrations for these sites on 6/28/22 were excluded from analysis (Figure 6 and Table 6).

DO is a very important variable in determining aquatic macroinvertebrate assemblage richness, diversity, and type. This is especially true for aquatic macroinvertebrates most of which cannot escape from the stream channel nor have mechanisms to incorporate atmospheric oxygen. An important metric included in all B-IBI calculations is the Hilsenhoff biotic index (HBI) (1977). This metric attempts to quantify individual species based upon empirical “pollution tolerance” values from 1-10. This quantification is determined by an individual aquatic macroinvertebrate species’

capability to survive in conditions of low DO (“pollution tolerant” with a correspondingly high HBI value) or if they have a requirement for higher DO concentrations (“pollution intolerant” with a correspondingly lower HBI value).

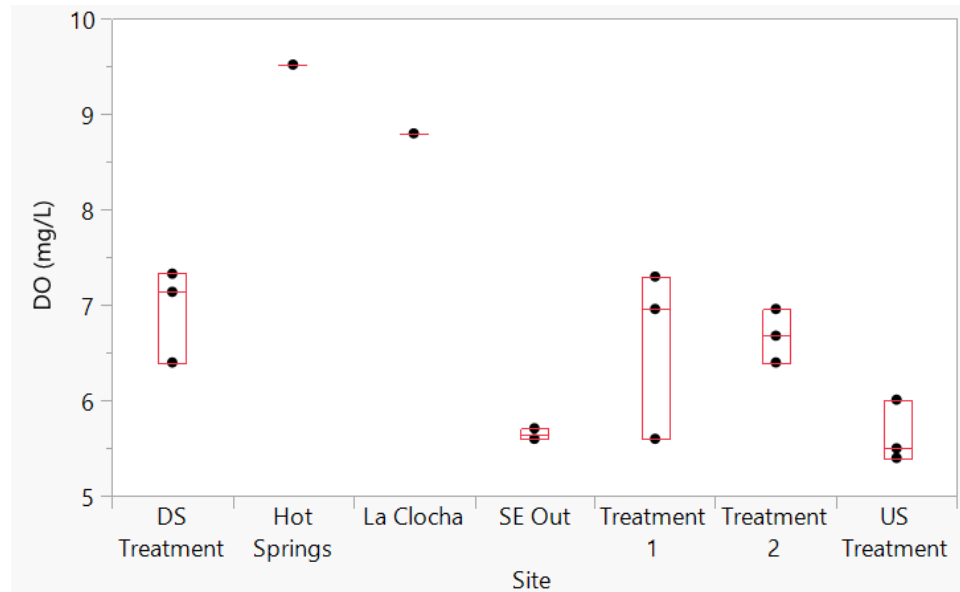


Figure 6. DO concentrations by site during the study period.

Table 6. Means and standard deviations of DO concentrations by site during the study period.

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
DS Treatment	3	6.956	0.491	0.283	5.736	8.177
Hot Springs	1	9.520	–	–	–	–
La Clocha	1	8.800	–	–	–	–
SE Out	2	5.655	0.077	0.055	4.956	6.353
Treatment 1	3	6.620	0.899	0.519	4.385	8.854
Treatment 2	3	6.680	0.280	0.161	5.984	7.375
US Treatment	3	5.636	0.327	0.188	4.823	6.449

Although variations existed by site, DO concentrations seemed adequate to sustain most types of aquatic macroinvertebrate species at all sites (Croijmans et al., 2021).

Levels of pH were relatively high for most sites but especially so for the La Clocha and SE-Out sites (Figure 7 and Table 7). An elevated pH, during the day, is usually an indication of increased algal primary productivity. As CO² is incorporated into algal biomass, this leaves CaCO₃ in excess in the water driving pH values higher. This is a common phenomenon and large swings in DO and pH are often observed from day to night in waters with elevated algal biomass (Diel patterns) (Staehr et al., 2010). Levels of pH, like that of DO, also often decrease on cloudy days when photosynthetic rate is

quelled. Given that the La Clocha site had low DO levels on 6/28/22, but had the highest pH levels of all sites, indicates that the low DO readings observed on 6/28/22 for a few sites was likely due to the membrane on the DO probe needing to be changed further confirming the removal of this DO data from analysis.

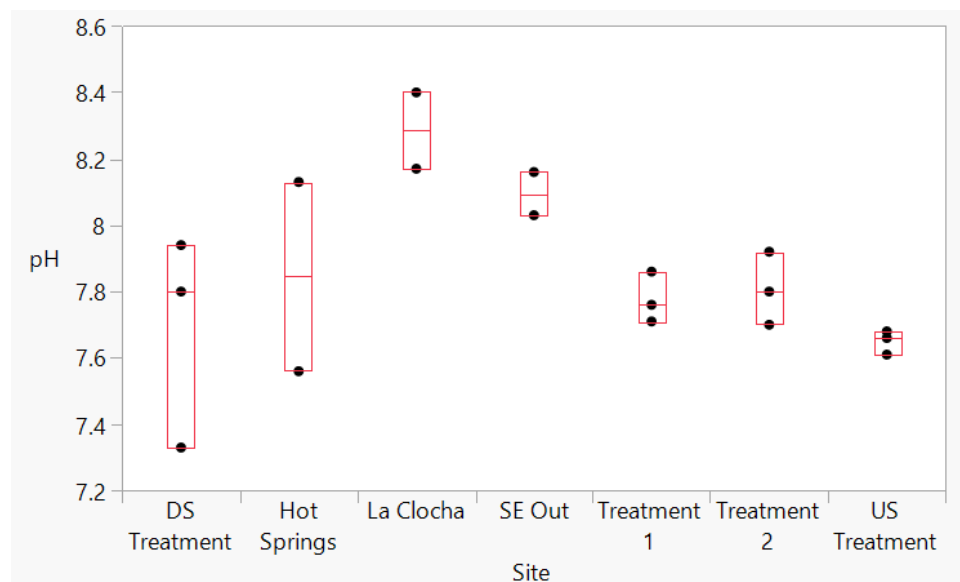


Figure 7. Levels of pH (SU) by site during the study period.

Table 7. Means and standard deviations of pH levels (SU) by site during the study period.

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
DS Treatment	3	7.690	0.319	0.184	6.896	8.483
Hot Springs	2	7.845	0.403	0.285	4.223	11.466
La Clocha	2	8.285	0.162	0.115	6.823	9.746
SE Out	2	8.095	0.091	0.065	7.269	8.920
Treatment 1	3	7.776	0.076	0.044	7.586	7.966
Treatment 2	3	7.806	0.110	0.063	7.533	8.080
US Treatment	3	7.650	0.036	0.020	7.560	7.739

The La Clocha and SE-Out sites also had the lowest relative streamflow rates (Figure 4 and Table 4). Low flow rates favor benthic algal growth and, therefore, elevated pH levels.

SE-Out had, by a significant margin, the highest levels of specific conductivity (Figure 8 and Table 8). Given the nature of this site as being either completely pooled or having very low flow, this isn't surprising as evaporative rate is also likely higher than sites with higher flow. Overall, levels of specific conductivity were generally high for all sites, however, this is not uncommon for aquatic ecosystems in arid regions due to high levels of evaporation, and abundant minerals in parent material, especially CaCO_3 .

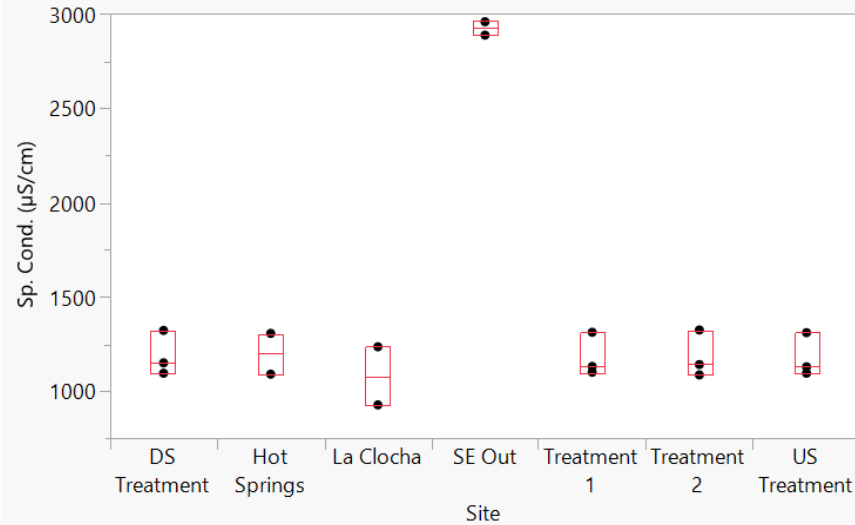


Figure 8. Mean levels of specific conductivity ($\mu\text{S}/\text{cm}$) by site during the study period.

Table 8. Means and standard deviations of specific conductivity levels ($\mu\text{S}/\text{cm}$) by site during the study period.

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
DS Treatment	3	1190.000	118.418	68.369096	895.831	1484.168
Hot Springs	2	1199.000	152.735	108.000000	-173.270	2571.270
La Clocha	2	1082.000	217.788	154.000000	-874.755	3038.755
SE Out	2	2923.500	50.204	35.500000	2472.429	3374.570
Treatment 1	3	1182.000	114.503	66.108000	897.558	1466.441
Treatment 2	3	1184.666	124.604	71.940000	875.131	1494.201
US Treatment	3	1179.000	115.572	66.725000	891.902	1466.098

Turbidity levels were relatively high. This is typical for lotic regions at lower elevations in the arid southwestern US, where a general lack of organic parent material combined with increased imperviousness of soils, relatively steep relief, and erosive summer storms, often lead to high levels of suspended sediment-caused turbidity (Dean et al., 2016) (Figure 9 and Table 9). Additionally, flow spates were observed during the sampling period adding to the variability of turbidity measurements observed.

It appears that the sites farthest away from the treatment area had lower levels of turbidity than those within or near this area. SE-Out, although mostly pooled or with low flow, had relative turbidity levels higher than would be anticipated for such a condition. However, most of the turbidity at SE-Out was likely due to phytoplanktonic algae rather than suspended sediment experienced at other sites.

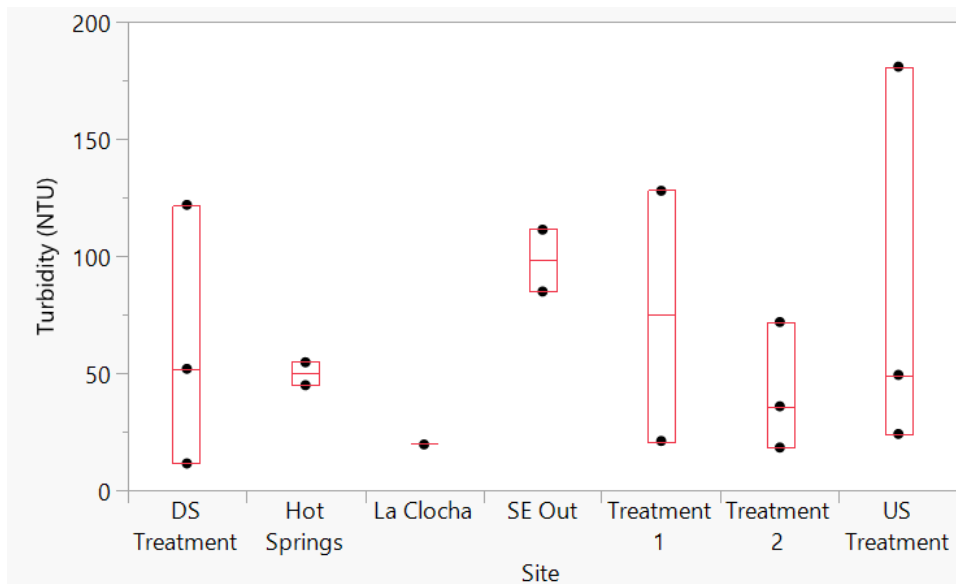


Figure 9. Levels of turbidity (NTU) by site during the study period.

Table 9. Means and standard deviations of turbidity (NTU) by site during the study period.

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
DS Treatment	3	61.866	55.8570	32.249	-76.890	200.624
Hot Springs	2	49.900	6.9296	4.900	-12.360	112.160
La Clocha	2	19.850	0.2120	0.150	17.944	21.755
SE Out	2	98.200	18.6670	13.200	-69.521	265.921
Treatment 1	2	74.600	75.5190	53.400	-603.911	753.111
Treatment 2	3	42.133	27.3210	15.773	-25.736	110.003
US Treatment	3	84.866	84.2010	48.614	-124.302	294.035

Aquatic macroinvertebrates

Aquatic macroinvertebrates were not collected from sites Treatment 1, Treatment 2, or DS-Treatment during the Arsenal® treatment on 5/30/22 and 5/31/22. For the other dates and sites combined, a total of 3,166 individual aquatic macroinvertebrates were found comprising 13 Orders, 33 Families, and 66 Genera (Figure 10, Figure 11, and Table 10). Dipterans of the Family Chironomidae were the most frequently found aquatic macroinvertebrate for all sites with the genera *Dicrotendipes* the most frequently observed chironomid. This is a relatively pollution-tolerant species of non-biting midge having an HBI value of 7. *Dicrotendipes* spp. were found at all sites, but were most prevalent at the La Clocha, SE-Out, US-Treatment, Treatment 2, and DS-treatment sites. The site with the highest overall numbers of aquatic macroinvertebrate species was La Clocha followed by Treatment 1 (Figure 11).

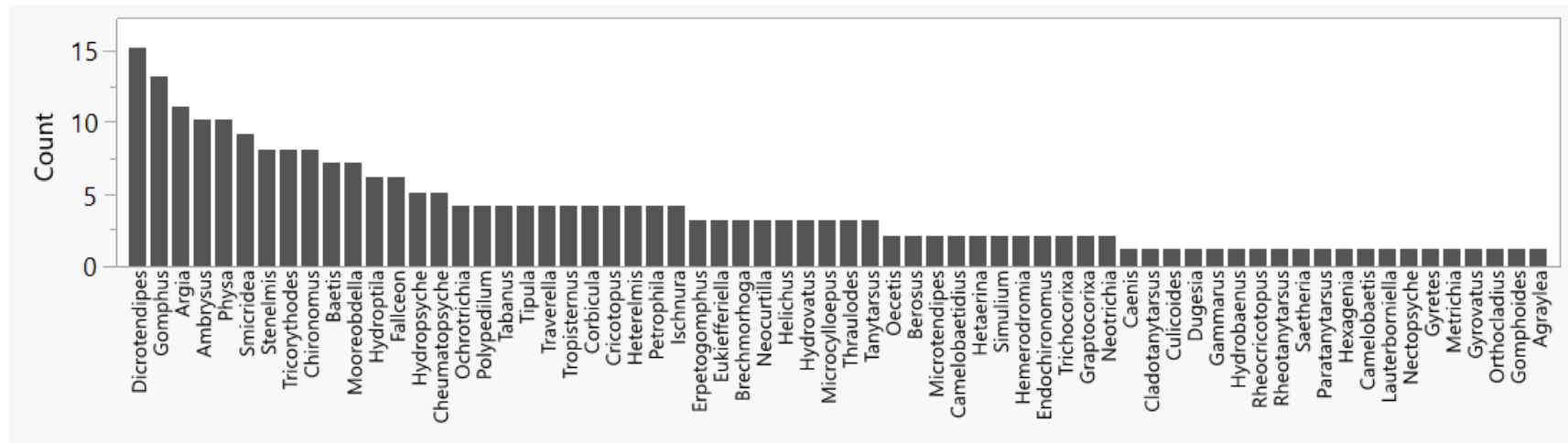


Figure 10. Incidence of aquatic macroinvertebrate Genera during the study period (not site specific). Counts are the sum incidence of each Genera found and not the total number of individual species counted.

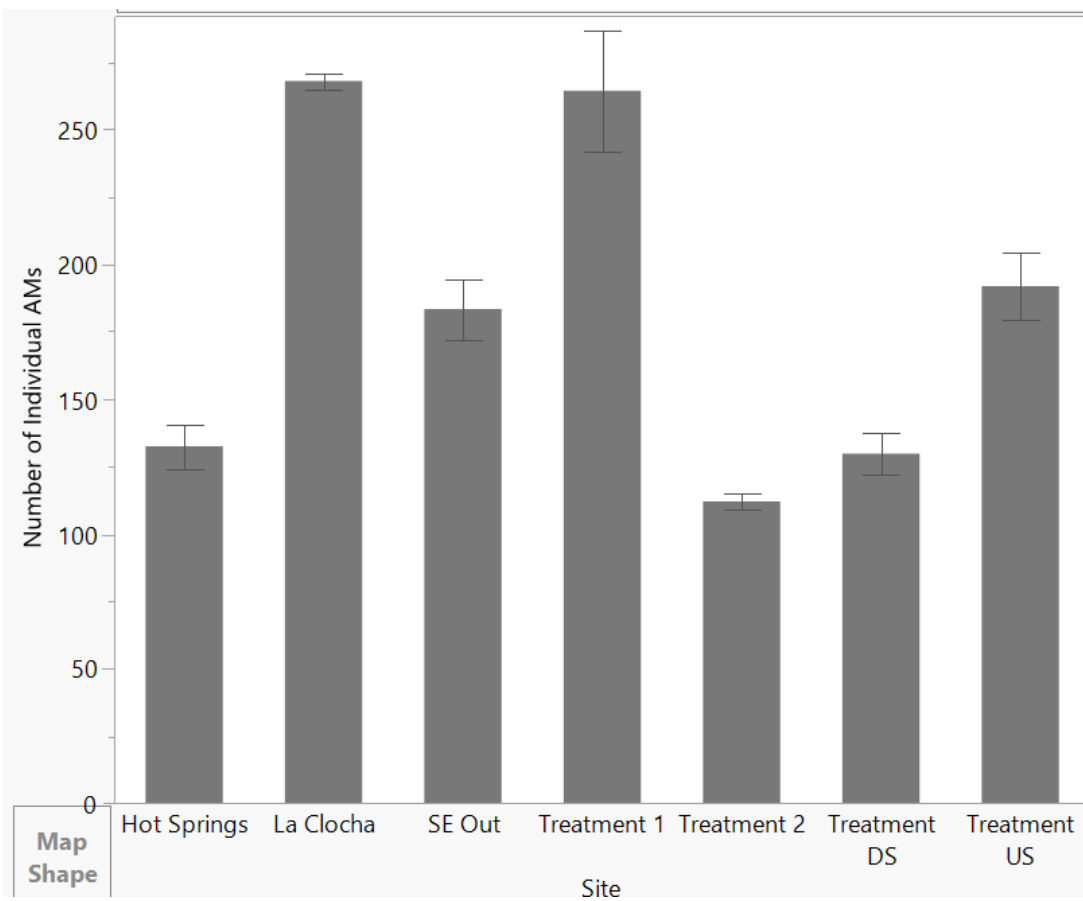


Figure 11. Mean and standard error of aquatic macroinvertebrate species found by site.

Table 10. List of aquatic macroinvertebrates found at each site, denoted with an X. HS = Hot Springs, LC = La Clocha, SE = SE-Out, US-T = Upstream Treatment, T-1 = Treatment 1, T-2 = Treatment 2, DS-T = Downstream Treatment.

Order	Family	Genus	HS	LC	SE	US-T	T-1	T-2	DS-T
Amphipoda	Gammaridae	<i>Gammarus</i>	–	X	X	–	–	–	–
Basommatophora	Physidae	<i>Physa</i>	–	X	X	X	X	X	X
Coleoptera	Dryopidae	<i>Helichus</i>	–	X	X	–	X	–	–
Coleoptera	Dytiscidae	<i>Hydrovatus</i>	–	–	X	–	–	–	–
Coleoptera	Elmidae	<i>Heterelmis</i>	X	X	–	–	–	–	X
Coleoptera	Elmidae	<i>Microcylloepus</i>	–	X	–	X	–	–	–
Coleoptera	Elmidae	<i>Stenelmis</i>	–	–	–	X	X	X	X
Coleoptera	Gyrinidae	<i>Gyretes</i>	–	–	–	–	–	–	X
Coleoptera	Gyrinidae	<i>Gyrovatus</i>	–	–	X	–	–	–	–
Coleoptera	Hydrophilidae	<i>Berosus</i>	–	–	–	–	X	X	–
Coleoptera	Hydrophilidae	<i>Tropisternus</i>	–	–	X	–	X	X	–
Diptera	Ceratopogonidae	<i>Culicoides</i>	–	–	X	–	–	–	–
Diptera	Chironomidae	<i>Chironomus</i>	–	–	X	X	X	X	X
Diptera	Chironomidae	<i>Cladotanytarsus</i>	–	–	–	–	X	–	–
Diptera	Chironomidae	<i>Cricotopus</i>	–	–	X	–	–	X	X
Diptera	Chironomidae	<i>Dicrotendipes</i>	X	X	X	X	X	X	X
Diptera	Chironomidae	<i>Endochironomus</i>	X	–	–	–	–	–	X
Diptera	Chironomidae	<i>Eukiefferiella</i>	–	X	–	X	–	–	–
Diptera	Chironomidae	<i>Hydrobaenus</i>	–	–	–	X	–	–	–
Diptera	Chironomidae	<i>Lauterborniella</i>	–	–	X	–	–	–	X
Diptera	Chironomidae	<i>Microtendipes</i>	X	–	–	–	–	–	–
Diptera	Chironomidae	<i>Orthocladius</i>	–	X	–	–	–	–	–
Diptera	Chironomidae	<i>Paratanytarsus</i>	–	–	X	–	–	–	–
Diptera	Chironomidae	<i>Polypedilum</i>	–	X	–	X	–	X	–

Table 10 (continued). List of aquatic macroinvertebrates found at each site, denoted with an X. HS = Hot Springs, LC = La Clocha, SE = SE-Out, US-T = Upstream Treatment, T-1 = Treatment 1, T-2 = Treatment 2, DS-T = Downstream Treatment.

Order	Family	Genus	HS	LC	SE	US-T	T-1	T-2	DS-T
Diptera	Chironomidae	<i>Rheocricotopus</i>	–	–	–	–	–	X	–
Diptera	Chironomidae	<i>Rheotanytarsus</i>	–	–	X	–	–	–	–
Diptera	Chironomidae	<i>Saetheria</i>	–	–	–	X	–	–	–
Diptera	Chironomidae	<i>Tanytarsus</i>	–	X	–	X	–	–	–
Diptera	Empididae	<i>Hemerodromia</i>	–	X	–	–	–	–	–
Diptera	Simuliidae	<i>Simulium</i>	–	–	–	X	–	–	–
Diptera	Tabanidae	<i>Tabanus</i>	–	–	X	–	X	X	X
Diptera	Tipulidae	<i>Tipula</i>	–	–	X	–	–	X	–
Ephemeroptera	Baetidae	<i>Baetis</i>	X	X	–	X	X	X	X
Ephemeroptera	Baetidae	<i>Camelobaetidius</i>	–	–	–	–	X	–	–
Ephemeroptera	Baetidae	<i>Camelobaetis</i>	–	X	–	–	–	–	–
Ephemeroptera	Baetidae	<i>Fallceon</i>	X	X	–	X	–	–	X
Ephemeroptera	Caenidae	<i>Caenis</i>	–	–	–	–	X	–	–
Ephemeroptera	Ephemeridae	<i>Hexagenia</i>	–	–	–	–	X	–	–
Ephemeroptera	Leptohyphidae	<i>Tricorythodes</i>	X	X	–	X	X	X	X
Ephemeroptera	Leptophlebiidae	<i>Thraulodes</i>	X	X	–	–	–	–	–
Ephemeroptera	Leptophlebiidae	<i>Traverella</i>	–	X	–	X	X	–	–
Hemiptera	Corixidae	<i>Graptocorixa</i>	–	–	X	–	–	–	–
Hemiptera	Corixidae	<i>Trichocorixa</i>	–	X	–	X	–	–	–
Hemiptera	Naucoridae	<i>Ambrysus</i>	X	X	X	–	X	–	X
Hirudinida	Erpobdellidae	<i>Mooreobdella</i>	X	X	X	X	–	X	X
Lepidoptera	Crambidae	<i>Petrophila</i>	X	–	X	–	X	–	X
Neophora	Dugessidae	<i>Dugesia</i>	–	X	–	–	–	–	–
Odonata	Calopterygidae	<i>Hetaerina</i>	–	–	–	–	X	X	–

Table 10 (continued). List of aquatic macroinvertebrates found at each site, denoted with an X. HS = Hot Springs, LC = La Clocha, SE = SE-Out, US-T = Upstream Treatment, T-1 = Treatment 1, T-2 = Treatment 2, DS-T = Downstream Treatment.

Order	Family	Genus	HS	LC	SE	US-T	T-1	T-2	DS-T
Odonata	Coenagrionidae	<i>Argia</i>	–	X	X	X	X	X	X
Odonata	Coenagrionidae	<i>Ischnura</i>	–	–	X	–	X	–	–
Odonata	Gomphidae	<i>Erpetogomphus</i>	X	X	–	–	–	–	–
Odonata	Coenagrionidae	<i>Gomphoides</i>	–	–	–	X	–	–	–
Odonata	Gomphidae	<i>Gomphus</i>	X	X	X	X	X	X	X
Odonata	Libellulidae	<i>Brechmorhoga</i>	–	X	–	–	–	X	–
Orthoptera	Gryllotalpidae	<i>Neocurtilla</i>	–	–	X	–	–	X	–
Trichoptera	Hydropsychidae	<i>Cheumatopsyche</i>	–	X	–	X	–	X	–
Trichoptera	Hydropsychidae	<i>Hydropsyche</i>	X	–	–	–	–	X	X
Trichoptera	Hydropsychidae	<i>Smicridea</i>	X	X	–	X	X	X	X
Trichoptera	Hydroptilidae	<i>Agraylea</i>	–	–	–	–	X	–	–
Trichoptera	Hydroptilidae	<i>Hydroptila</i>	X	X	–	X	–	–	X
Trichoptera	Hydroptilidae	<i>Metrichia</i>	–	–	–	–	–	X	–
Trichoptera	Hydroptilidae	<i>Neotrichia</i>	–	–	–	X	X	–	–
Trichoptera	Hydroptilidae	<i>Ocrotrichia</i>	–	X	–	X	X	–	–
Trichoptera	Leptoceridae	<i>Oecetis</i>	–	X	–	–	–	–	–
Trichoptera	Leptoceridae	<i>Nectopsyche</i>	–	–	–	X	–	–	–
Veneroida	Corbiculidae	<i>Corbicula</i>	–	X	X	X	–	–	–

B-IBI scoring for each site using TCEQ's metrics for ecoregion 24 showed that the sites with the highest mean scores were La Clocha and Hot Springs (Figure 12). Lumping the treatment and untreated sites into categories (Treated sites = Treatment 1, Treatment 2, and DS-Treatment; Reference sites = SE-Out, Hot Springs, La Clocha, and US-Treatment) there was a significant difference in mean B-IBI scores (Figure 13 and Tables 11 through 13) with treated sites having a lower overall mean ($\bar{x} = 16.33$) than untreated sites ($\bar{x} = 22.33$) ($p = 0.0226$).

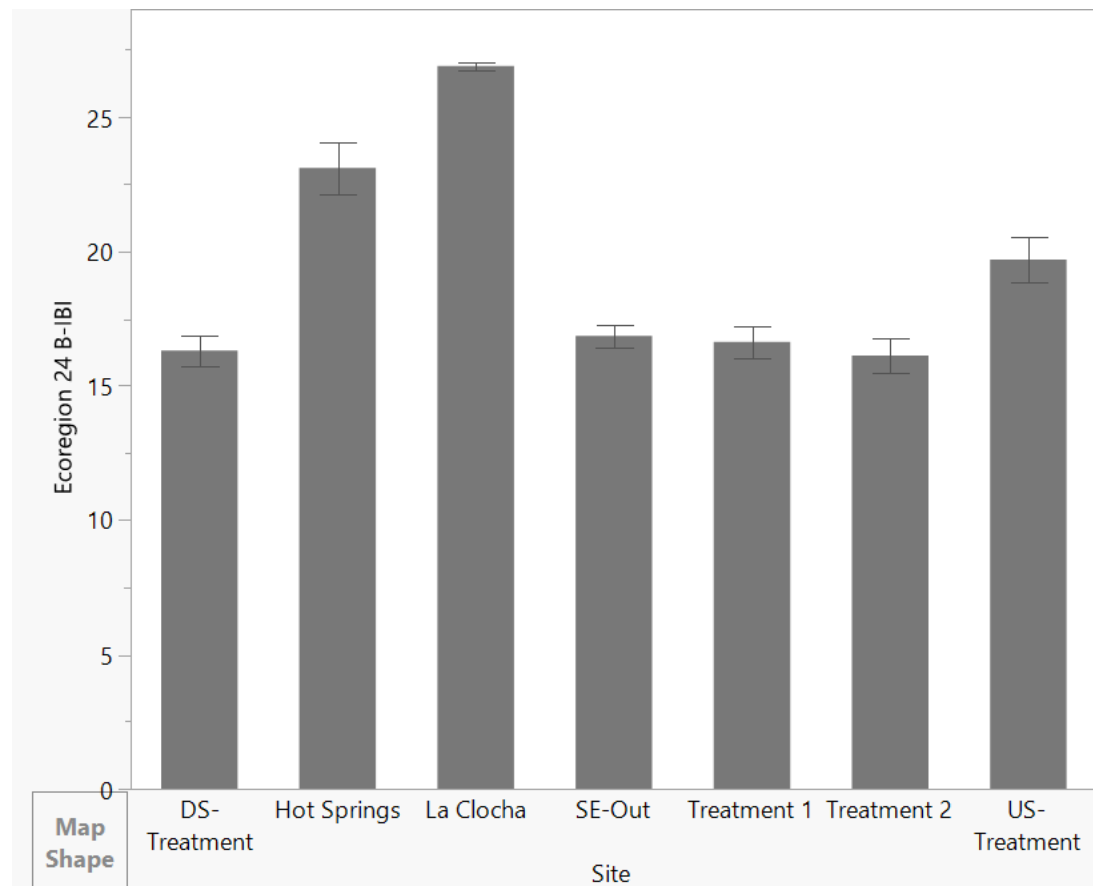


Figure 12. Mean B-IBI scoring by site during the study period.

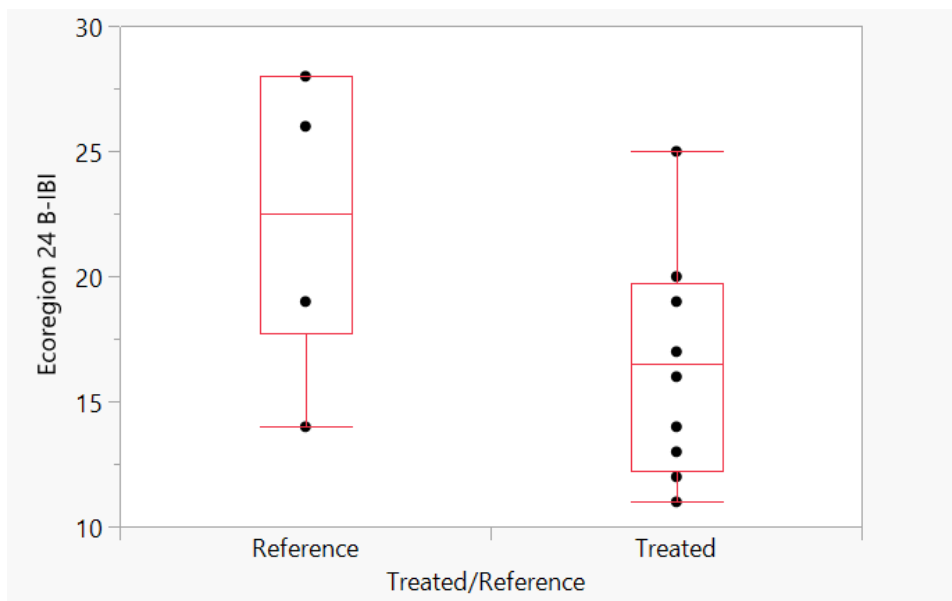


Figure 13. Ecoregion 24 B-IBI scoring for reference and treated sites.

Table 11. Summary of fit for Ecoregion 24 B-IBI scoring by reference and treated sites.

Summary of Fit	Value
Rsquare	0.284
Adj Rsquare	0.239
RMSE	4.756
Mean of Response	18.333
Observations	18

Table 12. Analysis of variance for Ecoregion 24 B-IBI scoring by reference and treated sites.

Analysis of Variance	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treated/Reference	1	144.000	144.000	6.364	0.0226
Error	16	362.000	22.625	—	—
C. Total	17	506.000	—	—	—

Table 13. Means for one way ANOVA for Ecoregion 24 B-IBI scoring by reference and treated sites.

Means for One way ANOVA	Number	Mean	Std. Error	Lower 95%	Upper 95%
Reference	6	22.333	1.941	18.217	25.450
Treated	12	16.333	1.373	13.422	19.244

Ecoregion 24 B-IBI scoring by date didn't reveal any distinct trends (Figure 14). B-IBI scores, and aquatic macroinvertebrate assemblages in general, are dynamic and often change due to a variety of reasons such as inherent variability and sudden changes in flow. Most sites decreased in B-IBI scoring during the study period, however, the Hot Springs and La Clocha sites increased during the same time period.

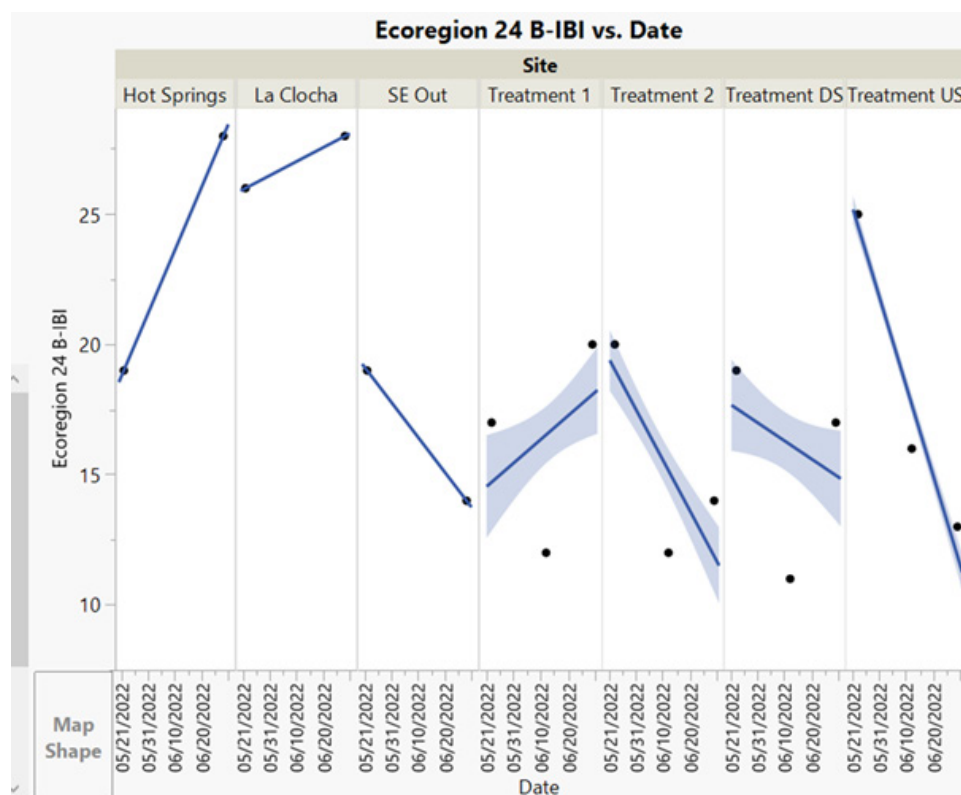


Figure 14. Ecoregion 24 B-IBI scoring by site and date.

Laboratory Toxicity Testing

Water toxicity using *Daphnia magna*

Although sample sizes were low, especially for the reference sites, there did appear to be a mean difference in toxicity testing of water using logMedian EC50 values of *D. magna* between treated and untreated sites ($p = 0.05$, Figure 15 and Tables 14 through 16). Treated sites had lower overall scores ($\bar{x} = 2.24$) than untreated sites ($\bar{x} = 2.90$). This indicates that it took less concentration of water collected from treatment sites to have an effect than water collected from reference sites. “Less concentration” for EC50 values equates to increased toxicity at treated versus reference sites.

All sites appeared to decrease in logMedian EC50 values throughout the study period (Figure 16).

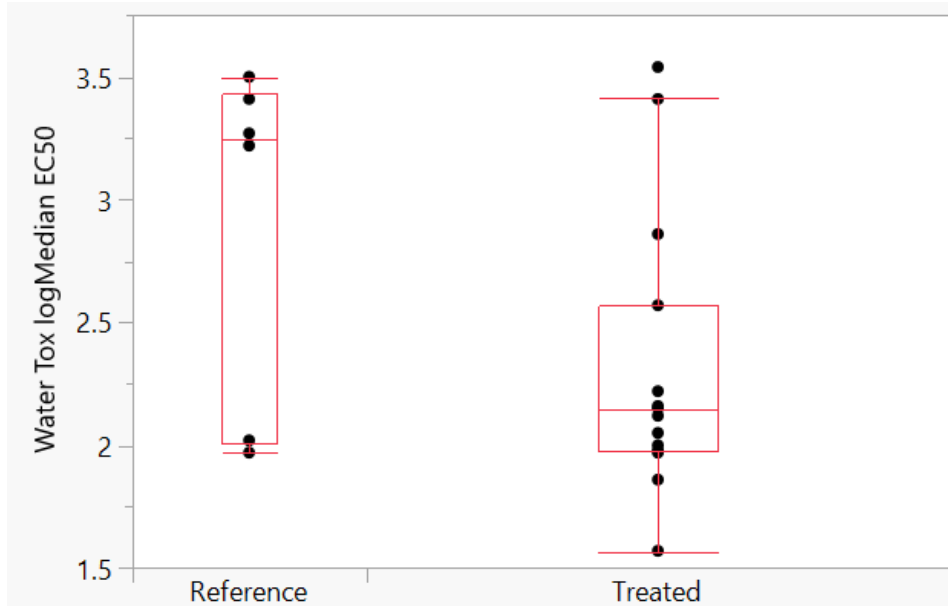


Figure 15. LogMedian EC50 values by reference and treated sites.

Table 14. Summary of fit for logMedian EC50 values of water by reference and treated sites.

Summary of Fit	Value
Rsquare	0.179
Adj Rsquare	0.136
RMSE	0.600
Mean of Response	2.476
Observations	21

Table 15. Analysis of variance for logMedian EC50 values of water by reference and treated sites.

Analysis of Variance	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treated/Reference	1	1.496	1.496	4.153	0.0486
Error	19	6.847	0.360	—	—
C. Total	20	8.344	—	—	—

Table 16. Means for one way ANOVA for logMedian EC50 values of water by reference and treated sites.

Means for One way ANOVA	Number	Mean	Std. Error	Lower 95%	Upper 95%
Reference	6	2.898	0.245	2.385	3.411
Treated	15	2.300	0.155	1.982	2.631

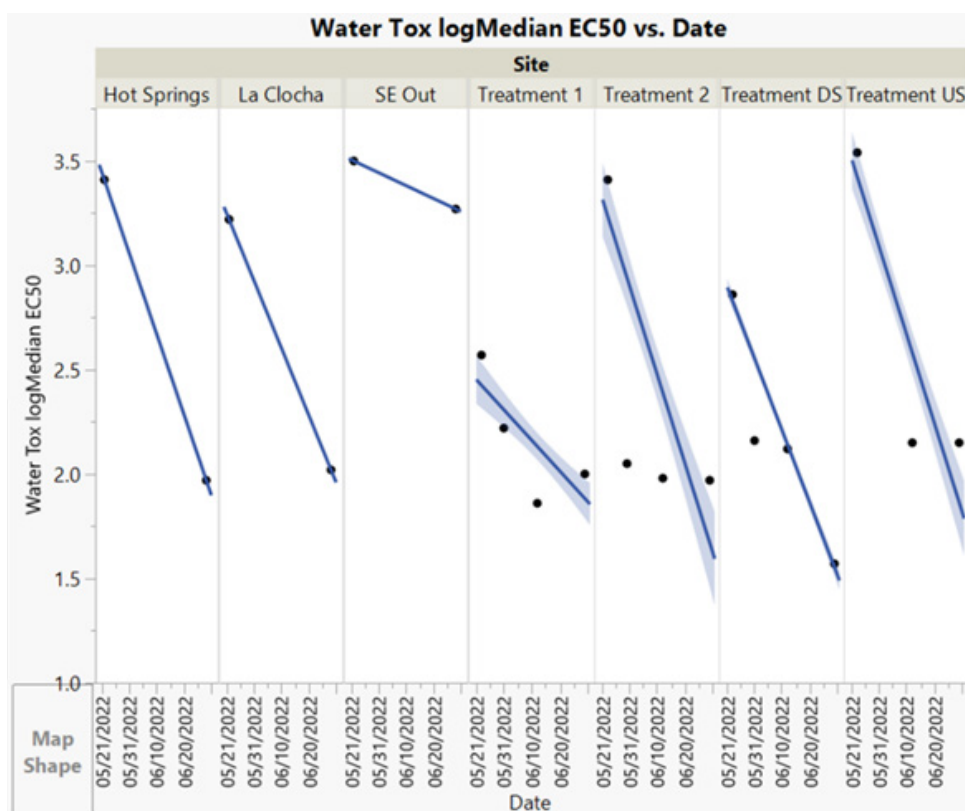


Figure 16. LogMedian EC50 values by site and date.

Sediment toxicity using *Heterocypris incongruens*.

There was a significant difference between sediment collected from treated and untreated sites in terms of percent growth inhibition ($\bar{x} = 14.47$ and 5.50 respectively) and percent mortality ($\bar{x} = 5.65$ and 2.81 respectively) of the ostracod *H. incongruens* ($p = 0.004$ and 0.016 respectively, Figures 17 and 18, Tables 17 through 22). Percent growth inhibition and mortality increased during the study period at all sites, except at Hot Springs, for growth inhibition which showed no difference between sampling dates (Figures 19 and 20).

There was an opposite trend between sediment and water toxicity testing overall in that the latter seemed to decrease over time and the former, increase. This could be due to nonylphenol (NPs) and nonylphenol ethoxylates (NPEs) strong affinity to bind to sediment and rapid dispersion in water (see Appendix B). Sediment may be a long-term repository of NPs and NPEs with these slowly being released in small concentration to overlying water over time. Given their chemical properties, it is likely that concentrations of NPs and NPEs in sediment are much greater than those in overlying water at any given time. Additionally, while water and sediment toxicity testing both showed statistically significant differences between reference and control sites, sediment toxicity showed a greater difference ($p = 0.004$ and 0.016 for growth inhibition and mortality respectively) than did water toxicity ($p = 0.0486$). Based upon p values, growth inhibition in sediment followed by mortality of *H. congruens* were the most significant followed by the logMedian EC50 of *D. magna* exposed to collected water.

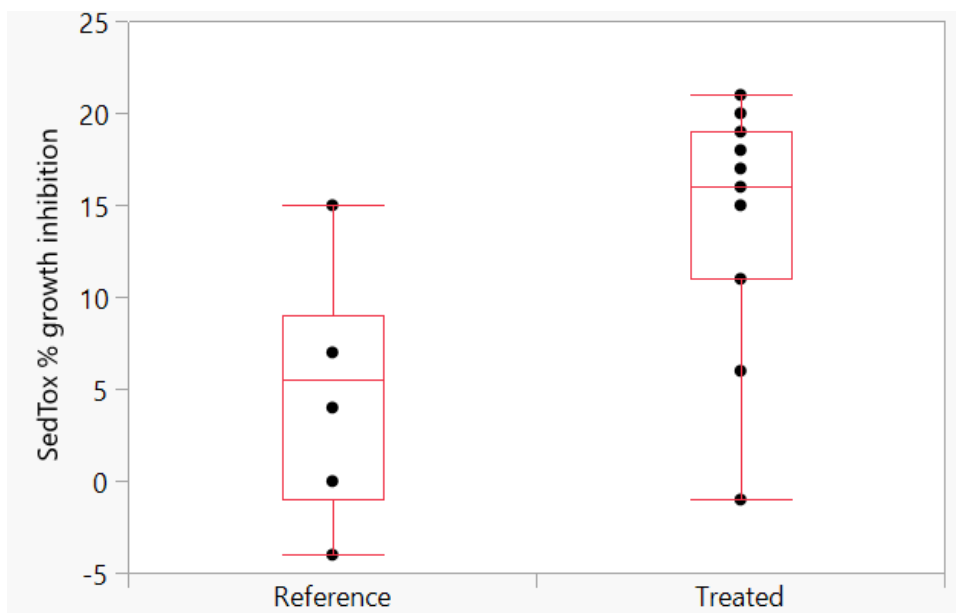


Figure 17. Mean percent growth inhibition in *H. incongruens* using sediment from reference and treated sites.

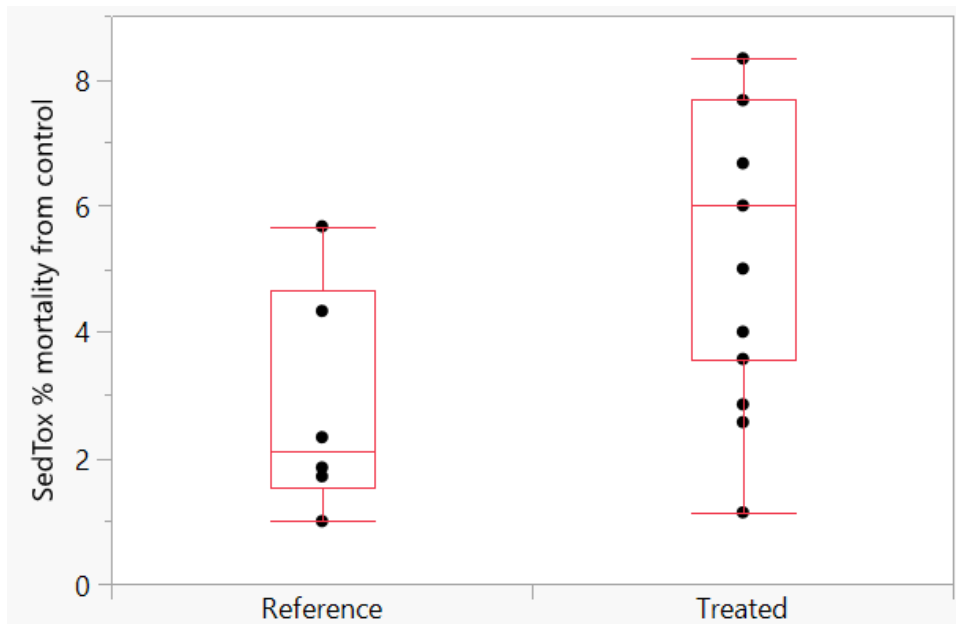


Figure 18. Mean percent mortality of *H. incongruens* using sediment from reference and treated sites.

Table 17. Summary of fit for percent growth inhibition of sediment to *H. congruens* by reference and treated sites.

Summary of Fit	Value
Rsquare	0.355
Adj Rsquare	0.321
RMSE	6.123
Mean of Response	11.666
Observations	21

Table 18. Analysis of variance for percent growth inhibition of sediment to *H. congruens* by reference and treated sites.

Analysis of Variance	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treated/Reference	1	392.233	392.233	10.460	0.0044
Error	19	712.433	37.496	–	–
C. Total	20	1104.666	–	–	–

Table 19. Means for one way ANOVA for percent growth inhibition of sediment to *H. congruens* by reference and treated sites.

Means for One way ANOVA	Number	Mean	Std. Error	Lower 95%	Upper 95%
Reference	6	4.833	2.499	-0.40	10.066
Treated	15	14.400	1.581	11.09	17.709

Table 20. Summary of fit of percent mortality of sediment to *H. congruens* by reference and treated sites.

Summary of Fit	Value
Rsquare	0.270
Adj Rsquare	0.231
RMSE	2.215
Mean of Response	4.842
Observations	21

Table 21. Analysis of variance of percent mortality of sediment to *H. congruens* by reference and treated sites.

Analysis of Variance	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treated/Reference	1	34.525	34.526	7.031	0.0157
Error	19	93.299	4.9105	—	—
C. Total	20	127.825	—	—	—

Table 22. Means for one way ANOVA of percent mortality of sediment to *H. congruens* by reference and treated sites.

Means for One way ANOVA	Number	Mean	Std. Error	Lower 95%	Upper 95%
Reference	6	2.815	0.904	0.921	4.708
Treated	15	5.653	0.572	4.455	6.850

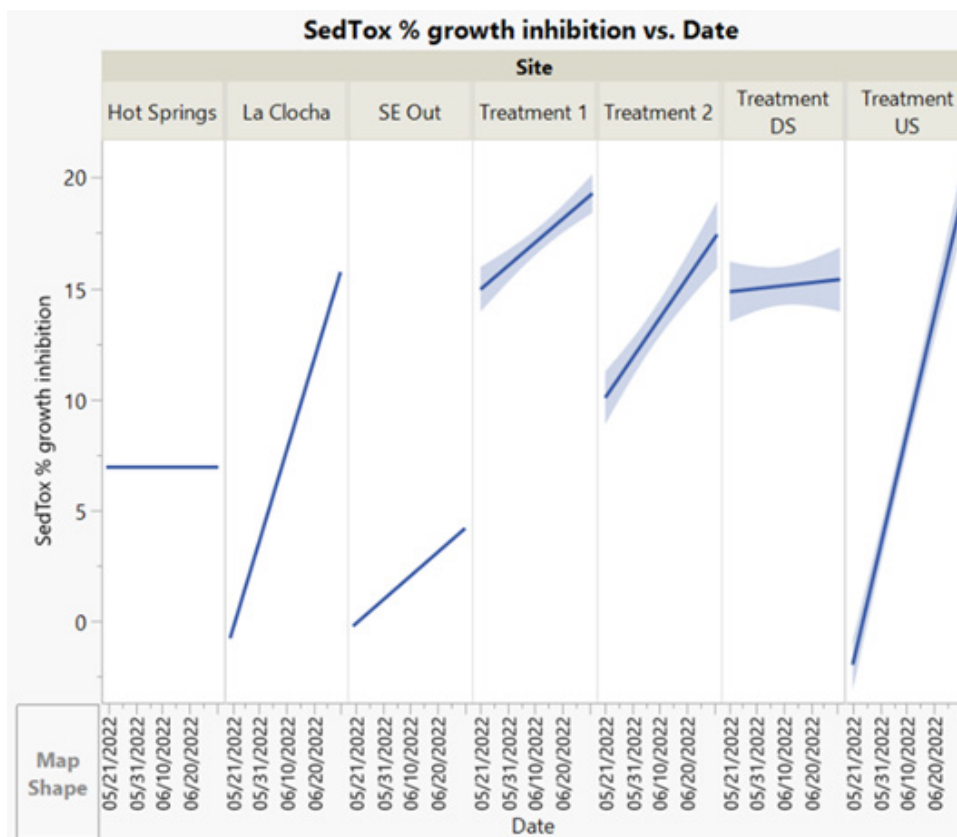


Figure 19. Percent growth inhibition by site and date of *H. incongruens* using sediment collected from reference and treated sites.

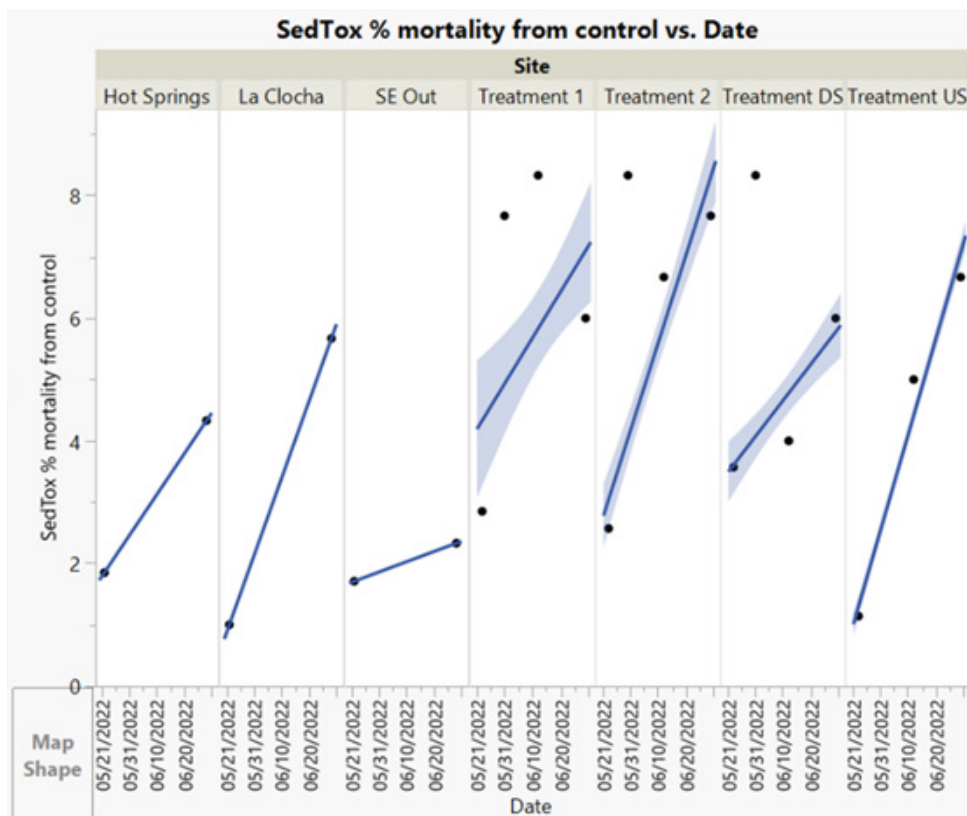


Figure 20. Percent mortality by site and date of *H. incongruens* using sediment collected from reference and treated sites.

Ordination Analysis

Ordination of highly dimensional data is often used to represent sample and species relationships as faithfully as possible in a reduced-dimensional space. Historically, ordination has often been used for data mining purposes but has also been used as a stand-alone statistical technique to view interactions among many variables simultaneously. However, it is still important to understand, and limit, the types of variables used in ordination analysis as adding too many could have a large effect on results that are often auto-correlative.

For this dataset, principal components analysis (PCA) was chosen as the most representative ordination technique employed to reduce dimensionality of selected variables. It is beyond the scope of this report to detail all aspects of PCA, rather, the reader is recommended to read Jolliffe and Cadima (2016).

Biplot rays in PCA indicate relationships among variables (Figure 21). The closer the biplot rays are to one another, the more positive the relationship whereas biplot rays that are farther apart show some degree of an inverse relationship.

Since the DO readings taken from the Hot Springs and La Clocha sites on 6/28/22 were suspect, this data was excluded from PCA analysis. Quick examination (Figure 21 and Table 23) reveals the positive relationship between B-IBI scores and taxa richness ($r = 0.77$). This is to be expected since

the latter is a specific metric in the calculation of the former. It's not surprising that the total number of aquatic macroinvertebrates is also positively related to the B-IBI scores ($r = 0.48$), but the strength of this association is not as great as with taxa richness.

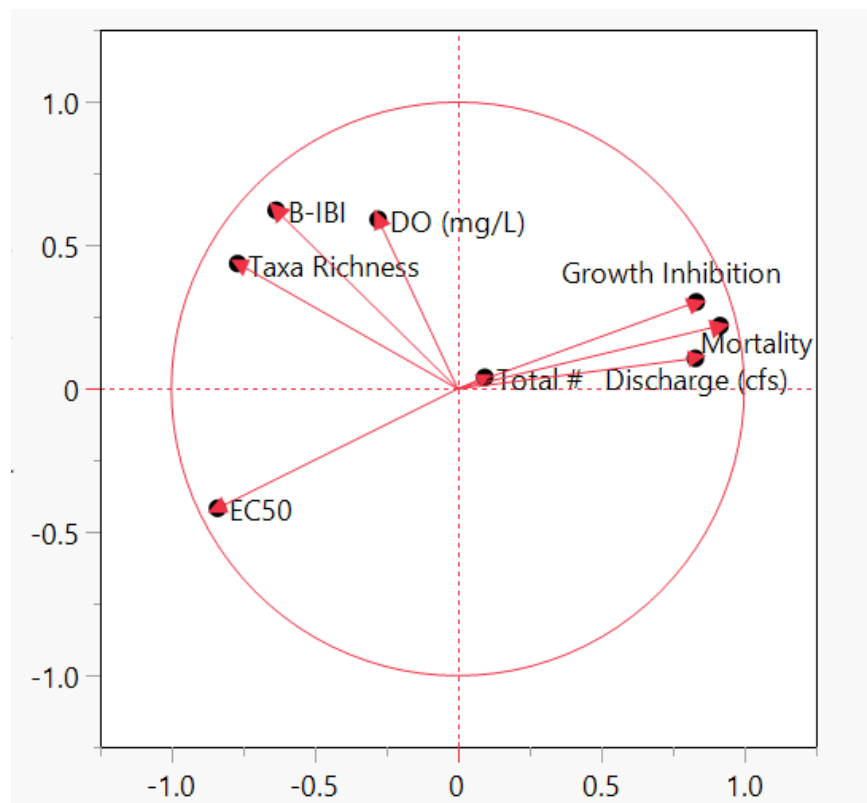


Figure 21. PCA biplot rays on correlations between biological, physical, physicochemical, and toxicological variables for all sites. Closeness of biplot rays indicates a positive relationship and distance, inverse. Note that increasing EC50 levels equals decreasing toxicity and vice versa.

There is a positive relationship between B-IBI values, taxa richness, and DO but inverse relationships between B-IBI values, sediment toxicology measurements (percent growth inhibition and mortality), total number of macroinvertebrates, and discharge (Figure 21 and Table 23). The discharge – B-IBI inverse relationship may indicate that scouring and downstream displacement of aquatic macroinvertebrates did occur during the study period ($r = -0.39$) however some if this may be auto-correlative because the sites with the highest toxicity also had the highest rates of flow. The positive relationship between DO and B-IBI values underscores the importance of dissolved oxygen to stream macroinvertebrates ($r = 0.30$).

The total number of aquatic macroinvertebrates and the inverse relationship with B-IBI scoring is due to the high number of relatively pollution-tolerant species, especially chironomids, found at treatment sites (Figures 10 and 11).

Table 23. Correlation matrix and *r* values between biological, physical, physicochemical, and toxicological variables. Abbreviation definitions are “B-IBI” = Ecoregion 24 benthic index of biological integrity, “EC50” = logMedian EC50 values of *D. magna* exposed to collected water, “Growth Inhibition” = Percent growth inhibition of *H. congruens* exposed to collected sediment, “Mortality” = Percent mortality of *H. congruens* exposed to collected sediment, “Total #” = the total number of aquatic macroinvertebrates collected at each site on each date. Taxa richness, discharge (cfs), and DO (mg/L) are self-explanatory. Note that increasing EC50 levels equals decreasing toxicity and vice versa.

Metric	B-IBI	EC50	Growth Inhibition	Mortality	Total #	Taxa Richness	Discharge (cfs)	DO (mg/L)
B-IBI	1.0000	0.2546	-0.3551	-0.4379	-0.0147	0.7671	-0.3868	0.2964
EC50	0.2546	1.0000	-0.7458	-0.9007	-0.0949	0.4320	-0.6612	0.0773
Growth Inhibition	-0.3551	-0.7458	1.0000	0.8068	0.0568	-0.4367	0.6452	-0.1184
Mortality	-0.4379	-0.9007	0.8068	1.0000	0.0805	-0.5405	0.6737	-0.2189
Total #	-0.0147	-0.0949	0.0568	0.0805	1.0000	-0.0042	0.0503	-0.0990
Taxa Richness	0.7671	0.4320	-0.4367	-0.5405	-0.0042	1.0000	-0.6464	0.2663
Discharge (cfs)	-0.3868	-0.6612	0.6452	0.6737	-0.6464	-0.6464	1.0000	-0.1198
DO (mg/L)	0.2964	0.0773	-0.1184	-0.2189	-0.0990	0.2263	-0.1198	1.0000

There was a positive relationship between B-IBI values and water toxicity to *D. magna* (EC50) ($r = 0.25$). It is important to remember that increases in EC50 values equate to lower levels of toxicity and vice versa. Therefore, a positive relationship between B-IBI scoring and EC50 values would translate to increasing levels of water toxicity having a negative effect on B-IBI scoring. Given that higher EC50 values equate to lower levels of toxicity, this would also explain the inverse relationship between this variable and measures of sediment growth inhibition ($r = -0.75$) and sediment mortality ($r = -0.90$).

In terms of toxicological variables that most affected B-IBI scoring, percent mortality of *H. congruens* had the greatest inverse relationship ($r = -0.44$) followed by percent growth inhibition of the same ($r = 0.35$) and EC50 values of *D. magna* exposed to water ($r = 0.25$).

The PCA results are in accordance with findings from parametric statistics; the effects of sediment toxicity (mortality and growth inhibition) are greater than toxicity of water (EC50). Sediments are likely a repository of NPs and NPEs that are more toxic to aquatic macroinvertebrates than the compound imazapyr. These NPs and NPEs may leach from the sediment into overlying water over periods of time where they quickly photo-degrade with half-lives of hours to days. Half-lives of nonylphenols in sediment, however, are on the order of years to decades (Soares et al., 2008). This means that sediments within treatment areas may be long-term sinks, and sources, of nonylphenols into the aquatic environment.

Conclusions

Based upon in situ biological monitoring of aquatic macroinvertebrates and laboratory toxicological testing, there did appear to be some effect of Arsenal® treatment on benthic organisms throughout the study reach compared to reference sites. Given that the parent compound, imazapyr, has been proven to be relatively non-toxic to a wide range of species, this effect may be caused by the primary surfactant, nonylphenol ethoxylate, and its degradation product, nonylphenol. Care should be implemented in this interpretation as 1) natural spatial variability between sites should be considered, 2) unequal sample sizes existed between reference and treatment areas and, 3) overall sample sizes are low. Also, site SE-Out was distinctly different than other sites due to having either a pooled or very low flow (8 cfs) condition while other sites had much higher flow rates. Aquatic macroinvertebrate assemblages are often distinctly different from a pooled/low flow condition compared to higher flows.

Sporadic and flashy streamflow did occur during the study period. This will influence aquatic macroinvertebrate assemblage dynamics due to scouring and flushing of individuals downstream. While trying to capture all inherent variability in a highly dynamic river ecosystem is impossible, sites within the treatment area did have higher flows than those of reference areas and this could have influenced B-IBI scoring and subsequent comparison.

The Arsenal® treatment that occurred on 5/29 to 5/31 near sites Treatment 1 and Treatment 2 resulted in high concentrations of product on the soil surface near the water's edge due to overspray and was readily observable, especially via the blue dye that is also included in the formulation (pers. comm. Jeff Renfrow). Results from this date, especially for soil/sediment toxicological testing, represented an extreme condition purposefully included as part of the study design. Sampling also occurred 2 weeks and one month following this treatment. It should be noted that concentrations of Arsenal® and its degradation compounds will typically not be as elevated in the water and soil/sediment as was observed during this sampling.

Many studies have examined the relative safety of imazapyr to aquatic organisms and amphibians yet hardly any have examined the effects of the surfactant identified in Arsenal®; nonylphenol ethoxylate (NPE). Lower toxicity was observed in water samples than soil. Soil adsorption of imazapyr is weak and it is readily photolyzed in water with a short half-life of 3-5 days. Therefore, its presence in water is short-lived as would be any toxic effect. NPEs, on the other hand, readily adsorb onto soil and sediment and have a much longer half-life than that of imazapyr. Also, a half-life degradation rate can be misleading as many compounds may exhibit a relatively short half-life with a much more persistent form following. Degradation of any compound is not always linear.

The parent compound, nonylphenol ethoxylate, is less toxic than the degradation product of nonylphenol (NP). NPs and NPEs both partition to sediments and can accumulate in the environment over time. They also bioaccumulate and bioconcentrate in aquatic biota causing significant endocrine-disrupting and reproductive effects (EPA, 2010). Although neither NPs nor NPEs biomagnify, it is possible for organisms at higher trophic levels to be exposed to these compounds through the food web.

The PCA results showed a greater inverse relationship between measures of soil/sediment toxicity and B-IBI scoring compared to water and B-IBI scoring. This could be due to environmental partitioning of the ingredients in Arsenal® with less-toxic imazapyr partitioning to the water and more-toxic NPs/NPEs partitioning to soil and sediment.

Naturally, the potentially toxic effects of Arsenal® use must be factored against the many negative effects of highly invasive *Arundo donax* in the region. Studies such as this report should be used as a guide in the decision-making process of how to best control a highly invasive species.

Future Recommendations

As mentioned, this study was hampered by low sample size. An increase in statistical power may make nebulous relationships clearer. The best method to encompass natural variability in highly dynamic ecosystems is by increasing sample size and repeatability. The reference sites should be numerically equal to treatment sites. Also, if possible, reference sites should be chosen that more closely mimic treatment sites in terms of flow and substrate quality.

Long-term monitoring of aquatic macroinvertebrates should be initiated. AMs can be highly dynamic assemblages, especially in areas with hydrologically flashy flow events. Long-term monitoring of selected sites would aid in determining how the structure and function of the Rio Grande may be temporally and spatially changing outside of any toxicity caused by Arsenal® use.

Sites within the study area have been established as part of a long-term botany and geomorphology monitoring effort conducted by NPS Inventory and Monitoring (Figure 22). These sites have legacy data and are sampled annually in April, an optimal time for aquatic macroinvertebrate sampling as the frequent flash floods that may cause scouring typically start in May. The combination of legacy data and timing make these excellent candidate sites for long-term AM monitoring. It is likely that the sampling could be conducted as a part of the larger Big River Monitoring effort. Basic water quality variables could easily be added to the data collected from these established sites and aquatic macroinvertebrate samples collected and sent for taxonomic identification and ecoregion 24 B-IBI calculation. Proposed sampling sites are provided in Figure 22.

NPEs and 4NPs are potent endocrine disrupting chemicals. Endocrine disruption in invertebrate species is difficult, if not impossible, to determine yet may have devastating effects on vertebrate species, such as fish. Non-lethal effects of endocrine disrupting compounds to fish species are well-documented (Walker et al., 2009) and include behavioral effects affecting the hypothalamic-pituitary-thyroid axis as well as overall fertility and fecundity via the hypothalamic-pituitary-gonadal axis. In situ monitoring of fish species is difficult due to their high mobility and potential to be affected in an area far removed from the collection site. Therefore, water and sediment should be collected from treatment and reference sites before, during, and after an Arsenal® treatment to be used in controlled and replicated laboratory experiments using aquaria and certified disease-free representative fish species such as fathead minnow (*Pimephales promelas*). Following an exposure period, samples from fish should be collected including 17 β estradiol, vitellogenin, 11-ketotestosterone, thyroid stimulating hormone, triiodothyronine (T3) and thyroxine (T4). Fish species are far more susceptible to the endocrine disrupting effects of NP/NPEs than are aquatic macroinvertebrates and

could have long-lasting effects on endangered fish species such as the Rio Grande silvery minnow (*Hybognathus amarus*) and the Big Bend gambusia (*Gambusia gaigei*).

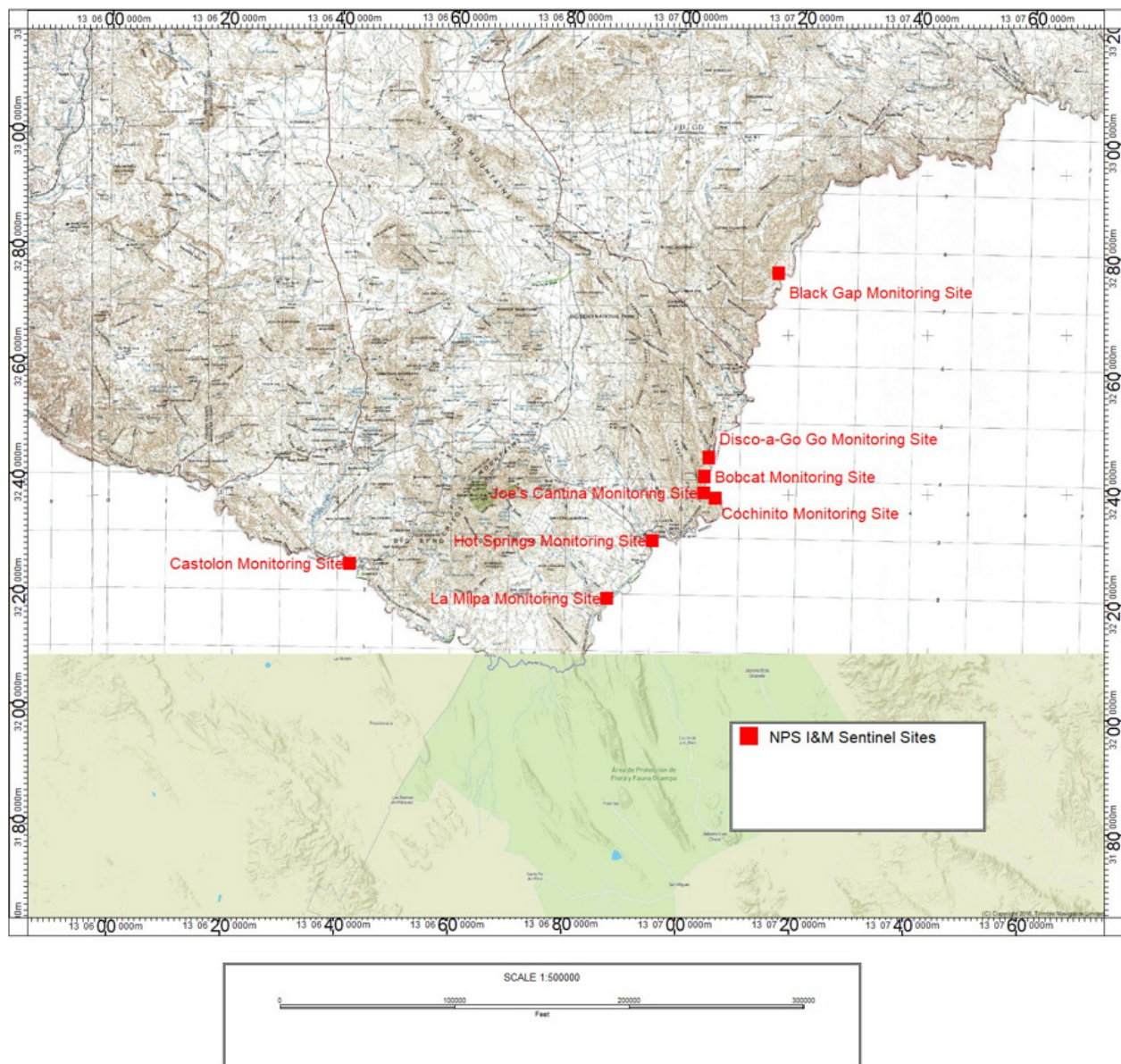


Figure 22. Proposed sampling sites for aquatic macroinvertebrate and basic water quality analyses. Note that these sampling sites already exist as part of a long-term botany and geomorphology monitoring effort.

This study performed no chemical analysis of water or sediment. Doing so for both imazapyr and NP/NPEs would enable more-precise estimation of concentrations within the river that had an effect versus those concentrations that may not.

In accordance with the above recommendation, chemical analysis of Arsenal®, and especially of NPEs and NPs, should be performed before, during, and after a treatment to determine downstream (flow-weighted) distance before these compounds become non-detectable in water and soil.

In addition to toxicity testing using the bioindicators *H. incongruens* and *D. magna*, kinetic luminescent testing using genetically engineered *Aliivibrio fischeri* is a well-established and sensitive toxicity test of water and sediment. This would allow increased resolution of any potentially toxic effect at a more-precise level than using EC50 of higher organisms.

The contribution of spring flow to the condition of aquatic macroinvertebrate populations in the study area is poorly understood. A better understanding of the hydrologic contribution of springs and their effect on biological integrity is needed. This is particularly true at low flows when springs contribute a significant percentage of the overall flow in the study reach.

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Appendix A. Toxicity Testing Methods

FILLING OF THE TEST PLATE

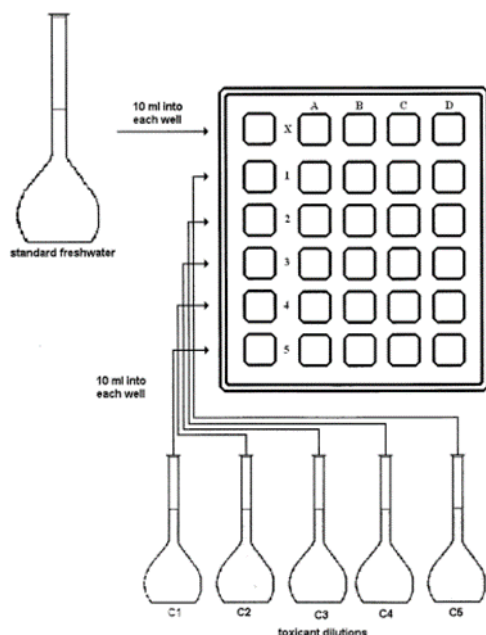


Figure A-1. Multiwell plate layout for EC_{50} values of *D. magna*. Effluent concentrations were C1 = 100%, C2 = 50%, C3 = 25%, C4 = 12.5%, and C5 = 6.25% corresponding to rows 1-5. Columns A-D were replicates. The first column was used only as a holding area for *D. magna* to be placed into corresponding cups of the multiwell plate. Image courtesy of EBPI Inc.

Name of operator :	David Walker											
Date of performance of test :	7/19/2022											
Sample or toxicant name:	DS Treatment 6.29.22											
Dilution series tested :	concentration C1	100	%									
	concentration C2	50										
	concentration C3	25										
	concentration C4	12.5										
	concentration C5	6.25										

Immobilized	Control		C5		C4		C3		C2		C1	
Exposure (h)	24	48	24	48	24	48	24	48	24	48	24	48
A	0	0	1	1	0	1	0	1	1	1	1	4
B	0	0	0	1	0	2	0	2	0	2	2	1
C	0	0	0	0	0	1	0	1	1	3	1	2
D	0	0	1	0	0	0	1	1	0	1	1	2
Total	0	0	2	2	0	4	1	5	2	7	5	9
% Effect	0	0	10	10	0	20	5	25	10	35	25	45

Validity criteria	YES	YES										
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Figure A-2. Example result sheet for dead/immobilized *D. magna* following 24- and 48-hour exposures to raw river water

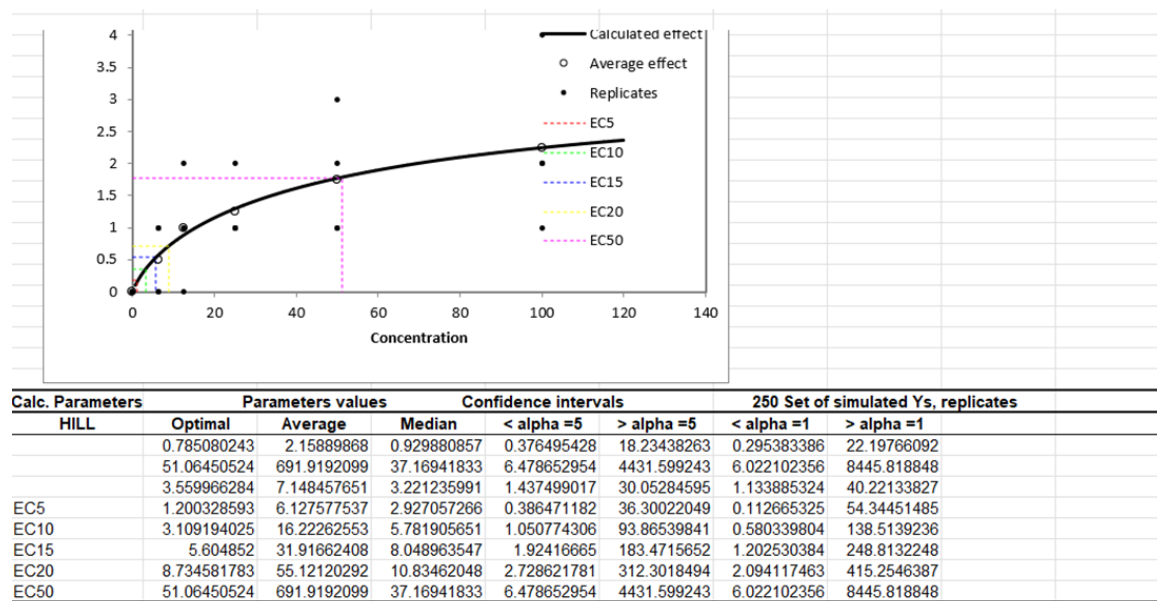


Figure A-3. Example of calculated EC50 values using the Hill slope equation

LENGTH MEASUREMENT OF OSTRACOD NEONATES

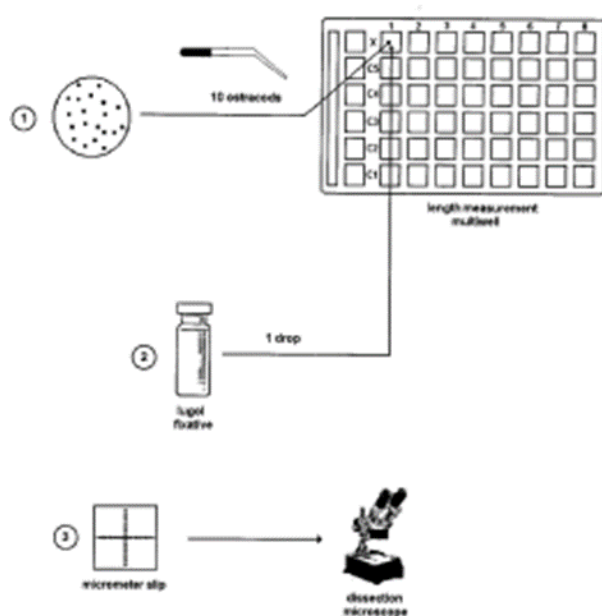


Figure A-4. Obtaining length measurements of day zero ostracod neonates. Image courtesy of EBPI Inc.

ADDITION OF STANDARD FRESHWATER, SEDIMENT, ALGAL FOOD AND OSTRACODS TO THE TEST WELLS

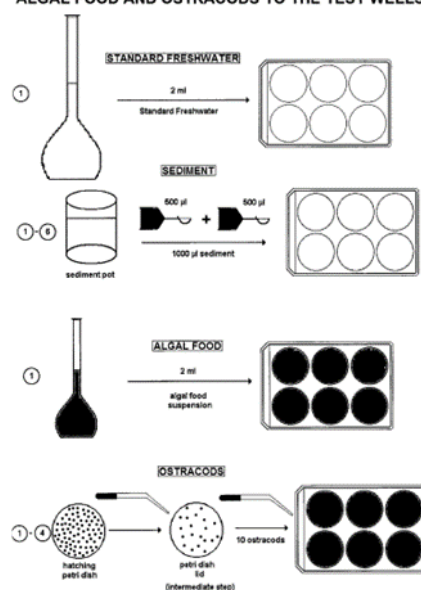


Figure A-5. Setup procedure for sediment toxicity testing using *H. incongruens*. Photo courtesy of EBPI Inc.

	Replicate	DAY 6						DAY 6					
		Replicate						Replicate					
		1	1	2	3	4	5	6	1	2	3	4	5
1	154	1087	988	753	890	870	880	665	882	629	963	745	707
2	198	1140	907	1050	866	770	867	934	701	749	804	682	671
3	158	916	781	763	895	1082	1113	874	843	984	659	794	645
4	194	964	1132	850	863	780	1038	722	968	625	768	681	853
5	146	959	843	921	939	1101	813	801	842	909	909	938	627
6	171	1174	760	1146	924	752	1005	847	690	685	838	887	667
7	217	1062	1081	937	892	1159	1064	870	896	856	781	715	778
8	180	1126	746	1095	739	803	962	884	704	947	664	918	918
9	218	865	947	1039	###	896	996	704	934	795	902	849	653
10	181	955	1164	1141	984	1035	1016	959	642	666	894	921	982
MORTALITY													
Number of dead ostracods per replicate	0	1	0	0	0	1	0	1	1	2	2	3	2
Mean Mortality for all replicates	0	0						2					
Mean % mortality for all replicates	0	3						18					
Standard deviation of mean % mortality	N.A.	1						1					
Variation coefficient of mean % mortality	N.A.	15						4					
GROWTH INHIBITION													
Mean length of ostracods per replicate	181.7	1025	935	970	911	925	975	826	810	785	818	813	750
Mean length for all replicates	184.778	957						800					
Mean length increment per replicate		840	750	785	726	740	791	641	625	600	633	628	565
Mean length increment for all replicates		772						616					
Mean growth inhibition (in μ) for all replicates								156					
Mean % growth inhibition in test sediment								20					

Figure A-6. Example calculation sheet for soil/sediment toxicity to *H. incongruens*

Appendix B. Comprehensive Literature Review of Imazapyr Properties and Toxicity to Organisms other than Aquatic Macroinvertebrates

The active ingredient in Arsenal® is an isopropylamine salt (2-[4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1H-imidazol-2-yl]-3-pyridinecarboxylic acid). Imazapyr is a member of the imidazolinone class of herbicides which also includes imazapic, imazamox, imazethapyr, imazamethabenz, and imazaquin. It is quickly taken up by the roots, leaves, and stems of plants and transported via xylem and phloem to meristematic tissue where it inhibits the enzyme acetohydroxy acid synthase (AHAS). This enzyme catalyzes the first two reactions in the synthesis of the branched chain amino acids isoleucine, valine and leucine (Osuna et al., 2003).

Imazapyr has a high hydrophilicity ($K_{ow} = 1.3$), low rate of soil sorption ($K_{oc} = 100 \text{ mL g}^{-1}$) and a high potential of groundwater infiltration (Gianelli et al., 2013). Soil sorption capacity is also pH dependent with highly alkaline soils showing lower rates (US EPA, 2005). It is highly mobile in surface waters. Degradation of imazapyr in soil is primarily via microbial degradation with a half-life ranging from 1-5 days depending upon environmental conditions (Dugdale et al., 2020). Degradation in surface water is rapid compared to soils with half-lives ranging from 2-5 days (Dugdale et al., 2020).

Acute imazapyr toxicity to birds (using mallard ducks and bobwhite quail as bioindicator species) on a dose-response level, is considered relatively “low” (Tu et al., 2001). Fletcher (1984a, 1984b) reported no mortality in duck or quail given diets dosed with 5,000 mg/L of imazapyr. This would be equivalent to 674 mg/kg body weight in quail and 1,149 mg/kg bw in ducks. Lethal dose concentrations required to cause mortality in 50% of a population (LC50) in was $> 2,150 \text{ mg/kg}$ for ducks and quail (Fletcher et al., 1984a, 1984b). During 18-week reproductive studies, no significant results were observed in egg production, hatching rate, or hatchling survivability when the F0 generation was fed a diet of up to 2,000 ppm imazapyr (Fletcher et al., 1995a, 1995b).

Imazapyr toxicity to mammals is similar to birds and relatively low. LD50 concentrations of acute toxicity $> 5,000 \text{ mg/kg bw}$ and $> 2,000 \text{ mg/kg bw}$ to rats and rabbits respectively (Hess et al., 2010). The effects of chronic toxicity are also reported as low with no observable effect levels in dogs fed 10,000 ppm imazapyr and rats 20,000 ppm over a 90-day period. No reproductive or developmental effects were reported in rats or rabbits exposed to 200 mg/kg bw/day in rats and rabbits. Imazapyr has been reported as non-mutagenic and non-genotoxic (Hess et al., 2010).

There appears to only be a single study examining imazapyr toxicity to terrestrial invertebrates with the honey bee being the bioindicator species. For honey bees, toxicity risk was very low and similar to birds and mammals. The weight adjusted LD50 was 1,000 mg/kg bw (SERA, 2004). Much more data is needed on the effects of imazapyr to terrestrial invertebrate species.

Although more studies have been performed on the toxicity of imazapyr to fish species than invertebrates, overall numbers of studies, especially field-based, are low with mixed results in terms

of overall toxicity. Golombieski et al. (2016) found that the combination of imazapyr and imazapic caused significant, acute effects on immunotoxicity, metabolism, ionoregulation and neuro-transmission in the silver catfish *Rhamdia quelen*. The US Environmental Protection Agency (USEPA) found that, on dose-response analyses, imazapyr was “practically non-toxic” to fish with LC50 and EC50 values >100 mg/L (US EPA, 2005a). However, the USEPA (2005a) did find that fish larval survival was decreased following chronic exposure to imazapyr. Patten et al. (2003) found that imazapyr concentrations up to 1,600 mg/L did not affect osmoregulation in Chinook salmon (*Oncorhynchus tshawytscha*) smolts.

There are a few reports of imazapyr, including Arsenal®, negatively affecting amphibian populations but, like fish, results are mixed. Babaloe et al. (2021) reported that Arsenal® was slightly toxic to developing *Xenopus laevis* larvae following a 96-h exposure at environmentally relevant concentrations. The authors developed a teratogenic index and found that Arsenal® provided a moderate risk of malformation in this species. Yet, Yahnke et al. (2013) found that Arsenal® caused no significant effect for any of the endpoints (behavior and body description) they measured in the Oregon spotted frog (*Rana pretiosa*).

Outside of a few studies, most of the toxicological effects of imazapyr have been of the active ingredient alone and not on adjuvants or surfactants commonly added to commercial mixtures. The Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) oversees the registration process of herbicides and pesticides. It does not require toxicological profiling of adjuvants or surfactants that are often proprietary. For example, the label for Arsenal® claims that 53.19% is imazapyr and 46.9% “other ingredients” that are proprietary. However, often the surfactant nonylphenol ethoxylate (a nonionic surfactant) is added to herbicides to enhance the effect(s) of the primary ingredient. Grisolia (2004, p. 124) positively identified that Arsenal® formulation contained “25 g/L Imazapyr, 186 g/L of ammonium hydroxide, 18g/L of nonylphenol ethoxylate (9 ethoxylated rings) and water”. Surfactants can greatly increase the toxicity of the parent compound in herbicides and pesticides and toxicological studies examining only the active ingredient may seriously underestimate overall environmental relevance and toxicity.

Nonylphenol (NP) is the breakdown product of nonylphenol ethoxylate (NPE). NP is believed to be more toxic than the parent compound NPE, however both are toxic xenobiotic compounds shown to cause significant endocrine disruption in a variety of species (Gaido et al., 1997; Soto et al., 1991). They are all hydrophobic and partition strongly to soils and sediments. NPs are commonly found in treated wastewater where they emanate from surfactants used in detergents, soaps, emulsifiers, lubricants, oil additives, and antioxidants (NPs are primarily found in sludges and biosolids). NPEs are commonly found in rivers and lake sediments at concentrations ranging from 0.13 to 5.1 mg/L and from 250 to 1020 mg/kg, respectively (Chen et al., 2010). Both xenobiotics moderately bioaccumulate and biomagnify.

Besides endocrine disruption along the hypothalamic-pituitary-gonadal axis (causing either feminization or androgenization), NPs are “highly toxic to fish, aquatic invertebrates, and aquatic plants” (US EPA, 2005). The 28-day no observed effect concentration (NOEC) for fish ranges from 0.05 to 0.07 mg/L and the 28-day lowest observed effect concentration (LOEC) ranges from 0.12 to

0.19 mg/L. A 33-day NOEC for fish is 0.007 mg/L and the 33-day LOEC is 0.014 mg/L. The 21-day NOEC for aquatic invertebrates ranges from 0.10 to 0.24 mg/L (US EPA, 2005). NPs are significantly more toxic to aquatic life than the active ingredient found in Arsenal® (imazapyr) yet does not factor into registration of the herbicide through FIFRA.

The most common degradant of NPE breakdown is 4-nonylphenol (4-NP). The effect(s) of 4-NP, besides that of endocrine disruption, is genotoxicity and perhaps mutagenicity. Soares et al. (2008) showed that 4-NP interferes with many regulatory pathways in cells of many organs. Park and Choi (2007) showed that 4-NP is genotoxic to the aquatic macroinvertebrate *Chironomus tentans* and Petridis et al. (2009) showed clear evidence of genotoxicity in the mollusk *Strobilus planus*. Frassinetti et al. (2011), using the bacterium *Saccharomyces cerevisiae* and D7 mutation assay, showed that 4-NP is mutagenic and genotoxic. In the only study found examining overall Arsenal® toxicity, separated by the primary ingredient of imazapyr and the NP surfactant, Grisolia et al. (2004) found clear evidence that 1) the NP fraction within Arsenal® was highly toxic to *Drosophila melanogaster* (fruit fly), *Mus musculus* (Swiss albino mice), and *Biomphalaria tenagophila* (snail) while imazapyr was not toxic to the same species and 2) the NP fraction was genotoxic and mutagenic to *Allium cepa* (onion) via mitotic spindle disturbance. Again, FIFRA does not require registering anything but the primary active ingredient, imazapyr, and not any surfactant such as nonylphenol yet, organisms are exposed to both in the environment, especially AMs.

The Department of the Interior protects and manages the nation's natural resources and cultural heritage; provides scientific and other information about those resources; and honors its special responsibilities to American Indians, Alaska Natives, and affiliated Island Communities.

NPS 155/193236, April 2024

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