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Hydraulic Fracturing Fluid Biocide, Tributyl Tetradecyl Phosphonium Chloride, Causes Mitochondrial Dysfunction that is Enhanced by Sodium Chloride in Chironomus Riparius

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HYDRAULIC FRACTURING FLUID BIOCIDE, TRIBUTYL TETRADECYL PHOSPHONIUM CHLORIDE, CAUSES MITOCHONDRIAL DYSFUNCTION THAT IS ENHANCED BY SODIUM CHLORIDE IN *CHIRONOMUS RIPARIUS*.

By

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Abstract

Tributyl tetradecyl phosphonium chloride (TTPC) is a biocide utilized in the hydraulic fracturing process to extract oil and natural gas from deep underground. This study used 4th instar Chironomus riparius to investigate the toxicity of TTPC, NaCl, and TTPC+NaCl. Our results show that the 24 h LC_{50}s for TTPC, NaCl, and TTPC+NaCl were 0.57 mg/L, >10,000 mg/L, and 0.32 mg/L, respectively, while the 48 h LC_{50}s for the same treatments were 0.48 mg/L, 9808 mg/L, and 0.22 mg/L, respectively. Additionally, TTPC's mechanism of action was investigated by measuring the levels of adenosine triphosphate (ATP), superoxide dismutase (SOD), and lipid hydroperoxides (LPO) as indicators of compromised viability. Red (cool) laser light, which stimulates cytochrome c activity and enhances ATP production, was utilized for a first time in macroinvertebrates to test the mitochondrial function. TTPC treatment resulted in significant increases in ATP levels, SOD activity, and levels of LPO. Additionally, there was a failure in the stimulation of ATP production in response to red laser exposure. Furthermore, TTPC+NaCl treatment resulted in significant increases in SOD activity, ATP, and LPO levels. These results showed increasing toxicity as a synergistic effect with the combined TTPC+NaCl treatment. However, results for NaCl alone showed no change in ATP levels but increased in SOD and LPO levels. These results indicated that radical oxygen species caused mitochondrial dysfunction by damaging the membrane. Adding NaCl to TTPC showed to increase the toxicity of TTPC, which enhanced the damage to mitochondrial membrane, even though the SOD activity was not detected. The toxicity of TTPC to macroinvertebrates was addressed for the first time with calculation

of LC_{50}. This shows that hydraulic fracturing fluids entering freshwater ecosystems could put aquatic organisms at risk.

A. Introduction:

1- What is Hydraulic Fracturing?

Nowadays with the development of industries and the growth of human populations, sources of environmental pollution have increased. These contaminants have significant impacts on human and wildlife, in particular aquatic biota. Contamination of water resources with industrial chemicals could lead to extinction of some aquatic species or their forced adaptation to new or altered environments. One current industrial development that concerns many environmental scientists is hydraulic fracturing. This modern technology extracts oil and gas from deep underground in areas of the United States where production was once considered impractical[1]. It is the process used to extract natural gas and oil from shale formations by using a pressurized drilling technique that injects fracturing fluids, a mix of additive chemicals, sand or ceramic beads (proppant) and water, into deep wells under high pressure conditions[2]. This fluid then penetrates the perforations in the pipe in the horizontal borehole and induces cracks in the rock formations. The purpose of the sand or ceramic is to ‘‘prop’’ the pores open after pressure release, when the liquid medium is withdrawn[3]. This process allows fracturing of geological formations, increasing permeability and extracting of oil and gas.

2- Hydraulic fracturing water sources:

Because of the improvement in the fracturing technique, the number of natural gas wells in the U.S. alone has increased by 200,000 in the last two decades, and it is projected to increase gas production to about 1065 billion per year by 2040 [2]. This technique was met with criticism and wrath of environmental organizations around the world, especially the U.S.A., because of fears of leakage of the fracking fluid chemicals and the processed water into nearby aquifers and surface water, which could have potential effects on drinking water and the environment.

Previous estimates in the oil and gas industry indicate that hydraulic fracturing uses about 100 million gallons of water in California annually[1]. Another source indicates that nearly 92 billion gallons of water were used for hydraulic fracturing throughout the time period studied: 36 billion gallons in 2011, 52 billion gallons in 2012, and 3.8 billion gallons in the first two months of 2013 [4]. Based on the analyzed data of hydraulic fracturing well disclosures in a 2015 EPA report, Pennsylvania had the third largest number of disclosures (2,467; 6.5% of disclosures) and the second largest cumulative total water volume (approximately 11 billion gallons)[4]. Water volumes in the Marcellus Shale, for example, have been reported to range from 3 to more than 5 million gallons per well[5]. Gas disclosures report a median total water volume of approximately 2.9 million gallons, and oil disclosures report a median total water volume of approximately 1.1 million gallons. Total water volumes reported in gas disclosures range from approximately 91,000 gallons to approximately 7.8 million gallons (5th to 95th percentile). Total water volumes reported in oil disclosures range from approximately 18,000 gallons to approximately 6.1 million (5th to 95th percentile)[4]. Most hydraulic fracturing disclosures do not indicate the exact source of water used in the fracturing fluid, while some of them state the water source as "fresh, surface, and recycled water" rather than indicate the specific identification of the body of water used[4]. Another source points out that the water used in fracturing processes comes from groundwater, surface water and reused hydraulic fracturing wastewater[6]. Most of the water used for hydraulic fracturing in the United States is freshwater: only 5% of the injected volumes come from reused wastewater according to the EPA report[4].

3- Hydraulic fracturing fluid composition:

Due to public concerns about the composition of hydraulic fracturing fluids, the Ground Water Protection Council (GWPC) and the Interstate Oil and Gas Compact Commission

(IOGCC) developed the FracFocus Chemical Disclosure Registry (subsequently referred to as "FracFocus"). FracFocus is a publicly accessible website (www.fracfocus.org) where oil and gas production well operators can disclose information about the ingredients used in hydraulic fracturing fluids at individual wells[4]. EPA analyzed FracFocus data for more than 39,000 individual oil and gas production well disclosures provided to the U.S.A. Each disclosure data has the list of additives used in the fracturing fluid of the well, trade names, concentrations, the purpose of each addictive, and the total water volumes. The most additive ingredients commonly reported were methanol, hydrochloric acid, and hydrotreated light petroleum distillates (reported in 71%, 65%, and 65% of disclosures, respectively). Among proppants, quartz was the most common material reported (present in at least 98% of disclosures that identified proppants), with a median maximum hydraulic fracturing fluid concentration of 10% by mass[4]. Some purposes of additive ingredients in fracturing fluids include: acetic acid as a buffer to reduce fluid volume and improve proppant carrying capacity[4], potassium hydroxide and potassium carbonate as crosslinkers to control the solution's pH and protect pH-dependent effectiveness of other chemicals[1], peroxydisulfuric acid and diammonium salt as a gel breaker, sodium chloride as a breaker, friction reducer, scale inhibitor, and clay control, and biocide[4]. One common biocide is tributyl tetradecyl phosphonium chloride (TTPC). It is used to minimize bacterial contamination of hydrocarbons, reduce bacterial production of corrosive by-products to maintain wellbore integrity and prevent breakdown of gellants[1]. Companies are willing to keep using these chemicals because they are all necessary to make the fracturing process efficient; however, there is not much information about the effects of these chemicals when they are used for fracking. Also, some of the companies do not disclose the chemicals

involved in their process so it is impossible to evaluate possible detrimental effects of some fracturing fluids[4].

Of the chemicals used in hydraulic fracturing fluid, 75% have effects in human skin, eyes, other sensory organs, and the respiratory and gastrointestinal systems, while 40-50% have serious impacts in nervous, immune and cardiovascular systems. In addition to that, 37% and 25% could affect the endocrine system and cause cancer and mutations, respectively[7].

4- Hydraulic fracturing water types:

There are three different types of water in hydraulic fracturing operation: fracturing fluid, flowback water, and produced water, and each of these water types could have different concentrations of the same compound. Fracturing fluid water is the mixture of chemicals, sand and water that is used in the fracturing operation. Produced water is the water resulting due to fracturing of the well. Because there is always confusion between the two terms, "flowback" and "produced water", some papers distinguished between them. Flowback is the immediate return of injected fluids and water, and produced water is fracturing fluid mixed with formation water native to the well. Other definitions distinguish these two types of water as the initial water that is recovered (for flowback) and the long-term water (for produced water) that comes back while the gas production is taking place[2].

5- Impacts on drinking water:

Several sources mention evidence of environmental issues related to the hydraulic fracturing. The first issue is related to sources of water used in the fracking process. The large volumes of water used in fracturing process are frequently withdrawn from groundwater, river, and streams. This can lead to a decrease in the amount of water in the streams thereby reducing

drinking water supplies as well as habitat for aquatic organisms[7]. "Approximately 9.4 million people live within one mile of a hydraulic fracturing well between 2000 and 2013 [6]. Furthermore, approximately 6,800 sources of drinking water for public water systems were located within one mile of at least one hydraulically fractured well during the same period. These drinking water sources served more than 8 million people annually in 2013" [6]. The fracturing operations in the eastern region of the United States rely on surface water, while operation in the western states, which are more semi-arid to arid, use mixed supplies of surface and ground water. For instance, the Marcellus Shale site in Pennsylvania uses fracturing water from surface water, while the Barnett Shale site in Texas uses a mix of surface and groundwater. However, in areas that lack surface water, groundwater is the only source for fracturing water supply[6]. According to FracFocus disclosures, water used in fracturing operations increased from 2010 to 2012 by 10% in some counties, whereas it increased by 50% in other counties[6]. The reviewers indicate that hydraulic fracturing operations could be one of the factors affecting drinking water availability. In Louisiana, the state requested switching from ground to surface water for hydraulic fracturing operations due to the concerns that ground water withdrawals could affect availability of drinking water supply in combination with other uses. Furthermore, the effects of withdrawal water in the availability of drinking water depend also on the amount of available drinking water and the number of hydraulic fracturing operations in the state. For instance, Sothern and Western Texas have high fracturing operations with low water availability[6]. Scanlon *et al* (2014) observed excessive drawdown of local ground water in a small proportion (approximately 6% of the area) of the Eagle Ford Shale in southern Texas[8]. In this case, withdrawal of groundwater not only affected the quantity of drinking water but also the quality of groundwater because the rate of withdrawals exceeded natural recharge rates

decreasing water storage in aquifers. This potentially mobilized contaminants or allowed the infiltration of lower quality water from the land surface or adjacent formations. Withdrawals could also decrease ground water discharge to streams, potentially affecting surface water quality. Areas with large amounts of sustained ground water pumping are most likely to experience impacts particularly drought-prone regions with limited ground water recharge[6].

Another issue related to sources of fracturing water is contamination of drinking water. Drinking water can become contaminated due to subsurface mechanisms of fracturing fluid injection and the creation and propagation of fractures. The two major mechanisms are the unintended movement of liquids or gases out of the production well or along the outside of the production well into a drinking water resource via deficiencies in the well's casing or cement, and the unintended movement of liquids or gases from the production zone through subsurface geologic formations into a drinking water resource. For instance, in North Dakota, an inner string of casings burst during hydraulic fracturing that resulted in a release of fluids on the land surface and possibly into the aquifer near the city of Killdeer[6]. Another instance occurred in Bainbridge, Ohio, where inadequately cemented casing in a hydraulically fractured well contributed to the buildup of natural gas and high pressures along the outside of a production well. This ultimately resulted in movement of natural gas into local drinking water aquifers[9, 10]. Also, in the Mamm Creek gas field in Colorado, inadequate cement placement in a production well allowed methane and benzene to migrate along the production well and through natural faults and fractures to drinking water resources[11, 12, 13].

Some studies have measured changes in ground and surface water at hydraulic fracturing sites. A 2015 EPA study focused on the potential impacts of hydraulic fracturing on drinking water resources in five sites across the United States (Washington County, Pennsylvania

(southwestern Pennsylvania); Bradford County, Pennsylvania (northeastern Pennsylvania); Wise County, Texas; Las Animas and Huerfano Counties, Colorado (Raton Basin); and Dunn County, North Dakota (Killdeer)[14]. The study focused on investigating reported instances of drinking water resource contamination in areas where hydraulic fracturing had already occurred and were intended to inform several of the primary research questions related to chemical mixing, well injection, and flowback and produced water. For instance, in Washington County, the Marcellus Shale ranges in thickness from less than 50 to about 150 feet, and varies in depth from about 5,000 to over 7,000 feet below land surface14. Water samples were collected from 16 domestic wells, three springs, and three surface water locations during three events in July 2011, March 2012, and May 2013. The domestic wells sampled in Washington County ranged in depth from 50 to 160 feet below land surface, with a median depth of 95 feet below land surface. The water quality data of the samples were compared with the data from previous sources for water samples collected before 2005, which means before the discovery of Marcellus Shale gas. The study found an elevation in the chloride level based on comparison with historical water quality data in the water samples collected from locations near the Yeager impoundment in Amwell Township, where drilling wastes and wastewater associated with the hydraulic fracturing water cycle were stored, and the concentration exceeded the EPA's secondary maximum contaminant levels (MCLs) for drinking water supply. In addition, methane, which is odorless and tasteless in water and can outgas and produce flammable or explosive environments at high concentrations, was found in 24% of the groundwater and spring water samples collected and the detected concentrations ranged from about 0.002 to 15.5 milligrams per liter (mg/L), with a median value of 0.045 mg/L. For the organic chemicals analysis, 133 organic compounds detected in the collected samples, were the same ones used in hydraulic fracturing fluids in Pennsylvania14.

Taken together this data indicates that there can be an impact of the fracturing process on freshwater water quality as well as drinking water supply.

Researchers of oil and gas companies state that there is a physical space separating the production zone and drinking water zone that protects drinking water resources. However, there are some hydraulic fracturing operations that occur in zones not that far from drinking water zones. For example, "hydraulic fracturing in the Antrim Shale, Michigan site and New Albany Shale, Illinois, Indiana and Kentucky sites take place at shallower depths (100 to 1,900 ft or 30 to 579 m, respectively) with less vertical separation between the formation and drinking water resources. Moreover, an EPA 2009 to 2010 survey of oil and gas production wells found that 20% of 23,000 wells had less than 2,000 ft (610m) of measured distance between the point of shallowest hydraulic fracturing and the base of the protected ground water resources reported by well operators". Another statement mentioned that there are some places where the oil and gas production zone and drinking water resources co-exist in the same formation, which could result in the introduction of the fracturing fluid into formations that may currently, or in the future, be a drinking water resource[6].

Darrah and colleagues (2014) demonstrated that while upward flow of leaking hydraulic fracturing fluids would be substantially slower than that of buoyant natural gas, production well failure in the proximity or above an aquifer is a more likely potential pathway for groundwater contamination by fracturing fluid components[15]. A recent study by Eaton (2013) pointed out that some of the actual regulatory frameworks for hydraulic fracturing are not adequate to prevent contamination of water supplies in the city of New York[16]. Another study by Ziemkiewicz *et al* (2014) proposed protective measures in field construction and maintenance in order to minimize

exposure of hydraulic fracturing activities in waste streams that were found to contain exceeding limits of contaminants[17].

6- Pathways of fracturing fluid chemical additives and potential spills:

There are multiple pathways through which hydraulic fracturing fluids could contaminate surface and drinking water. The pathways include: transportation of chemicals to well pads, mixing of chemical additives with the bulk of the fracturing fluid, injection of the mixed fluid into the borehole (often occurs simultaneously with in-line mixing), handling, collection, and storage of chemical-containing produced water, and reuse, treatment, recycling, and/or disposal of the produced water. Some of the modes of potential environmental exposure of chemicals used in hydraulic fracturing fluid could be due to several issues. The first potential issue is accidental surface spills during transportation of chemicals to or off the site via pipelines, trains, or trucks, or well blowouts and casing failures. Any of these could be a cause of contamination of soil and runoff into surface water. The second issue is surface spills leaching into shallow aquifers. They are often a result of use of lined pits to temporarily store and evaporate flowback brine in order to reduce the volume of waste, contamination of shallow groundwater via borehole leakage, fault lines, and abandoned wells, and contamination of shallow groundwater via induced fractures[18].

While complete data of surface spills are lacking and difficult to ascertain due to oil and gas company discretion, some robust databases of spills in the State of Colorado are published. According to the Colorado Oil and Gas Conservation Commission (COGCC), "in 2013, there were 591 reported spills, which released a total of 14,067 barrels (i.e., ~2,200,000 L) or 0.004% of all produced water. This relates to a total of 50,067 active oil and gas wells in Colorado, including 4,025 new wells that had been drilled in 2013" [18]. According to EPA data analysis, if

the national hydraulic fracturing fluid spills estimates are representative, the number of spills nationally could range from approximately 100 to 3,700 spills annually, assuming 25,000 to 30,000 new wells are fractured per year[6]. Based on a study of 151 cases of fracturing fluid or chemical spills occurring between January 2006 and April 2012 in 11 states, the median volume of a spill is 420 gal (1,600 L) per spill. Causes for spills include equipment failure, human error, and failure of container integrity. The most known cause of spills, 34%, was equipment failure, specifically blowout preventer failure, corrosion, and failed valves. Leakage of fracturing fluid from storage units accounted for 30% of the spills[6].

Generally, the closer the geographical proximity of a susceptible ecosystem to a drilling site or location of related industrial processes, the higher the risk that the ecosystem will be impacted by the operations[1]. The result of these operations could lead to increase erosion and sedimentation, risk to aquatic ecosystems as a result of chemical spills or runoff, and the availability of surface and water volume may decrease due to withdrawal-induced lowering of local groundwater levels[1]. In a study of 151 spills by EPA, fracturing fluids reached surface water in 13 (9% of 151) cases and soil in 97 (64%) cases[6]. Researchers, Papoulias and Velasco, demonstrated that in Kentucky a spill impacted a surface water body relatively quickly when hydraulic fracturing fluid entered a creek, significantly reducing the water's pH and increasing its conductivity[19].

7- Wastewater of hydraulic fracturing components:

Produced water of oil and gas production wells usually contains high levels of total dissolved solids (TDS) and ionic constituents such as: bromide, calcium, chloride, iron, potassium, manganese, magnesium, and sodium. Also, it may contain metals such as: barium, cadmium, chromium, lead, and mercury, and organic compounds such as benzene[6]. The EPA

identified 134 chemicals in produced water some of them came from hydraulic fracturing fluid, while other occurred naturally such as: organic chemicals, radionuclides and metals and other chemicals mobilized by the hydraulic fracturing process. Exact concentrations of most chemicals in produced water are limited due to the difficulties in obtaining samples from actual produced water from wells as well as the inadequacy of methods available to analysis the produced water samples[6].

8- Management of fracturing wastewater:

Disposal of fracturing fluid waste water is another environmental concern. Clark and Veil (2009) estimated that, in 2007, approximately one million active oil and gas wells in the United States generated 2.4 billion gal per day (9.1 billion L per day) of wastewater[20]. The EPA recommends several ways used to manage hydraulic fracturing wastewater such as: 1) disposal in Underground Injection Control (UIC) wells, also called disposal wells through evaporation ponds, 2) treatment at Centralized Waste Treatment Facilities (CWTs) followed by reuse or by discharge to either surface waters or a Publicly Owned Treatment Works (POTWs), 3) reuse with minimal or no treatment, and 4) land application or road spreading[6]. According to a survey of state agencies in 2007, more than 98% of produced water from oil and gas companies was managed via underground injection[20]. Another common way to manage wastewater is land application. For example, wastewater is spread on roads for purposes of deicing or dust suppression. This process could potentially introduce wastewater into surface and ground water due to runoff and migration of brines. Studies of road spreading of conventional oil and gas brines have found elevated levels of metals in soils and chloride in ground water[6]. Hydraulic fracturing wastewater treated at commercially operated industrial wastewater treatment plants and Municipal Wastewater Treatment Plants (MWTPs) has also raised concerns. MWTPs are

designed for common compounds (nutrients and organic matter) and not intended to treat the multitude and amounts of chemical species that resurface with flowback brine. Furthermore, effluents from both types of facilities are typically released into natural streams and waterways. Unfortunately, the Wastewater Treatment Plants (WWTPs) and commercial treatment plants are not effective at reducing contaminants found in the highly saline, hydraulic fracturing wastewater. Release of this inadequately treated wastewater threatens drinking water supplies to drinking water treatment facilities as they may contain high concentrations of total dissolved solids (TDS), bromide, chloride, and iodide. In particular, bromide and iodide are precursors of disinfection byproducts (DBPs) that can form in the presence of organic carbon in drinking water treatment plants or wastewater treatment plants. Drinking water treatment plants are required to monitor for certain types of DBPs because some are toxic and can cause cancer[6].

9- Hydraulic fracturing biocide (TTPC):

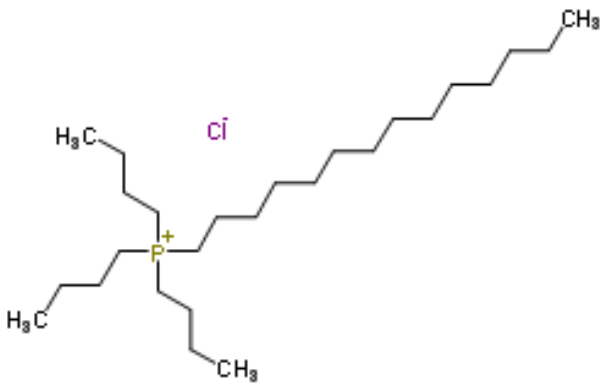
Biocides are a common component of hydraulic fracturing fluids. At total concentrations of up to >500 mg/L and total fluid volumes surpassing 10 million L per horizontal well, biocides can exceed 1,000 gallons per hydraulic fracturing events[18]. The goal of biocide application in fracturing fluids is to minimize bacterial contamination of hydrocarbons and to reduce bacterial production of corrosive by-products in order to maintain wellbore integrity and prevent breakdown of gellants. Presence of bacteria may cause bioclogging and inhibit gas extraction, produce toxic hydrogen sulfide and induce corrosion leading to downhole equipment failure[21, 18]. TTPC is a common biocide added to fracturing fluid, and there is limited information pertaining to its environmental toxicity. The molecular formula of TTPC is $C_{26}H_{56}ClP$, its molecular weight is 435.15, its pH is 6-8, and its boiling point is 100 °C [22]. TTPC is stable and not reactive to water[21]. No literature sources have determined its persistence and bioaccumulative potential;

however, several sources have confirmed that TTPC is extremely toxic[21]. In terms of acute toxicity, its 96 h LC_{50} (lethal concentration affecting 50% of the exposed population) in fish is 0.58 mg/L, while its 96 h TLM (median tolerance limit) in caridean shrimp (*Crangon crangon*) is 1.6 mg/L and its 48 h TLM in water flea (*Daphnia magna*) is 0.025 mg/L [22]. From the Halliburton company website, the percentage of TTPC in hydraulic fracturing fluid used in Pennsylvania wells is 0.3 gal/1000 gal or 0.00333 % by mass in 2013, which is equal to 33.3 mg/L. This concentration is several orders of magnitude above its acute toxicity. Also, TTPC concentrations used in North Dakota, East Texas, West Texas, South Texas, West Virginia and Oklahoma wells are 0.25 - 0.5 gal/1000 gal of fracturing fluid, while in North Texas the concentration is 0.25 - 0.6 gal/1000 gal[23].

TTPC is a quaternary phosphonium biocide with a tetradecyl chain which causes the molecule to have surface-active properties[24]. It is one type of lytic biocide or "amphiphilic surfactants". The surface acting properties of TTPC causes severe damage to microbial cell membranes and deactivates cell enzyme processes[25]. The biological activity of amphiphilic surfactants is generally based on dissolution and subsequent disruption of the bacterial cell wall. Specifically, their known mode of action involves binding to anionic functional groups on the membrane surface and subsequent perturbation and dissolution of the lipid bilayer, resulting in loss of osmotic regulation capacity and eventual lysis of the cells[18]. TTPC is chlorine-compatible and will enhance ability of chlorine to penetrate the polysaccharide slime layer found in microbial biofilms. In the hydraulic fracturing environment, TTPC is cationic with high surface activity but low foaming tendency. It has a high level of hydrolytic stability, and functions over the entire pH range of open and closed cooling water systems. Several studies report that TTPC in processed water causes strong sorption of cationic organic amines to the surfaces and

interlayers of clay beyond the clay's cation exchange capacity, causing extensive clay aggregation[18]. Research also shows that cationic surfactants such as didecyl dimethyl ammonium chloride (DDAC) and TTPC absorb strongly to soil reducing their bioavailability and biodegradation rates and thereby potentially increasing their persistence in the environment[18]. Xue *et al* (2015) proposed that the mechanism of antimicrobial action for quaternary phosphonium biocides involves penetration into the cell wall and destructive interaction with the cytoplasmic membrane, followed by the leakage of intracellular components and consequent cell death. This study provides intuitive and persuasive evidence that supports the hypothesis about the antimicrobial mechanism of action for cationic biocides such as TTPC. "The electrostatic interactions between the cationic polymers and the lipid head groups result in the formation of interfacial complexes within the outer leaflet. The interaction also induces flip-flop of anionic lipid molecules from the inside to the outside leaflet, followed by significant distortions and phase separation of the phospholipid bilayer"[26].

Although research is scarce, developmental toxicity (i.e., teratogenicity) has been observed from several of the lytic biocides used in fracturing fluids, such as DDAC and TTPC, as well as the conventional chlorine dioxide. Preliminary EPA studies suggest TTPC exhibits some developmental toxicity with an oral LD^{50} of 1,002 mg/kg and inhaled LC_{50} of <0.9 mg/L[18]. The final report has not yet been published. Santillan (2015) states that TTPC not only has important implications to public safety due to its toxicity, but it may also have the unintended consequences of adding further toxicity to produced water and this should be a consideration when determining biocide use and produced water disposal. Furthermore, the study indicates that water analyses of samples containing TTPC show higher Cl concentrations than non-biocide samples. This is due to combining TTPC, a phosphonium cation, with chloride, a counterion[27].



Fgure 1: Structure of Tributyl Tetradecyl Phosphonium Chloride [TTPC] adapted from (Kahrilas, 2015).

10- <u>Hydraulic fracturing brine:</u>

A study by Haluszczak *et al* (2012)[28] assessed the components of flowback (produced) water from Marcellus shale in Pennsylvania. The study found that most inorganic components of the produced water increased with time following hydraulic operations: inorganics included Cl, Br, Na, K, Ca, Mg, Sr, Ba, Ra, Fe, Mn, and total dissolved solids (TDS). Median concentrations of Cl and Na in injected hydraulic fracturing fluid were 82 mg/L and 80 mg/L, respectively, compared to concentrations in flowback of 98,300 mg/L and 36,400 mg/L, respectively[28]. Concentrations of chloride in fracturing fluid were 82 mg/L compared to 0 to 2 mg/L in rainwater and 0 to 100 mg/L in freshwater lakes and streams[30]. As a result, if oil or gas well brine was entering the waterways, TDS and Cl levels would rise, along with other constituents of the brine[28]. Furthermore, according to the analysis of water samples from the Monongahela River, there are elevated of levels of TDS and Cl as well as associated golden algal blooms,

which could produce toxins that can suffocate aquatic organism[29]. The PA DEP determined that the hydraulic fracturing processes for extraction of natural gas could be contributing Cl and TDS to the water system[28].

Freshwater organisms only tolerate certain ranges of water salinity. Therefore, secondary salinization has an impact at the individual, population, community and ecosystem levels, which ultimately leads to a reduction in aquatic biodiversity and compromises the goods and services that rivers and streams provide[31]. It has been shown that different concentrations of NaCl can affect the normal osmo-regulatory and physiological processes of individual aquatic invertebrates. In spite of these findings, the responses of invertebrate communities to this stressor in the natural environment have not been well-defined[32].

With all these chemical compounds and brine, the chemical and physical quality of freshwater will be changed. The change in freshwater components will affect water ecosystems. Therefore, freshwater biota could be at risk due to hydraulic fracturing contamination. Thus, there is a need to study major constituents of hydraulic fracturing such as brine and biocides on biota including alone and in combination because the fracturing fluid contains a mixture of these components. The mixture may be more toxic than the individual constituents. To avoid the risk of these chemicals and help natural resource managers understand potential hazards of the hydraulic fracturing process, the molecular and physiological impacts of these components should be study. These studies should provide information about the sensitivity of aquatic biota to components of hydraulic fracturing as well as toxic mechanisms of action that can help distinguish effects of TTPC from other environmental stressors.

11- Superoxide dismutases (SOD), reactive oxygen species (ROS), and Lipid peroxidation (MDA):

Toxic chemicals are associated with oxygen radicals that cause damage to lipid membranes. An increase in lipid peroxidation indicates damage to cell membranes of animals. TTPC has been shown to damage the lipid bilayer of microbes; therefore, this project will investigate its ability to damage membranes by generating oxygen free radicals in a common freshwater organism called chironomid[18]. Studies with NaCl have shown a relationship between NaCl salt and SOD due to the osmotic stress that the salt produces[33]. Therefore, measuring lipid peroxidation and SOD will allow us to investigate the molecular target of TTPC and NaCl.

Lipid peroxidation is the oxidative degradation of lipids, which process as free radical electrons from the lipids in cell membranes, which leads to damage cells[34]. "Lipid hydroperoxides are a group of non-radical intermediates that derived from unsaturated fatty acids, phospholipids, glycolipids, cholesterol esters and cholesterol". Their formation can occur in both enzymatic or non-enzymatic reactions, which would involve activating chemical species known as "reactive oxygen species" (ROS). ROS are responsible for toxic effects occur in the body due to various types of tissue damage. ROS also include other free radicals such as: hydroxyl radicals, lipid oxyl, and peroxinitrite formed from nitrogen oxide (NO)[35].

Membrane lipids are particularly susceptible to lipid peroxidation. Many cellular organelles form by membranes such as: mitochondria, lysosomes, and plasma membranes. So, any damage in lipid peroxidation would affect the function of these organelles, which would lead to damage the cell. The main feature of fluidity in the bilayer of biological membranes comes from the presence of polyunsaturated fatty acids (PUFAs) in the phospholipids of this bilayer. Because of the function of lipid peroxidation is to attack the components that responsible for

these properties, the biophysical properties of membranes will be affected. Lipid peroxidation decreases the membrane fluidity, changes the phase properties of the membranes and decreases electrical resistance[36]. Also, cross-linking of membrane components restricts mobility of membrane proteins. The attacking of peroxidative to the biological membrane on PUFAs will lead to effect of these membrane as barrier. Therefore, lipid peroxidation may cause lysosomes to have a decreased, which make it leaky[37].

Exposure to NaCl has been associated with lipid membrane damage. According to a study conducted by Chen *et al* (2015)[36] in *Microcystis aeruginosa*, NaCl significantly increased the activity of superoxide dismutases (SOD) in treated versus control cultures. Antioxidant enzymes such as SOD in cyanobacteria were sufficient to remove reactive oxygen species (ROS) and prevent oxidative damage under normal conditions. In this study, ROS levels increased significantly and oxidative stress occurred since the antioxidant response was not adequate to remove all ROS under salinity stress. In addition, oxidative stress resulted in the release of microcystins (MCs) into surrounding water systems. MCs are powerful toxins that can destroy the liver structure and cause health hazards to humans and animals and even cause death in acute cases[36].

In *M. aeruginosa*, ROS levels were significantly changed by both the NaCl treatment and the incubation period, with both factors having a significant interaction. The increase was concentration-dependent. Moreover, elevated ROS concentrations caused membrane lipid peroxidation. This was demonstrated in *M. aeruginosa* by higher malondialdehyde (MDA) values in treated compared to control. MDA content was significantly altered by both the NaCl treatment and incubation period. MDA is a natural biomarker produced in the reaction of lipid peroxidation, which can be quantified and used for the evaluation of this process. It is used as

mutagenic in bacteria and mammalian cells and as DNA bases modifier[38]. Membrane lipid peroxidation also aggravated cellular stress and caused more MC production by the cyanobacteria since MC may act as a defense mechanism under stressful conditions. Consequently, this study in *M. aeruginosa* showed that treatment with NaCl, or brine, can cause membrane lipid peroxidation and that this type of cellular damage can be measured using SOD activity and levels of MDA[36].

TTPC is a cationic surfactant and, therefore, likely shares the same mechanism of action with other members of biocides in this group such as DDAC. DDAC is a cationic surfactant biocide used in fracturing process. A study on the acute toxicity of DDAC was done by Johnston *et al* (1998) in several aquatic species. The study tested DDAC with four fish and four aquatic invertebrate species. The 48-h LC_{50} values for *Daphnia magna*, *Mysidopsis bahia*, *Hyalella azteca*, and *Neomysis mercedis* were 0.037 ppm, 0.039 ppm, 0.106 ppm, and 0.947 ppm, respectively[39]. The mechanism of action for DDAC was not studied by Johnston *et al*. However, DDAC toxicity in bacteria cells has been associated with disruption of intermolecular interactions and dissociation of lipid bilayers[18]. The cell membrane damage caused by DDAC in *Escherichia coli* involved leakage of intracellular molecules and subsequent death of the cells[40]. Also, Kwon *et al* (2014) proved that DDAC increased intracellular reactive oxygen species (ROS) production while it decreased glutathione (GSH) activity in human lung epithelial cells, even if the exposure concentration was very low[41].

It is anticipated that because TTPC is structurally similar to DDAC, it will share the same mechanism of action with DDAC, which is to cause lipid bilayer membrane disruption. Consequently, it is anticipated that TTPC should have molecular mechanisms of action related to cell membrane disruption in animals as in microbes.

12- <u>Adenosine triphosphate (ATP) and Cytochrome *c* oxidase:</u>

Chemicals that damage and disrupt cell membranes have affected mitochondria that depend on their double membranes for ATP production. Effects on mitochondria have been detected by changes in levels of ATP and release of cytochrome *c*. Cytochrome *c* is an essential component of the mitochondrial respiratory chain. Its location in the inner membrane of mitochondria as a soluble protein. When the mitochondria damaged, cytochrome *c* releases as a key for apoptosis[42].

According to Sun *et al* (2012)[43], extracellular ATP (eATP) regulated a wide range of cellular processes required for salt adaptation, including vacuolar Na+ compart mentation, Na+/H+ exchange across the plasma membrane, K+ homeostasis, reactive oxygen species regulation, and salt-responsive expression of genes related to K+/ Na+ homeostasis and plasma membrane repair[43]. The purpose of their study was to know whether eATP might mediate salinity tolerance. The authors found that "the eATP signaling was mediated by H_2O_2 and cytosolic Ca^{2+} released in response to high salt in *Populus euphratica* cells, a type of plant". Therefore, they concluded that salt-induced eATP was sensed by purinoceptors in the plasma membrane (PM), and this led to the induction of downstream signals like H_2O_2 and cytosolic Ca^{2+}, which are required for the upregulation of genes linked to K+/Na+ homeostasis and PM repair[42].

Michea *et al* (2002)[44] demonstrated that mitochondrial dysfunction is an early event in high NaCl induced apoptosis[44]. The study utilized murine inner renal medullary collecting duct (mIMCD3) cells as a model to study the effects by raising osmolality to 700 mosmol/kg H_2O. Evidence of mitochondrial dysfunction included: 1) decreased tetramethylrhodamine methyl ester perchlorate (TMRM) fluorescence, as evidence of mitochondrial membrane depolarization,

2) increased cellular ADP/ATP ratio within 1-6 h, 3) changed mitochondrial morphology as nuclear hypercondensation became evident, 4) decreased mitochondrial Bcl-2/Bax within 1-3 h, and 5) reduction in mitochondrial p53 at any osmolality. All these results led the authors to conclude that increasing brine leads to mitochondria dysfunction and subsequently the initiation of apoptosis[44].

Some research indicates that TTPC could be a mitochondrial toxicant. For example, a TTPC-like compound called DDAC has been shown to injure mitochondria[41]. The study utilized lung epithelial cells as a model because DDAC can cause lung inflammation and fibrosis. "The 3-(4,5-dimethylthiazol-2- yl)-2,5-diphenyl-tetrazolium bromide (MTT), neutral red (NUR), and lactate dehydrogenase (LDH) assays were used to determine the damage to the mitochondria, lysosomes, and cell membranes, respectively. In MTT assay, they found that DDAC significantly decreased cell viability in a dose-dependent manner ($P < 0.01$) with a half-maximal inhibitory concentration (IC_{50}) of 16.64 μM at 24 h of exposure". To assess the cellular damage through lysosomal injury, DDAC-treated cells were analyzed using the NUR assay. Lysosome activity was significantly decreased at 24 h ($P<0.01$), with an IC_{50} value of 30.17 μM. Results of the LDH assay showed that the level of LDH was significantly increased at a DDAC concentration of 20-40 μM compared to that of control ($P<0.01$). These results from cytotoxicity screening suggested that the disruption of function by DDAC in the mitochondria was more sensitive than that in the lysosome or cell membrane for lung epithelial cells[41]. In addition, it is anticipated that TTPC will impact mitochondria due to its cationic properties. Research has shown that cationic nanoparticles (NPs) cause more pronounced disruption of plasma-membrane integrity, stronger mitochondrial and lysosomal damage, and a higher number of autophagosomes than anionic NPs[45]. The major source of radiation-induced ROS production in human lung carcinoma A549

cells was associated with mitochondrial damage measured by the release of cytochrome *c* into cytosol[46].

Therefore, several sources prove that brine and TTPC- like biocide have an impact on mitochondria function. Their outcomes were based on measuring mitochondrial biomarkers such as ATP and cytochrome *c;* therefore, these two biomarkers could be used to study mitochondrial dysfunction as a mechanism of action of fracking fluid components.

13- <u>Red (cool) laser light</u>:

Low-level laser (light) therapy (LLLT) is a new fast technique used as a therapy for various conditions that require stimulation of healing, relief of pain and inflammation, and restoration of function. One organelle responding to this technique is mitochondria. The red and near-infrared wavelengths are utilized to induce production of ATP from mitochondria. The photons are absorbed by mitochondrial chromophores, in particular cytochrome *c* oxidase which is contained in the respiratory chain located within the mitochondrial membrane, that consequently increase electron transport, ATP generation, nitric oxide release, and blood flow[47].

The technique will be used to induce mitochondria to release more ATP and thereby protect the cell. The method involves shining red laser light on chironomids during TTPC, NaCl and TTPC+NaCl exposure. This method will be used to "rescue" mitochondria in the presence of chemicals and thereby confirm mitochondria as a cellular target for one or both chemicals. In addition, ATP will be monitored to determine its effectiveness. This approach is a novel way to validate mitochondrial toxicity as a mechanism of action.

14- <u>Model organism for freshwater biota:</u>

Invertebrates is utilized as an appropriate tool to assess the aquatic ecosystems, especially insect, due to their specific characteristics like short life cycles, high sensitivity, small size, and availability[48]. Species of the Family Chironomidae are very suitable invertebrates for risk assessment in aquatic ecosystems[48]. They are widely distributed, sensitive to many pollutants, some of them can be cultivated, they have a short life cycle and their larvae are of great importance in aquatic food chains[48].

In the proposed studies, larvae of *Chironomus riparius* will be used as the model organism for freshwater biota. Larvae are called chironomids, and they are widely used model for toxicity assessment of aquatic environments[49]. Chironomids show a wide spectrum of adaptation to aquatic habitats of varying salinity levels, ranging from freshwater to oligohaline (1-5 ppt)[49]. A major challenge faced by chironomids is the tendency for their habitats to fluctuate greatly in salinity due to natural causes such as rainfall, evaporation or global warming[49]. The species, *C. riparius*, has been used extensively as a model for genome structure analysis in insects and for functional developmental genetic studies[48]. Both their adult and larval forms have an important role in freshwater food chains, and they are easy to maintain in a laboratory environment[50].

A study by Zheng *et al* (2011)[51] supports the premise that biomarkers for lipid peroxidation can be measured in 4th instar Chironomidae. They studied enzyme activities as protective mechanisms against exposure to environmental contaminants including cadmium[51]. They measured antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione (GSH), catalase (CAT), and an index of lipid peroxidation (malondialdehyde, MDA). They found a significant increase in MDA levels and a change in GR and GPx activities in Cd^{2+}- treated *P. akamusi*. All doses of Cd^{2+} significantly suppressed GR

activity compared with the findings for the control dose, with an inhibition rate up to 0.55-fold in the 25.0 mmol/liter Cd^{2+}treatment. SOD and GST activities were not altered. The results indicate that "Cd^{2+} can induce oxidative stress as indicated by the changes in lipid peroxidation and antioxidant status. For *P. akamusi*, an increase in the dose that the threshold needed for defense (namely, MDA level and GPx activity) activation was achieved". From this, organisms can be hypothesized to enable cells to avoid oxidant stress up to a certain extent where damage is again measurable (higher Cd^{2+} concentration)[51]. Thus, modulation of one of these enzymes in the presence of TTPC or NaCl may indicate a toxic mechanism of action in chironomid.

According to the literature reviewed above, 4th instar *C. riparius* will be the best model organism for our study of hydraulic fracturing fluid components. This is because there are several previous studies done using similar animals to assess the biomarkers proposed for this study. In addition, there are no published studies in freshwater biota that evaluate the impact of NaCl and TTPC combined.

A search of the published literature yielded little information about the toxic mechanism of action of TTPC in animals and nothing that evaluated the combined effects of hydraulic fracturing biocides mixed with salts; therefore, further study is needed.

In the present study, larvae of *Chironomus riparius* were used as a model test organism for freshwater biota to study the acute toxicity of the biocide, TTPC, alone and in combination with brine, represented by NaCl. These larvae, called chironomids, have been widely used in aquatic toxicity testing[48,49] and were also recently used as an invertebrate indicator for oxidative damage in temperature and dissolved oxygen perturbed environments[52]. Given the known effects of amphiphilic surfactants on microbial membranes[18,25] and mitochondria in lung

epithelial cells[40,41], the present study focused on measures of mitochondrial dysfunction in chironomids. Acute toxicity was determined for TTPC alone and mixed with NaCl as well as DDAC for comparison. Mechanisms of action were evaluated by measuring ATP, SOD activity and lipid hydroperoxides (LPO). The hypothesis was that the toxicity of TTPC and NaCl is due to changes in the integrity of cells membranes, especially those of mitochondria, due to the generation of ROS caused by salts and lipid bilayer disruption.

B. Materials and Methods:

Model Organism

Chironomus riparius was used as a model test organism (Family Chironomidae, Order Diptera). Both adult and larval forms play an important role in freshwater food chains, and they are easy to maintain in a laboratory environment[49]. *C. riparius* has been used extensively as a model for ecotoxicology studies, especially for freshwater[49]. The 4th instar larvae of a laboratory strain of *C. riparius* (Environmental Consulting & Testing, Superior, WI) was maintained under controlled conditions in our laboratory and was used in all experiments.

Acute toxicity test

Six concentrations were prepared for each chemical under investigation. For NaCl (CAS No. 7647-14-5), concentrations were 0, 2000, 4000, 6000, 8000 and 10,000 mg/L. For TTPC (CAS No. 81741-28-8), concentrations were 0, 0.15, 0.3, 0.6, 0.8 and 1 mg/L. In the interaction experiments, NaCl was held constant at 3500 mg/L and TTPC was varied at 0, 0.0725, 0.15, 0.3, 0.6 and 0.8 mg/L. Each treatment was done in triplicate. Test vessels were 1 L polypropylene containers with give total water volume in mL, ten, 4th instar *C. riparius*, food (rabbit chow, Kaytee Products, Inc, Chilton, WI) and aeration. The hardness of the reconstituted water ranged from 160 to 180 ppm, pH ranged from 7.2 and 7.4, temperature ranged from 20 to 21 °C, and dissolved oxygen ranged from 7 to 9 ppm. Experimental conditions for acute toxicity tests were used throughout the study.

ATP modulation level and Red laser light exposure

Samples were collected at 24 h. Ten 4th instar *C. riparius* were placed into two containers per concentration (designated A and B). In container A, larvae were exposed to treatment but not red laser light and analyzed for ATP levels, n=4. In container B, larvae were exposed to treatment, followed by 10 m of red laser light before analyzing them for ATP levels, n=4. Mortality was determined for each replicate and the mean was calculated, n=2. The red laser light was provided by LED Red Light Therapy Panels from RadLites LED Lighting, model INFRARED LED THERAPY PAD. The light was suspended 5 inches above test vessels. To control the temperature during the red laser exposure, test vessels were placed in a 25 °C waterbath. After 24 h exposure, 2 µL of hemolymph from individual larvae were dissolved in 48 µL molecular grade H_2O (Sigma Aldrich). Then 5 µL of the mixture was used in the ATP assay. ATP was measured using a luciferase-based, ATP determination kit according to manufacturer's instructions (Molecular Probes, Eugene, OR). ATP concentrations were calculated using a standard curve, then samples were normalized as percent control of their respective time point and experiment.

Superoxide dismutase (SOD) activity

The effects of NaCl, TTPC and interaction of TTPC with NaCl on SOD activity were assessed in larval hemolymph at 24 h. Samples consisted of whole body homogenates from 3 larvae combined, n=4. SOD activity was measured using an SOD assay kit in accordance with the manufacturer's instructions (Dojindo Molecular, Rockville, MD). Samples were normalized by dividing by larval weight. Responses were represented as percent control of their respective

time point and experiment. Mortality was determined for each replicate and the mean calculated, n=2.

Lipid Hydroperoxide Assay

Samples were collected at 24 h, n=4. The same experimental conditions and sample collection method for SOD activity were used. Lipids were extracted and lipid hydroperoxides (LPO) were measured using the lipid hydroperoxide assay kit in accordance with the manufacturer's instructions (Cayman Chemical, Ann Arbor, Michigan). Samples were normalized by dividing by larval weight. Responses were represented as percent control of their respective time point and experiment. Mortality was determined for each replicate and the mean calculated, n=2.

Statistical analysis

IBM Statistics SPSS program (version 22) was used for all data analyses. For acute toxicity, SSPS Probit Analysis was used to calculate LC_{50} and 95% CI for each treatment, significance level was ≤ 0.05, heterogeneity factor = 0.15, maximum iterations = 20, and the step limit = 0.1. For ATP levels, significant differences between samples exposed or unexposed to red laser light at the same concentration were determined by independent T-test, $p \leq 0.05$. Significant differences between control and other treatments for ATP, SOD activity and LPO was determined using One-Way ANOVA and Tukey post hoc test, $p \leq 0.05$.

C. Results:

Acute toxicity and Mortality rate

Acute toxicities of NaCl, TTPC, TTPC plus NaCl, and DDAC were determined by calculating LC_{50}s at 24 and 48 h (Table 1). Results for NaCl showed that at 24 h the LC_{50} was greater than the highest concentration tested, 10,000 mg/L, and at 48 h, it was 9808 mg/L. For TTPC, the 24 h LC_{50} was 0.57 mg/L, and decreased marginally to 0.48 mg/L at 48 h as most larvae died within 24 h. When combined with a non-lethal concentration of NaCl (3500 mg/L), the 24 h and 48 h LC_{50} for TTPC was 0.32 and 0.22 mg/L, respectively, showing greater toxicity then TTPC alone. The 48 h LC_{50} of DDAC was 0.71 mg/L indicating that it was less toxic than TTPC. In addition, percentage mortality for TTPC, NaCl, TTPC+NaCl and DDAC at were measured at 24 and 48 h (Figure 1,2). Results for concentration-related increases in all treatments. However, samples treated with TTPC+NaCl at 24 h and 48h showed dramatically increase in mortality compared to TTPC or NaCl treatments alone as a prove of toxicity of synergistic effect. The percentage mortality of TTPC was higher than DDAC treatment. Table 2 showed the mortality rate of samples out of 10 for all treatments at 24 h and 48 h.

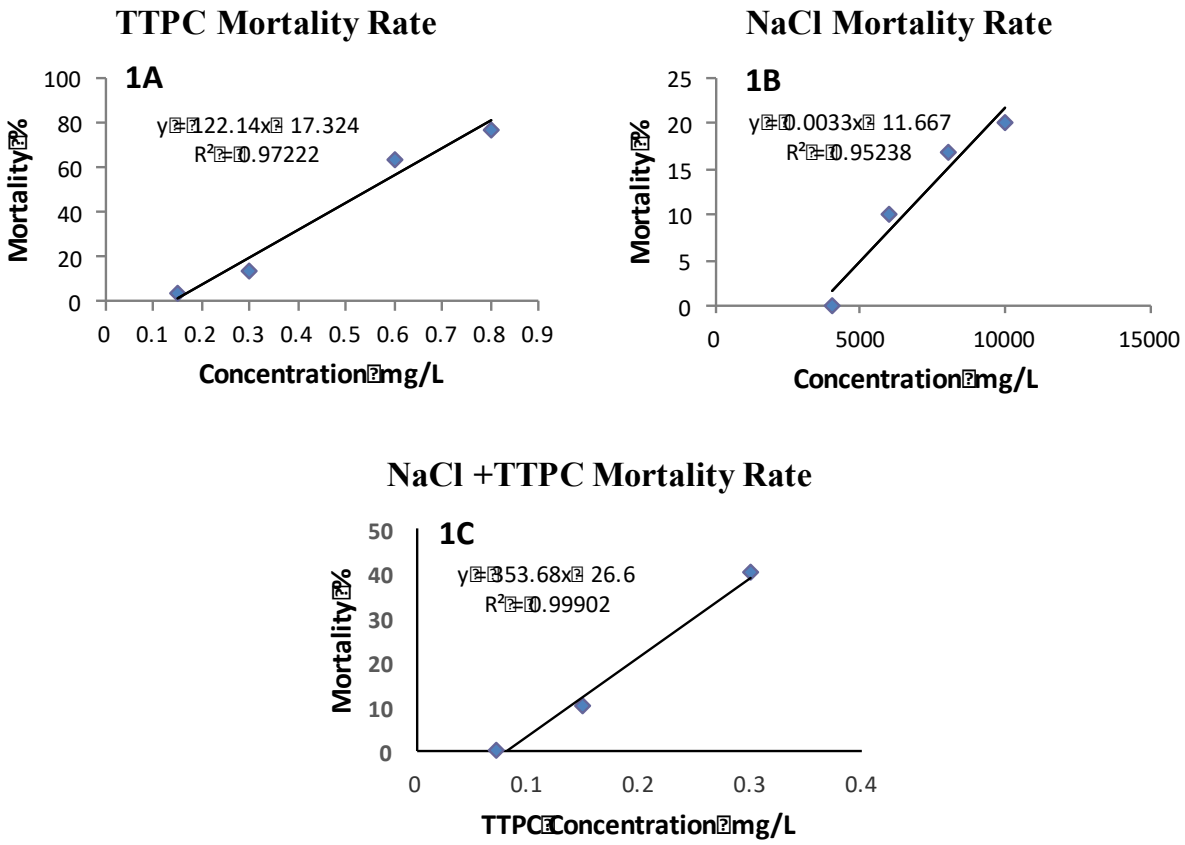


Figure 1. Percentage mortality of TTPC, NaCl and TTPC + NaCl at 24 h. Figure 1A shows percent mortality of TTPC. Figure 1B shows percent mortality of NaCl. Figure 1C shows percent mortality of NaCl (3500 mg/L) combined with varying TTPC concentrations (TTPC+NaCl).

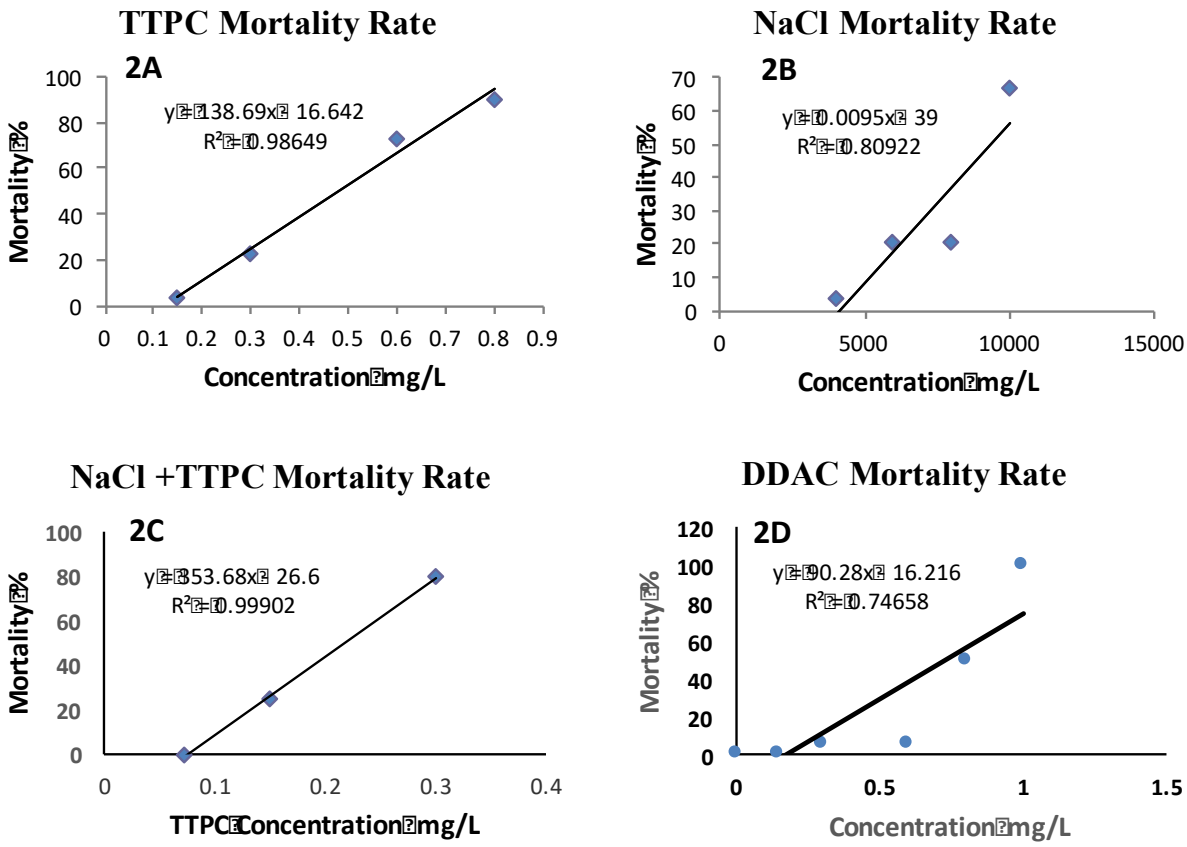


Figure 2. Percentage mortality of TTPC, NaCl, TTPC + NaCl and DDAC at 48 h. Figure 2A shows percent mortality of TTPC. Figure 2B shows percent mortality of NaCl. Figure 2C shows percent mortality of NaCl (3500 mg/L) combined with varying TTPC concentrations (TTPC+NaCl). Figure 2D shows percent mortality of DDAC.

Table 1. Acute toxicity for NaCl, TTPC, TTPC combined with NaCl and DDAC at 24 and 48 h (95% confidence level) in 4th instar of *C. riparius*.

Treatment	24 h LC_{50} mg/L	48 h LC_{50} mg/L
NaCl	>10,000	9808 (7843−15558)
TTPC	0.57 (0.50−0.63)	0.48 (0.37−0.59)
TTPC and NaCl*	0.32 (0.27−0.40)	0.22 (0.18−0.29)
DDAC		0.71 (0.60−0.86)

*NaCl, 3500 mg/L, was added to each concentration of TTPC.

Table 2. Average mortality rate out of 10 animals for NaCl, TTPC, TTPC combined with NaCl and DDAC at 24 and 48 h in 4th instar of *C. riparius*.

NaCl mg/L	**24 h**	**48 h**
0	0	0.7
2000	0	0.3
4000	0	0.3
6000	1	2
8000	1.7	2
10000	2	6.7

TTPC mg/L	**24 h**	**48 h**
0	0	0.3
0.15	0.3	0.3
0.3	1.3	2.3
0.6	6.3	7.3
0.8	7.7	9
1	10	10

TTPC+ NaCl * mg/L	**24 h**	**48 h**
0	0	0
0.0725	0	0
0.15	1	2.5
0.3	4	8
0.6	10	10
0.8	10	10

*NaCl, 3500 mg/L, was added to each concentration of TTPC.

DDAC mg/L	48 h
0	0
0.15	0
0.3	0.5
0.6	0.5
0.8	5
1	10

ATP modulation and red laser light exposure

Levels of ATP were modified by TTPC exposure and red laser light (Figure 3A). Treatment of chironomids with TTPC for 24 h caused concentration-related increases in ATP levels. The two highest concentrations, 0.3 and 0.5 mg/L, were significantly increased compared to control. When chironomids were exposed to red laser light, ATP levels significantly increased in control and 0.0725mg/L TTPC compared to the same samples without red laser light. This indicated that the light enhanced ATP production as anticipated. Interestingly, ATP levels of all the higher TTPC concentrations ($\geq$ 0.15 mg/L) were not increased by the red light, which indicated that mitochondria had stopped producing additional ATP. Percent mortality increased with TTPC concentration but was relatively low overall, $\leq$ 30 %, and not changed by red light exposure. Percent mortality was 0, 0, 10, 20, and 30 % in control, 0.0725, 0.15, 0.3, and 0.5 mg/L, respectively.

ATP results for the interaction showed increased toxicity when NaCl was combined with TTPC (Figure 3B). Increasing concentrations of TTPC plus 3500 mg/L NaCl corresponded with increasing ATP levels with 0.3 mg/L significantly higher than controls; however, 0.5 mg/L was not different from control indicating an inhibition of ATP production not seen in the absence of NaCl (Figure 3A). Chironomids exposed to red laser light showed significant increases in ATP for control, NaCl alone and the lowest concentration of TTPC (0.0725 mg/L). At higher concentrations, there was no increase in response to red laser light indicating mitochondrial dysfunction. Increased toxicity of the mixture was shown by higher percent mortalities at all TTPC concentrations when combined with nonlethal levels of NaCl. The mortality was 0, 0, 5, 25, 35 and 60 % in control, 3500 mg/L NaCl, 0.0725, 0.15, 0.3 mg/L TTPC + NaCl, and 0.5

mg/L TTPC + NaCl, respectively. Results for NaCl alone showed no change in ATP levels with concentrations ranging from 2000 to 10,000 mg/L. (data not shown).

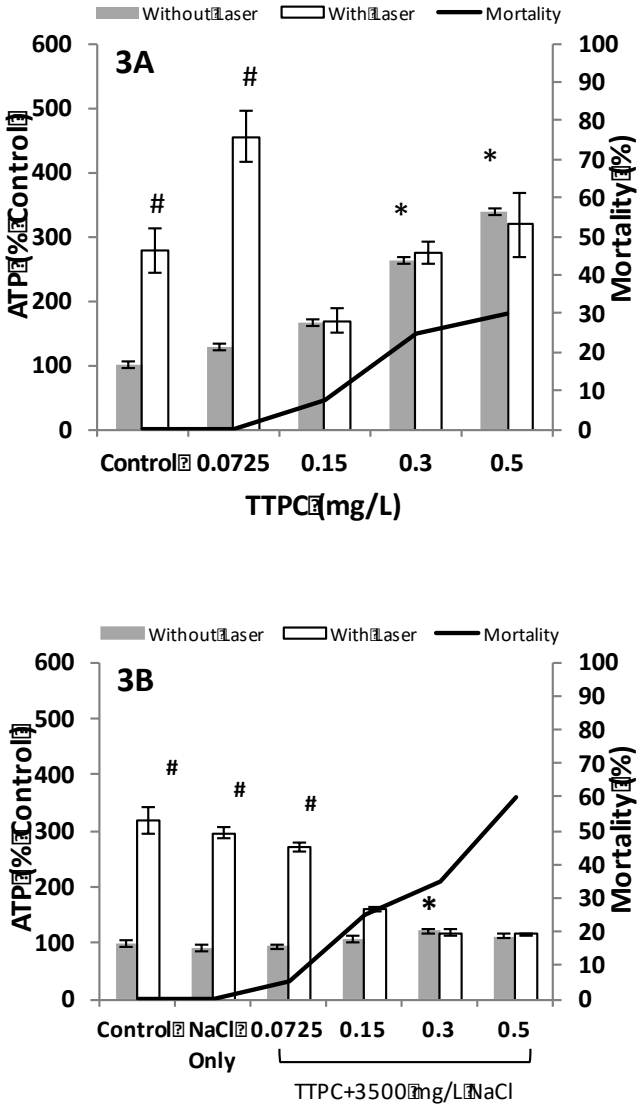


Figure 3. Effect of TTPC and TTPC + NaCl on ATP levels and % mortality in the presence and absence of red laser light at 24 h. Figure 3A shows results of TTPC (mg/L). Figure 3B shows results for NaCl (3500 mg/L) and NaCl (3500 mg/L) combined with varying TTPC concentrations. Statistical differences between control and treatments (*) were determined by One-way ANOVA followed by Tukey's post hoc test, $p \leq 0.05$. Differences between exposed and unexposed levels of the same concentration were determined by independent T-test (#). n= 8. Error bars represent standard error.

SOD activity

SOD activity was measured as an indicator of oxygen radical formation. For NaCl, there was a concentration-related increase at 24 h with statistically significant increases between control and treatments of 4000 mg/L and 10,000 mg/L, $p < 0.05$ (Figure 4A). Percent mortality was 0, 0, and 27% in control, 4000, and 10,000 mg/L, respectively. For TTPC, there was a concentration-related increase at 24 h with statistically significant differences between control and treatments of 0.3, 0.6 and 0.8 mg/L, $p < 0.05$ (Figure 4B). Percent mortality was 0, 7, 20, 33, and 60% in control, 0.15, 0.3, 0.6 and 0.8 mg/L, respectively. However, for the interaction treatment, there were no significant differences between control and treatments (Figure 4C). The mortality was 0, 20, 13, 13, and 60 % in control, 3500 mg/L NaCl, 0.15 mg/L TTPC + NaCl, and 0.3 mg/L TTPC + NaCl, respectively. The interaction experiment was run three times with similar results.

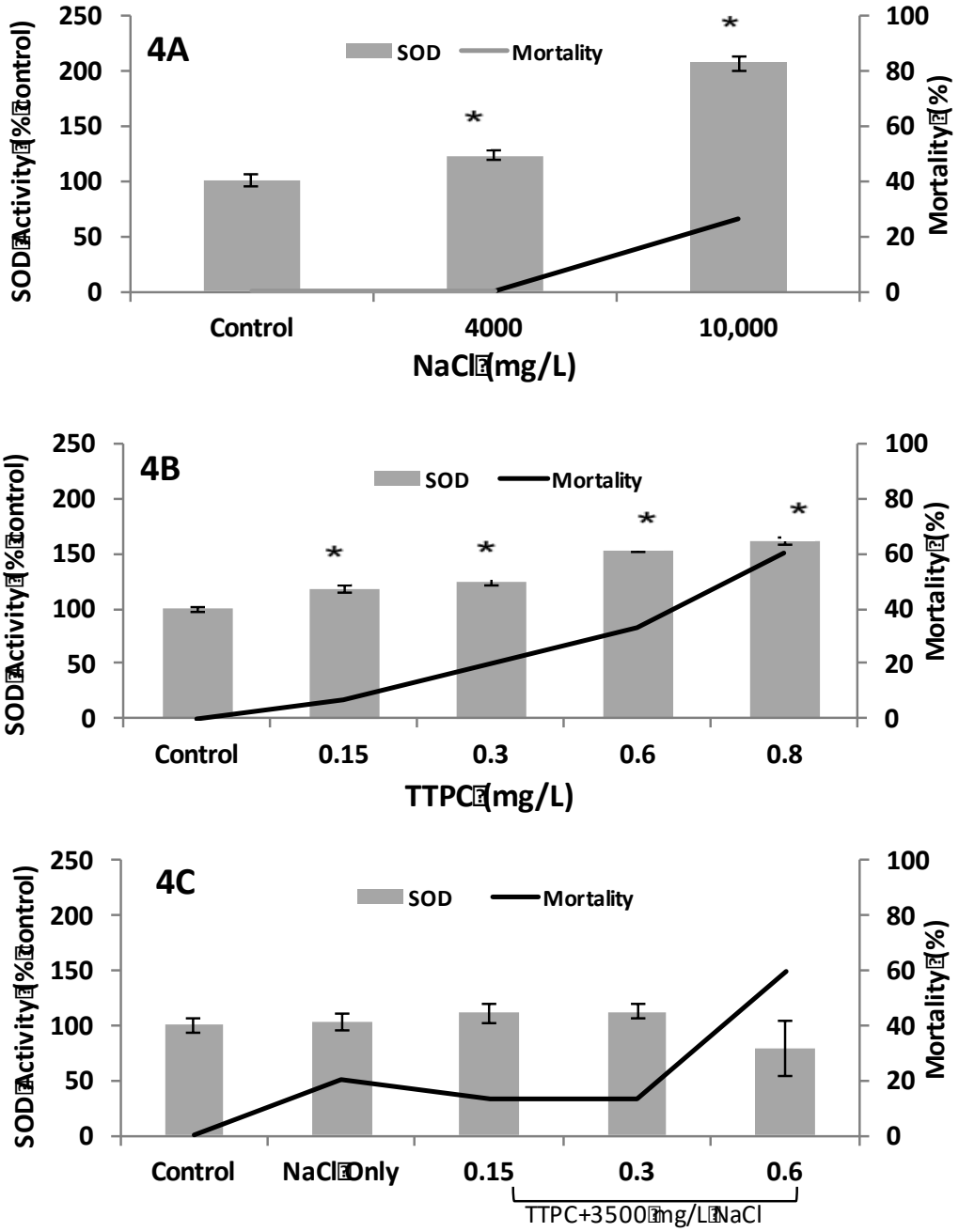


Figure 4. Effect of NaCl, TTPC and TTPC + NaCl on SOD activity and % mortality at 24 h. Figure 4A shows results of NaCl (mg/L), Figure 4B shows results for TTPC (mg/L), while Figure 4C shows NaCl (3500 mg/L) combined with TTPC. Statistical differences from control (*) were determined by One-way ANOVA followed by Tukey's post hoc test, $p \leq 0.05$, $n=4$. Error bars represent standard error.

Lipid Hydroperoxide Assay

Levels of lipid hydroperoxide (LPO) were measured as an indicator of ROS damage to membranes. When larvae were treated with NaCl (2000-6000 mg/L), only the highest concentration tested (6000 mg/L) was significantly higher than control (5A). Percent mortality was 0% in all treatments, except 7% for 6000 mg/L. TTPC showed concentration-related increases with 0.3 and 0.5 mg/L significantly higher than control (figure 5B). Percent mortality was 0, 3, 10, and 80 % in control, 0.15, 0.3, and 0.5 mg/L, respectively. The NaCl and TTPC interaction experiment also showed concentration-related increases for 0.15 mg/L and 0.3 mg/L; however, toxicity was greater in the presence of NaCl (figure 5C). This was demonstrated by a lower observable apparent effect level (LOAEL) of 0.15 mg/L TTPC + 3500 mg/L NaCl and higher levels of % mortality than found with TTPC only. The mortality was 0, 0, 7, and 50 % in control, 3500 mg/L NaCl, 0.15 mg/L TTPC + NaCl, and 0.3 mg/L TTPC + NaCl, respectively.

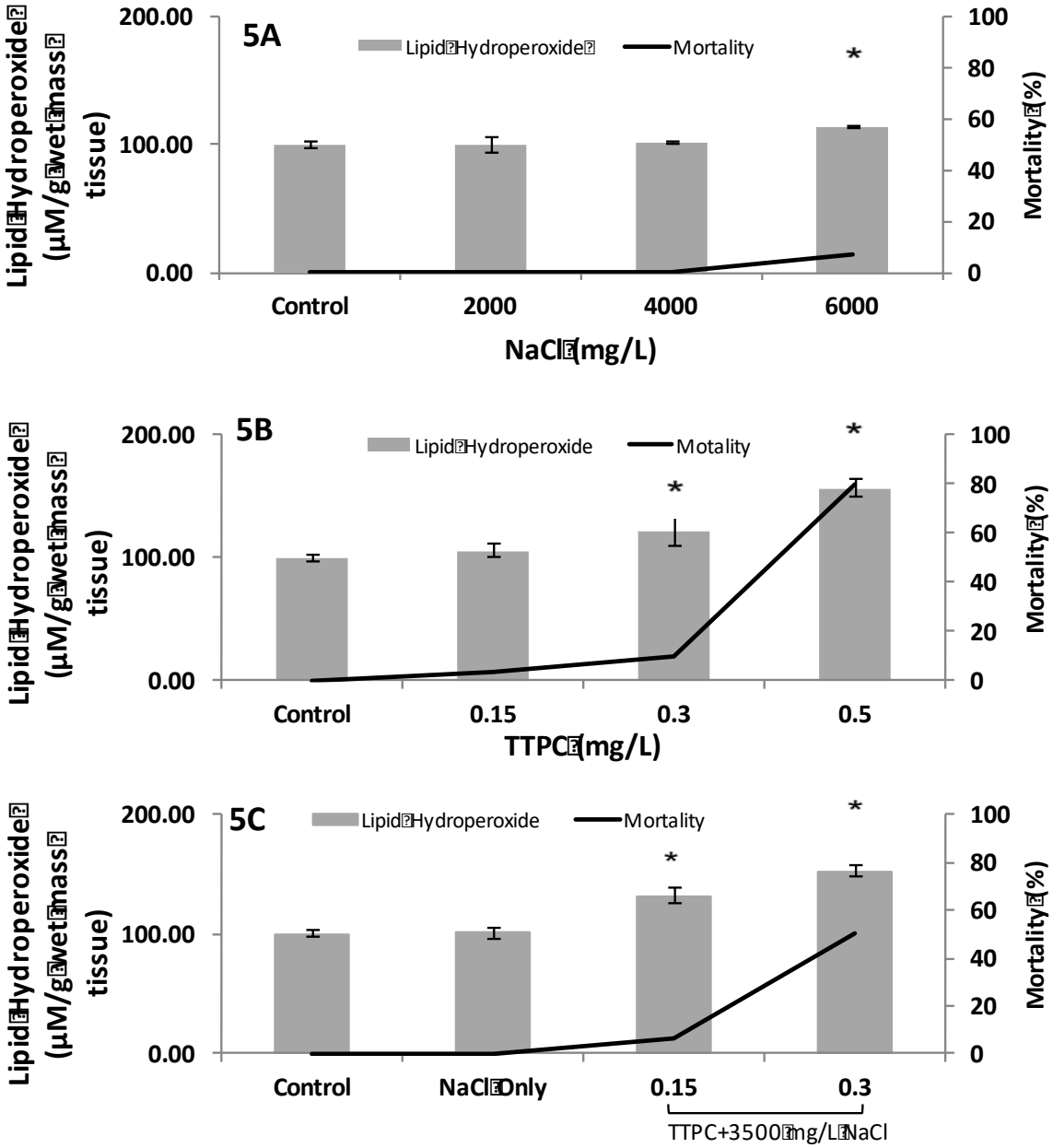


Figure 5. Effect of NaCl, TTPC, and TTPC+NaCl treatments on lipid hydroperoxide levels at 24 h. Figure 5A shows results of NaCl (mg/L) and Figure 5B shows results for TTPC (mg/L), while Figure 5C shows NaCl only (3500 mg/L) and combined with TTPC. Statistical differences from control (*) were determined by One-way ANOVA followed by Tukey's post hoc test, $p \leq 0.05$, n=4. Error bars represent standard error.

Synergistic Effect

The relationship between TTPC and TTPC+NaCl treatments were determined by comparing data from the same experiment. For acute toxicity test, Figure 6A showed a significant decrease between LC_{50} 0f TTPC and TTPC plus NaCl for 24h and 48h. For ATP results, figure 6B demonstrated significant decrease among all concentrations, except the lowest concentration 0.0725 mg/L, in comparison of TTPC and TTPC plus NaCl treatments without the presence of red laser treatment. Furthermore, when samples were exposure to red laser light, there were significant reduction between TTPC and TTPC plus NaCl treatments for 0.0725 mg/L, 0.3 mg/L, and 0.5 mg/L (figure 6C). In figure 6D, the results showed a significant increase between TTPC and TTPC plus NaCl for samples treated with 0.3 mg/L. Overall, results suggested that there is a synergistic effect when TTPC biocide combined with NaCl brine.

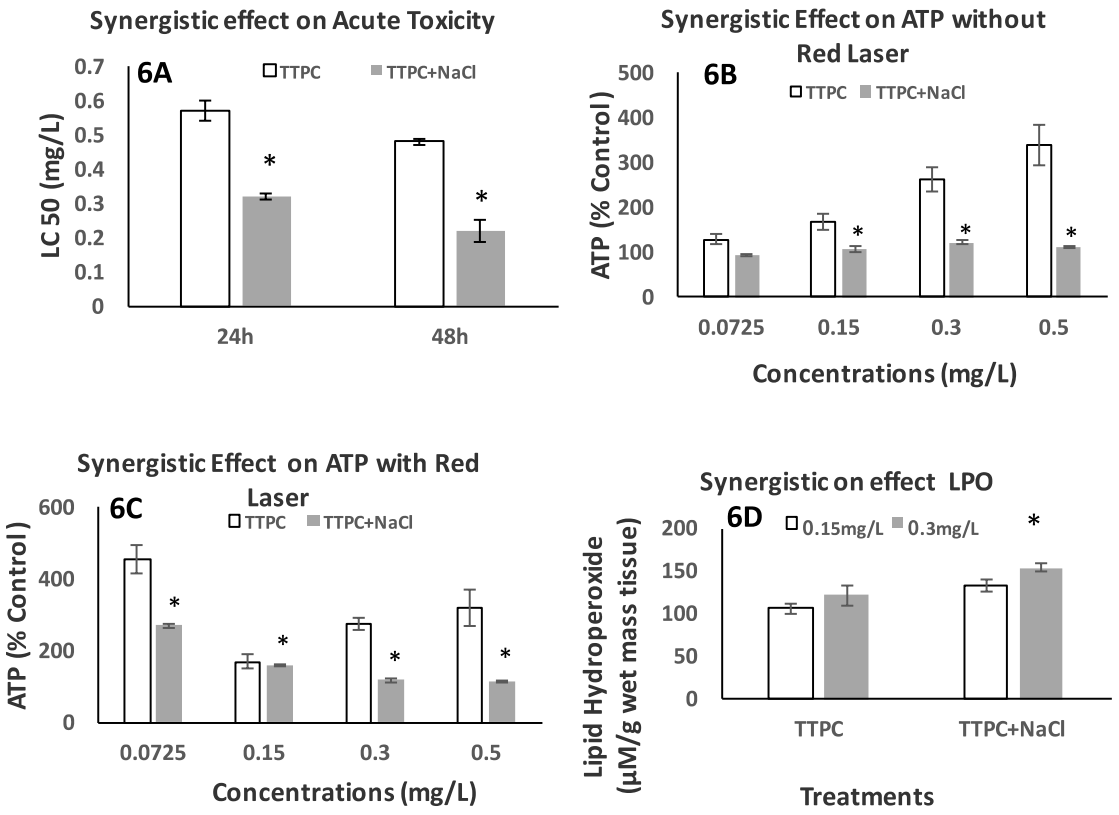


Figure 6. Synergistic Effect of LC_{50}, ATP and LPO levels at 24h. TTPC and TTPC+NaCl data for LC_{50}, ATP and LPO levels are shown to demonstrate the synergistic effect of the combination of biocide and brine of hydraulic fracturing fluid. Figure 6A shows comparison of LC_{50} for TTPC alone and TTPC+NaCl. Figure 6B shows ATP results for TTPC alone and TTPC+NaCl treatments for samples without exposure to red laser light, while Figure 6C shows results for the same treatments but with samples exposure to red laser light. Figure 6D shows comparison of results for samples treated with TTPC alone and TTPC+NaCl for LPO levels. NaCl was held constant at (3500 mg/L). Statistical differences between samples of TTPC alone and TTPC+NaCl treatments in the same concentration of TTPC (*) were determined by independent T-test, $p \leq 0.05$, n=4. Error bars represent standard error.

D. Discussion:

Hydraulic fracturing is one of the new techniques used to increase production of gas and oil in the United States. The technique utilizes a mixture of chemicals and brine that can contaminate freshwater during the hydraulic fracturing process. Biocides are one of the major components of fracturing fluids for which there is limited information on their molecular mechanisms of action especially in aquatic organisms. This paper studied the effect of TTPC biocide and NaCl brine in *C. riparius,* a freshwater animal model. The goal was to clarify the level of toxicity of chemicals under investigation, study their effects on mitochondrial function as a toxic mechanism of action, and determine if TTPC was more or less toxic when combined with NaCl. We hypothesized that TTPC and NaCl are more toxic when combined and that toxicity is due to changes in the integrity of cells membranes, especially those of mitochondria, due to the generation of ROS and lipid bilayer disruption.

1- Acute toxicity and Mortality rate:

Acute toxicity and mortality rate tests were performed for NaCl, TTPC, TTPC+NaCL, and DDAC (Table 1, 2; Figure 1,2). Results for NaCl showed a 24 h LC_{50} > 10,000 mg/L and a 48 h LC_{50} of 9808 mg/L, which is similar to the literature value of 9995 mg/L for chironomid[53]. In addition, the percentage mortality showed concentration-related increase in both 24 and 48 h (Figure 1B, 2B). The response to TTPC was approximately 5 orders of magnitude more sensitive than NaCl with 24 and 48 h LC_{50}s of 0.57 mg/L and 0.48 mg/L, respectively. For comparison, the 96 h LC_{50} in fish (*Oncorhynchus mykiss*) was 0.46 mg/L, and the 48 h EC_{50} in water flea (*Daphnia magna*) was 0.025 mg/L[22]. Surprisingly, TTPC percentage mortality results showed dramatically concentration-related increase in both 24 and 48 h (Figure 1A,2A). Combining a non-lethal concentration of NaCl (3500 mg/L) with TTPC doubled its toxicity,

which prove the rise in the toxicity of the chemical mixture compared to each component alone. The LC_{50}s for TTPC+NaCl at 24 and 48 h were 0.32 and 0.22 mg/L, respectively. The percentage mortality of TTPC+NaCl was doubled of the percentage mortality of TTPC alone (Figure 1C,2C). DDAC is another biocide used in hydraulic fracturing fluid that is structurally similar to TTPC. The acute toxicity of DDAC biocide at 48 h was measured for the purpose of compression with TTPC, especially due to the lack in TTPC literature information. Results showed a 48 h LC_{50} of 0.714 mg/L indicating that it was less toxic than TTPC in chironomid. However, chironomid proved less sensitive to DDAC than water flea, 48 h EC_{50} of 0.057 mg/L DDAC[54]. Taken together, data showed that all chemicals under investigation had concentration-related increases in mortality at 48 h. The mixture of TTPC biocide and NaCl brine was more toxic than either alone. When the two biocides were compared, TTPC was more toxic than DDAC. The difference between the 48 h LC_{50} for TTPC (0.48 mg/L) and its concentration in hydraulic fracturing fluid (33.3 mg/L) suggested that environmental exposure to hydraulic fracturing fluid containing this compound could be environmentally hazardous. Furthermore, the increase of toxicity of the combination of TTPC with NaCl light up a concern about the environmental toxicity effects of the hydraulic fracturing fluid as a big mixture of hundreds of chemicals.

2- <u>ATP modulation and red laser light exposure:</u>

The influence of TTPC and NaCl on mitochondrial function was investigated by measuring ATP without and with exposure to a red (cool) laser light. Without red laser light, TTPC showed concentration-related increases in ATP, which contradicted expected outcomes (Figure 3A). Mitochondrial dysfunction should have decreased not increased ATP levels. One possible explanation is that TTPC, which is structurally similar to fatty acids, was catabolized

through β-oxidation pathways thereby providing an alternative source of energy[55]. NaCl toxicity has been associated with mitochondrial dysfunction due to hypertonic stress. Toxicity in murine inner renal medullary collecting duct cells (mIMCD3) corresponded with reduced Δp as well as rapid increases in cellular ADP/ATP ratios at 1-6 h[44]. In the current work, NaCl alone showed no concentration-related effect on ATP production (data not shown). Similarly, Pastor and colleagues (2009) showed that there is no depletion of the cellular ATP pool during osmostress in yeast as a result of the elevation of NaCl concentration[56]. In the present study, when combined with TTPC, a sub lethal concentration of NaCl (3500 mg/L) suppressed the increase in ATP observed with TTPC alone and increased overt toxicity observed by the higher % mortality (Figure 3B). This could be because NaCl was effected the mitochondria due to the increase in osmostress, however, the effect was not appeared until another stress caused by TTPC presence. Both components crease a stress to the level that caused a damage in the mitochondria and prevent it from creating more ATP as we saw in TTPC treatment alone.

Red laser light proved an effective means of measuring mitochondrial function. Exposing larvae to red laser light tripled ATP production in control as well as low concentrations of TTPC (0.0725 mg/L) and NaCl (3500 mg/L) (Figures 3A and 3B). surprisingly, in the case of TTPC+NaCl treated samples with red laser light, the lower concentration (0.0725 mg/L) was not increased to the same level of TTPC alone. This can show the toxic effect of the presence of both components compared to the presence of one component only. In addition, when the concentrations of TTPC increased, in the presence of red laser light, the level of ATP in both exposure and non-exposure samples were equal. Other studies showed that red laser light stimulated cytochrome c oxidase thereby increasing electron transport, raising mitochondrial membrane potential (Δp), and increasing ATP production[47]. The inability to stimulate

cytochrome c oxidase in TTPC-treated chironomids might be explained by electron leakage across the mitochondrial membrane. Fatty acids have reduced membrane potential (Δp) of mitochondria by inducing ROS-associated, proton leakage across the lipid bilayer through adenine nucleotide translocase (ANT) and uncoupling proteins (UCPs)[57]. This mode of mitochondrial toxicity was supported by fungicide studies in bumble bee (*Bombus terrestris*). Some fungicides caused uncoupling of mitochondrial respiration in flight muscle detected as reduced Δp and increased ROS levels[58].

3- <u>SOD activity and LPO level:</u>

The proposed mechanism of action for TTPC, electron leakage, was supported by biomarkers indicative of ROS presence (SOD activity) and lipid peroxidation (LPO). Results showed SOD activity and levels of LPO increased in a concentration-dependent manner when chironomids where exposed to TTPC alone or NaCl alone (Figures 4 and 5). These findings are supported by studies with DDAC, which increased ROS while decreasing glutathione (GSH) activity in human lung epithelial cells at 1.8 mg/L[41] as well as studies with Cd, which enhanced SOD activity and measurable levels of lipid peroxidation in chironomid[51]. The response of SOD to TTPC plus NaCl was unexpected (Figures 4C). Our data showed no concentration-related increase in SOD activity for the mixture. This experiment was repeated three times with a similar outcome suggesting that the mixture inhibited SOD but not LPO production. The main function of SOD enzyme is to catalyze the dismutation of the superoxide radical to either ordinary molecular oxygen or hydrogen peroxide[59]. This could be explained by that SOD enzyme was presented in the TTPC treated samples due to the damaged in mitochondrial membrane as an antioxidant defensive manner against reactive (O2−). However, in some cases when the damage was very extensive and a large number of the superoxide radical presence, SOD enzyme fails to

control the damage, which can cause a reduction in the level of SOD[59] (as shown in TTPC+NaCl treatment) (Figures 4C). Overall, mitochondrial membrane damage caused by ROS in chironomids could account for the inability of red laser light to increase ATP levels.

4- Synergistic Effect:

In the present study, we have shown that TTPC biocide mitochondrial dysfunction is enhanced by NaCl brine (Figure 6). Figure 6A shows an increase in acute toxicity (as measured by LC_{50}) for TTPC+NaCl as compared to TTPC alone. In addition, the levels of ATP production in response to red laser exposure also support the acute toxicity results (Figures 6B, 6C). Furthermore, LPO levels also increased when chironomids were treated with TTPC+NaCl (Figure 6D). These results demonstrate that there is a synergistic effect when chironomids are treated with TTPC+NaCl as compared to TTPC biocide alone. Interestingly, the levels of SOD increased when exposed to NaCl alone and to TTPC alone, but did not increase in a synergistic fashion as was seen for acute toxicity, LPO levels and ATP levels (Figure 4C). This is probably because in the presence of NaCl alone, the mitochondria were compromised due to the hypertonic stress[12], but not to a level that completely disrupted SOD function. However, in the presence of NaCl+TTPC, the extent of mitochondrial damage was increased so that mitochondrial SOD production failed. This is a reasonable conclusion in light of the dramatic increase in LPO levels, acute toxicity and the decrease in ATP production as compared to TTPC alone.

E. Summary:

The practice of hydraulic fracturing to generate fossil fuels could pose environmental risks. Results from the current study show that biocides, such as TTPC, are acutely toxic to eukaryotic organisms at levels 1000x lower than concentrations found in fracturing fluids and

that its toxicity increases when combined with NaCl (representing the brine found in fracturing fluid). Mitochondrial dysfunction is supported as a mechanism of toxicity for TTPC by the increase in SOD activity, increased LPO levels, and reduced ATP production in the presence of red laser light. Induction of ATP by red (cool) laser proved to be a valuable technique for studying mitochondrial toxicity in chironomids. This work represents the first study of TTPC toxicity in freshwater macroinvertebrates. This is also the first published report of LC_{50} for TTPC. Future work will investigate target organs of TTPC and will evaluate its chronic toxicity. Furthermore, studies with combinations of more than two chemicals found in hydraulic fracturing fluid is important as well as using other model organisms.

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