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Dispersing Agents Prevent Negative Impact of Oil on Uptake of Zinc by Duckweed (*Lemna*).

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Summary.

Oil spills have had extremely negative effects upon the environment, affecting both animal and plant species living in and around the contaminated water. It is known that the oil can interfere with certain plant and animal functions, potentially causing death. Means developed to remove the oil from the water include physical methods such as skimmers and chemical approaches such as adsorbents and dispersants. In the cases of the chemical treatments, some people question whether the remedy may also cause problems. Here, we confirm that the aquatic duckweed plant (*Lemna*) can take up zinc from its environment and show that oil in the water will inhibit that uptake. Further, we demonstrate that the negative affect the oil has upon zinc uptake by duckweed can be ameliorated by treatment with a dispersant and that the dispersant itself does not inhibit zinc uptake by duckweed. We conclude that, treatment of oil contaminated water by dispersant may be a good method to clean up the water.

Introduction.

Duckweed is a hardy aquatic plant that consists of a round, slightly oval-shaped body, called a frond. A small root-like structure, sometimes called a "rootlet," hangs down from underneath the frond of many species; however, some species are rootless. Fronds are small, generally being no bigger than 10 millimeters, but tend to adhere to one another and form thick mats over water. The flower of the duckweed genus *Wolffia* is the smallest known flower in the world, measuring merely 0.3 mm long. Duckweed is found in a wide rand of marine and freshwater ecosystems, providing food and protective habitat for numerous animals. The common name was derived from the fact that these plants provide food for many species of water fowl, especially ducks (Stutzenbaker 2010). Duckweed grows and produces biomass faster than any other flowering plant and is sometimes considered to be a menace, especially in the fishing community because a thick surface layer of duckweed can prevent sunlight from reaching the deeper parts of the water column so that underwater plants and algae cannot photosynthesize and produce oxygen. The lowered oxygen levels can stress or even kill fish and other aquatic species (Stutzenbaker 2010; Sengupta and Medda 2010). Nonetheless, duckweed is considered to be a good source of protein and is used as an abundant animal feed supplement (Brown et al., 2011; Zetina-Cordoba et al., 2013; Xu et al., 2012). It also contains high levels of starch that can be fermented into ethanol and is being researched as a potential source of biofuel (Blackburn et al., 2013). Further, because of its ability to uptake pollutants (e.g., metals such as cadmium, lead, and zinc) from water, it is also being researched for use as a tool for phytoremediation (Brooks and Robinson 2012; Khellaf and Zerdaoui 2009; El- Kheir et al., 2007; Boniardi et al., 1998).

Oil spills occur in both fresh and salt water and are damaging to the environment and the organisms that live in it (Fitzgerald and Gohlke 2014; Kazlauskiene et al., 2004; La Peyre et al., 2013; Sanchez et al., 2005; Kazlauskiene et al., 2004; Walker et al., 1995). Oil spills in aquatic ecosystems affect a wide range of organisms linked to humans through complex food webs and these spills can affect the quality of fresh water available to humans and other organisms (Walker et al., 1995). There are numerous ways oil can be removed from water biomes. Some options include traditional mechanical techniques (e.g., skimmers to contain and remove the surface oil) and newer techniques such as the practices of in-situ burning or using chemical agents to disperse or adsorb the oil (El-Zahaby et al., 2011; Allen 2008).

This project uses a dispersing agent (i.e., DISPERSIT™; Chestnut Ridge, NY), which is a mixture of detergents used to treat oil spills, to determine if this method of cleaning up the spill actually interferes with the damage done by oil contamination and if it does so without exacerbating the environmental problem. Indeed, the U.S. Environmental Protection Agency also found that DISPERSIT™ was helpful in dispersing the oil in environments without bringing harm to the organisms occupying it (Environmental Protection Agency 2013). Here we report that motor oil does indeed interfere with zinc uptake by duckweed but that this inhibition can be ameliorated by the dispersing agent.

Results.

Study 1. Determination of zinc concentration absorbed by duckweed over time. We needed to confirm that duckweed uptakes zinc from the environment and also what exposure time to zinc gives good measurable uptake results. About 0.5 g of duckweed was added to 18 small Ziploc™ (Ziploc; Racine, Wisconsin) bowls containing DI water and Miracle Grow™ (100 mL) as described above. All bowls sat next to a large kitchen window at about 22-26°C with roughly 12/12 light cycles. After 3 days, the water was changed in all bowls except for three bowls from which the plants were harvested as controls. The remaining 15 bowls received the solution containing 10 ppm zinc as described above; this solution was changed after 24 hours and then every 2 days to maintain a constant concentration of zinc. The plants were harvested at days 3, 7, 11, 15 and 19 after the initial exposure. All harvested plants were gently rinsed with DI water using a wire sieve and then dabbed with paper towels. These were then placed in Ziploc™ bags and frozen at -20°C until digested and assayed for zinc at the same time. The data show here that, consistent with the literature, duckweed will uptake zinc from its aquatic environment (Figure 1; Khellaf and Zerdaoui 2009; Boniardi et al., 1998). The amount of zinc detected in the duckweed exposed to 10 ppm zinc increased with exposure time, being barely above detection limits at Day 3 and increasing dramatically between Days 3 and 7 (15.2%). The increase continued through Day 19, although the rate of increase slowed over time (as noted by the decreased slope), appearing that the increase would plateau shortly after 19 days. We determined that 7 days of exposure to 10 ppm zinc would be a good measure of zinc uptake for the remainder of our studies because this timeframe allows for a quick, strong uptake of zinc and minimizes exposure time.

Study 2. Determination of motor oil concentration which interferes with zinc absorption by duckweed over time. We needed to know what concentration of motor oil would inhibit zinc uptake by duckweed. Small buckets (n=6) containing 3.0 L of DI water (with Miracle Grow™ as above) were placed in a window-lit garage. Aliquots of unused motor oil were added to 5 of these to yield the following concentrations: 2.5, 5.0, 10, 25, and 50 ml/L motor oil in water. These were allowed to sit in the garage in front of a large window at ~ 25-30°C. After 7 days, water was syphoned off the bottom of the bucket to avoid motor oil. Aliquots (150 mL) of this water were added to 15 small Ziploc™ (Ziploc; Racine, Wisconsin) bowls. Fresh DI water (with Miracle Grow™ as above; 100 mL) was added to another 18 bowls. Three of these were used as controls. Aliquots of oil were added to the other bowls to yield 3 bowls of each: 2.5, 5.0, 10, 25, and 50 ml/L motor oil in water. Aliquots of duckweed (~0.5 g) were added to each bowl and all bowls sat in front of a large kitchen window as in Study 1. In this study, the water was not changed throughout the experiment. After 7 days, all plants were harvested as described above except that the rinsing of the plants was a bit more vigorous in this study than in study 1; this was necessary to remove the oil. All plants were first rinsed with DI water containing Dawn™ detergent (5 mL per L; Proctor and Gamble; Jackson, MO) and then with DI water. All rinsed plants were dabbed and frozen at -20°C prior to being assayed for zinc. The data show that the motor oil did interfere with zinc uptake by duckweed at all concentrations with a maximum of a 53.8% decrease in zinc uptake by plants treated with the highest concentration of oil (50 ml/L) after 7 days. This interference did increase as the concentration of oil increased, demonstrating that the inhibition of zinc uptake by motor oil was concentration dependent (Figure 2). Interestingly, the oil treated water (oil incubated in water for 7 days and then oil removed) also significantly interfered with duckweed uptake of zinc (decreased 36.9%), but only at the highest concentration of oil treatment (50 ml/L oil in water for 7 days). We determined that 50 ml/L motor oil would be used in our next study because it induces a strong inhibition of zinc uptake by duckweed and creates a physical barrier that would best test the power of the dispersal agent.

Study 3. Determination of the effect of motor oil, dispersant and a combination of the two upon zinc uptake by duckweed. Sixteen small Ziploc™ (Ziploc; Racine, Wisconsin) bowls were placed into four columns, each column containing four bowls. A 150 mL aliquot of the zinc solution was added to each container. The containers in the first column served as the control. To the next column, 7.5 mL of Mobil Premium Motor Oil was added to each container (50 ml/L) and stirred. To the third column, 0.15 mL of the DISPERSIT™ (Poly Chem; 1 ml/L, according to maunfacturer recommendations) was added to each container and stirred. Aliquots of 7.5 mL motor oil and 0.15 mL DISPERSIT™ were added to the bowls in the fourth column. Duckweed (0.5 g) was added to each bowl and allowed to sit in the containers in front of a large kitchen window (as in Study 1) for seven days. The light:dark cycle was approximately 11:13 because the study was done in September. The bowls were agitated for 1 minute in the morning and again in the evening. In this study, the water was not changed throughout the experiment. After seven days, all duckweed samples were harvested onto a small screen and cleaned by gentle rinsing with a solution of water and Dawn dishwashing liquid and then water (as above). The plants were then dabbed and placed in appropriately labeled Ziploc bags and frozen (-20°C). As shown in study 2, the oil (50 ml/L) significantly inhibited the uptake of zinc by 28.3% (Figure 3). This inhibition is not as great as in Study 2, although it is still significant. We suggest that this could be explained by variability in the plants used or by the decreased amount of light to which the plants were exposed in Study 3. However, the zinc uptake was not statistically different from that of control samples when the oil treated samples were also treated with the oil dispersing agent. Further, the dispersing agent itself did not significantly interfere with zinc uptake by duckweed. The data indicate that the dispersing agent will prevent the oil from having a negative effect on zinc uptake by duckweed and not negatively impact the plant uptake of zinc itself.

Discussion.

Duckweed is an aquatic plant that can remove pollutants from the water in which it grows (Brooks and Robinson 2011; El- Kheir et al., 2007; Boniardi et al., 1999); specifically, it is known that this plant will remove zinc from the environment when conditions are appropriate (Khellaf and Zerdaoui 2009). The experiment described here was performed to determine if dispersing agents (e.g., DISPERSIT™) are beneficial to plants whose environment has been contaminated by motor oil. We hypothesized that motor oil would interfere with zinc uptake by duckweed and further that the dispersing agent, DISPERSIT™, would indeed disperse the contaminating motor oil and allow the duckweed to function more normally in spite of the oil contamination. Initially, we determined that 7 days of exposure to 10 ppm zinc would allow duckweed to incorporate a reliably measurable concentration of zinc, yielding a reliable measure of duckweed health (Study 1). As expected, the oil had a negative impact on zinc uptake and this effect became worse as the concentration of oil increased. In fact, we show that a 50 ml/L motor oil exposure has a substantial negative effect on zinc uptake by duckweed (Study 2). We suspect that zinc uptake was actually inhibited (to some extent) by a physical barrier created by the oil because we observed that the oil did cling to parts of the plant clusters, creating a physical barrier between parts of the plant and the water. However, the plants did not appear truly damaged by the oil once they were rinsed with the Dawn™ and water solutions, having a similar shape and only mildly muted color in comparison to the non-oil treated plants. We suggest the decreased green color was caused by the block of some sunlight to these plants caused by the oil coating. It is certainly noteworthy that duckweed grown in water incubated for 7 days with the highest concentration of oil (water from which the oil was removed before adding the plants, Figure 2) also had a (milder) negative effect on the zinc uptake. This suggests that the inhibition of zinc uptake is not completely a result of physical inhibition by the thick oil, but that there may be some water soluble chemical extracted from the oil that has a negative effect upon duckweed's ability to extract zinc from water. Using recommended concentrations of the dispersing agent, we found that the mean zinc concentrations in duckweed treated with zinc only (as control), with DISPERSIT™ and zinc, and with DISPERSIT™, oil and zinc were not significantly different from each other, but were all significantly greater than the plants treated with the motor oil and zinc alone. Thus, the DISPERSIT™ was able to disperse the oil in the Ziploc bowls without having a negative impact on the plant's ability to uptake zinc itself. It can be concluded from these results, that when searching for a resource to clean environmental aquatic oil spills, DISPERSIT™ will be beneficial in dispersing the oil without negatively impacting the health of duckweed.

Materials and Methods.

Plants: Duckweed plants (*Lemna)* were bought from William Tricker, Inc. (Cleveland, OH) and cared for as recommended by Landolt and Kandler (1996). The plants were placed in small Ziploc™ (Ziploc; Racine, Wisconsin) bowls containing 100-150 ml of DI water (~pH 7.0) containing a recommended 2.5 ml/L of Miracle Grow™ house plant food. The studies were performed indoors within a controlled temperature (~22-26°C) environment with the plants being placed on a table in front of a large window. The studies 1 and 2 were performed in June (of different years) so that the light:dark cycle was approximately 12:12 hrs. Study 3 was performed in September so the light:dark cycle was approximately 11:13 hrs.

Zinc Solution: A 10 ppm zinc solution was made by adding 25 mL of a 1000 ppm zinc solution (Hach Company; Loveland, CO) to 2.5 L of deionized (DI) water. A 6.25 mL aliquot of Miracle Grow™ (Scott's Miracle-Gro; Marysville, Ohio) was also added and mixed. The Miracle Grow label reports that it contains 0.06% zinc, an amount negligible to this study as diluted.

Motor Oil: Mobil Premium Motor Oil (MobilExxon, United States) was used. The company reports that this product contains no water soluble zinc

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Oil Dispersive Additive: DISPERSIT™ by Poly Chem (Chestnut Ridge, NY) was added to disperse motor oil. This product contains no reported zinc.

Plant Digestion for zinc extraction. Sample plants were thawed at room temperature and approximately 0.5 gram of each sample was placed in individual, acid-washed test tubes. The exact weights of the samples were recorded. Aliquots of 65% suprapur nitric acid (5 mL) and 35% hydrochloric acid (6 mL) were added to each test tube. The samples were then heated at 93°C using a heating block (Fisher Scientific; Barrington, Illinois) under a ventilated fume hood until the samples were clear of plant material. Each sample was then diluted to 20 ml with DI water and the pH adjusted to 4 using sodium hydroxide pellets.

Zinc Assay: The zinc concentration of each sample was determined using a commercial Zinc Determination Kit (Hach Company; Loveland, CO). Zinc standard were prepared by diluting a 100 ppm zinc solution with DI water to: 0.1, 0.25, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 ppm. A Zincover 5 Reagent pillow was dissolved in 10 mL of each standard. A 0.5 mL of cyclohexanone was added and mixed for 30 seconds. After 3 min, the absorbances were read at 620 nm using a Genesys 5 Spectrophotometer (ThemoFisher Scientific; Waltham, MA). All standards were read against a treated blank. The standard concentrations and absorbances were used to create a line graph and a simple linear regression was performed to determine sample zinc concentrations (extrapolation of sample sample absorbances against the standard curve). Zinc concentration per plant (µg Zn/g plant) was calculated using the weight of the starting plant material.

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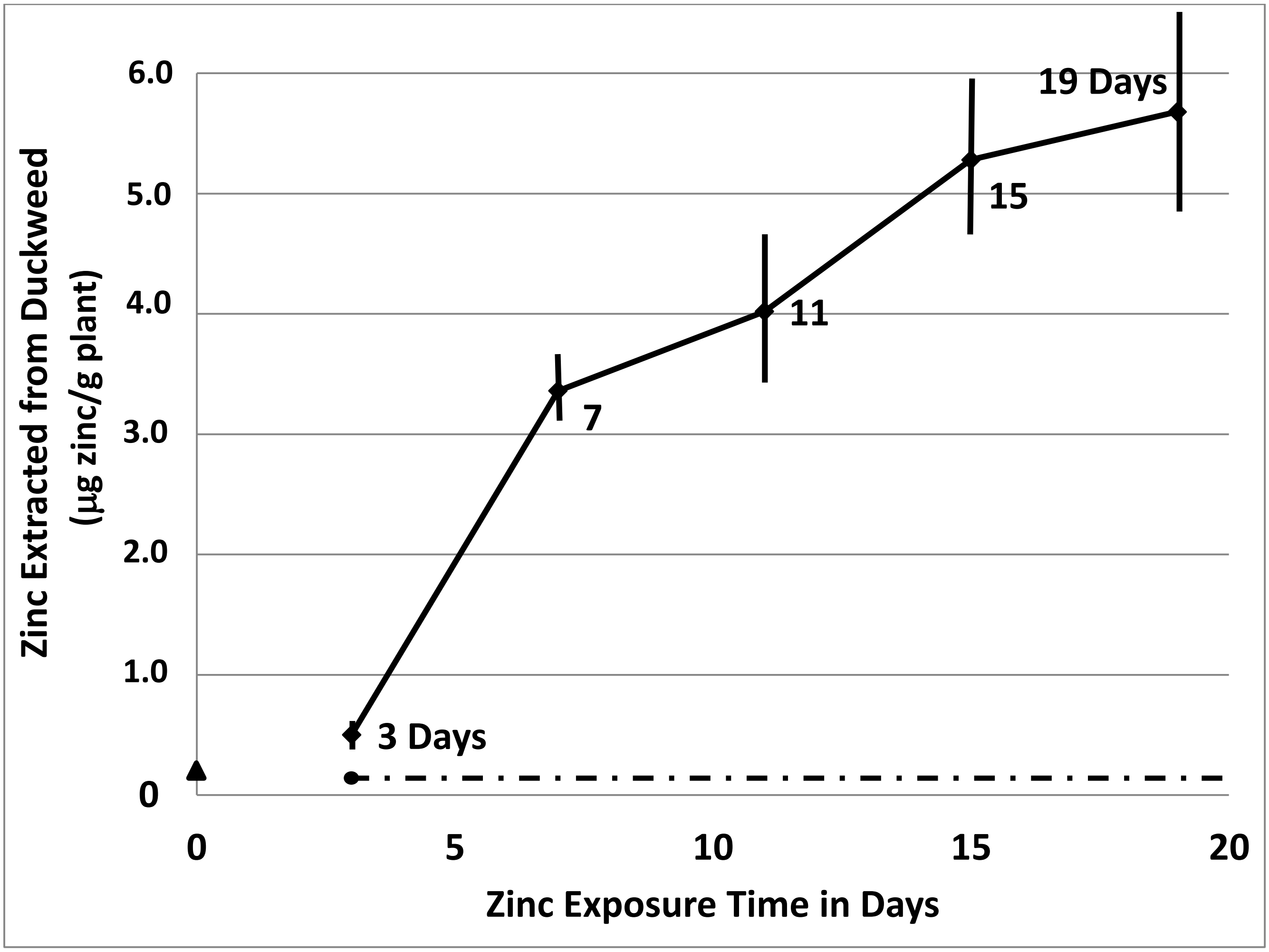

Figure 1. Zinc uptake by duckweed (*Lemna*) increases over time. n = 18, 3 replicates per time point. ——— = Change in Zinc concentration. ◆ = mean ± standard error of the mean (SEM). ▲ = The detection limit of our assay was 0.25 µg zinc/g duckweed. — · — = Zinc concentrations in control plants (no zinc treatment) were all below detection limit.

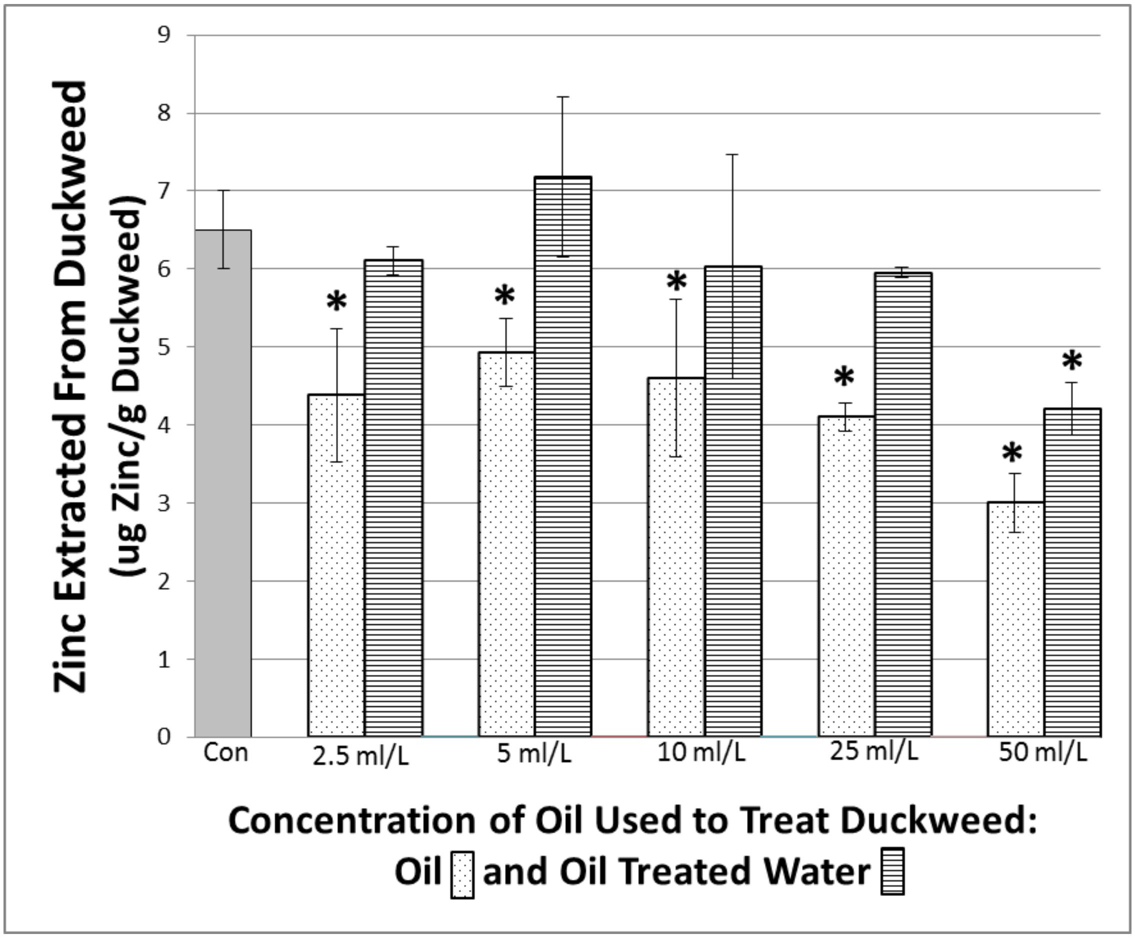

Figure 2. Motor oil treatment interferes with the uptake of zinc by duckweed (*Lemna*) in a concentration dependent manner. n = 33, 3 replicates per 11 treatments. Bars represent mean. Error bars represent the standard error of the mean. "*" $p<0.05$ and indicates that the noted sample groups are significantly different from the control group.

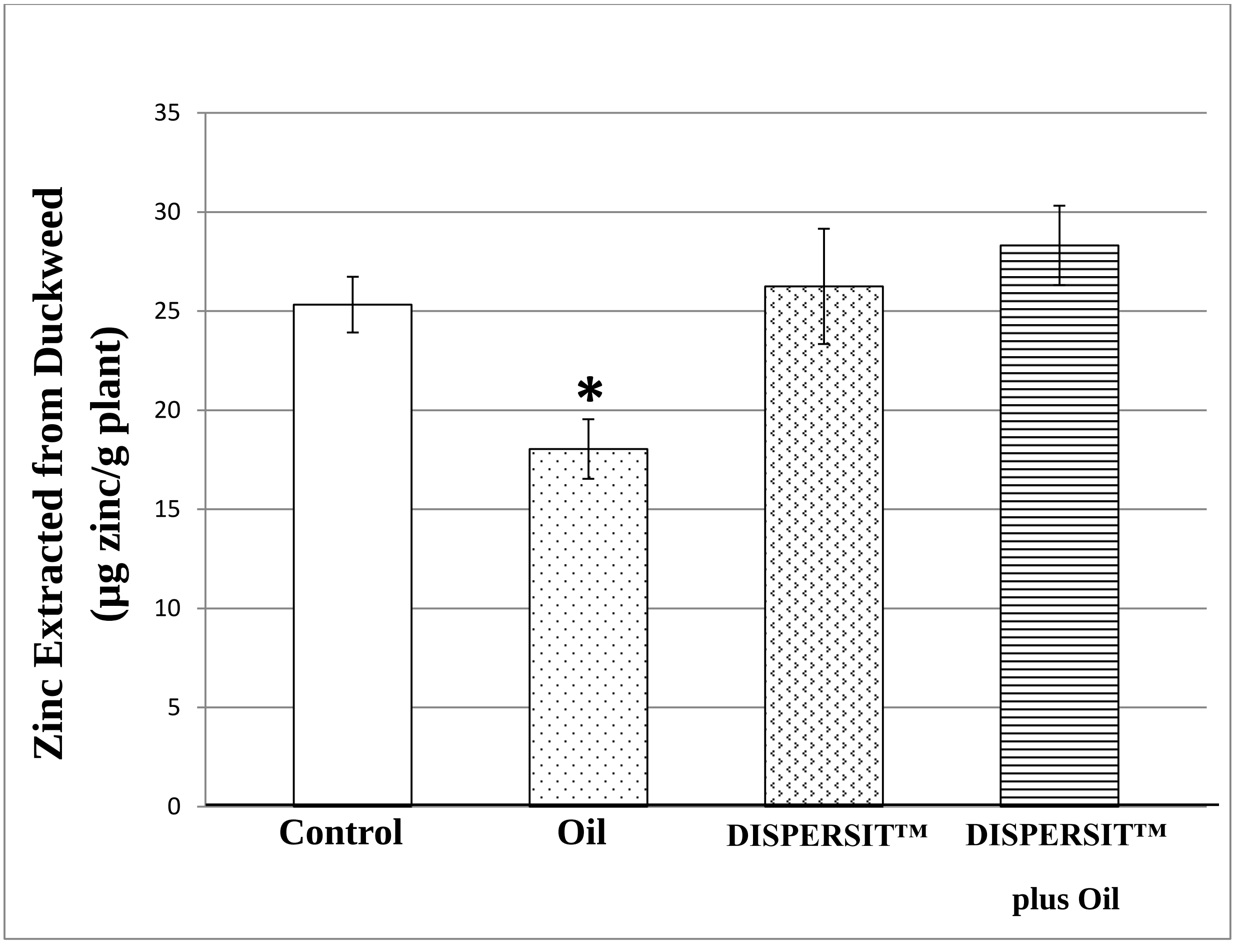

Figure 3. Motor oil treatment inhibits zinc uptake by duckweed, but this inhibition is ameliorated by treatment with dispersing agent. Bars represent the mean zinc concentrations of duckweed treated for 7 days with: 1) zinc only (control, 10 ppm zinc); 2) oil (50 ml/L) and zinc (10 ppm); 3) Dispersit™ (1.0 ml/L) and zinc (10 ppm); and 4) Dispersit™ (1.0 ml/L), oil (50 ml/L) and zinc (10 ppm). n= 16 samples, 4 replicates per 4 treatments. Error bars represent the standard error of the mean.

"*" = $p \leq 0.05$ and indicates that the oil treated group removed significantly less zinc from the water than the other treatment groups.