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Embryotoxicity of Corexit 9500 in mallard ducks (*Anas platyrhynchos*)

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Abstract Embryotoxicity of the oil dispersant Corexit 9500 was examined using fertilized mallard duck eggs. Corexit 9500 was topically applied below the air cell to eggs in volumes ranging from 0 to 100 μL on day 3 of incubation. The highest incidence of mortality occurred at developmental stage 4, one day post-Corexit 9500 application. Hatching success was significantly decreased among eggs treated with ≥20 μL of Corexit 9500 as compared to controls ($P \leq 0.047$). No egg treated with ≥40 μL successfully hatched. The application volume resulting in 50% mortality (corrected for control survival) was determined to be 15.5 μL. Developmental stage at embryo death was also significantly decreased compared to controls in eggs exposed to 40 μL ($P = 0.0042$) and above.

Keywords Corexit 9500 · Mallard duck · *Anas platyrhynchos* · Dispersant · Oil spill

Introduction

Dispersants are commonly applied to oil spills to enhance dispersion of oil into small droplets and subsequently increase microbial degradation and reduce amounts of crude oil on the surface of affected aquatic systems. The dispersant Corexit 9500 was the primary dispersant used during the Deepwater Horizon oil spill throughout the spring and summer of 2010 in the Gulf of Mexico. Corexit 9527, a dispersant of similar chemical composition, was used to a lesser extent early in the spill response (hereafter, unless a formulation is specified, "Corexit" refers to both 9500 and 9527). During the Deepwater Horizon spill Corexit was applied on the surface of the Gulf and near the damaged wellhead at a water depth of 1500 m in an attempt to disperse oil before it reached the surface. Overall, the amount of Corexit used in the Deepwater Horizon spill totaled over 8,000,000 L—approximately 5,300,000 at the surface and 2,915,000 L in subsea applications between May 15 and July 12, 2010 (Kujawinski et al. 2011).

Corexit 9500 is approved for use in a variety of oil spill situations and is effective at dispersing oil, yet its chemical fate in aquatic ecosystems is not well characterized (Place et al. 2010). Due to the proprietary nature of many dispersants, formulation-specific analytical methods have been slow to emerge. While Singer et al. (1996) speculated on the identity of Corexit 9500 and 9527 ingredients, the chemical constituents were not confirmed until information was released by NALCO, the producer of Corexit, in the summer of 2010 (NALCO 2010). Ingredients include both anionic and nonionic surfactants as well as organic solvents. These components are thought to quickly dissociate in aquatic environments. Early studies examining the fate of Corexit components following the Deepwater Horizon oil spill have focused on individual components, which have been identified in the Gulf at various depths (Kujawinski et al. 2011) and in near shore environments where direct application was not permitted (OSAT 2010).

Application of Corexit to oil spills in aquatic systems has prompted a number of aquatic toxicity evaluations. A review by George-Ares and Clark (2000) contains the most comprehensive review of acute toxicity endpoints for both Corexit 9500 and 9527. Overall, both formulations were of low to moderate toxicity to aquatic organisms, with EC_{50}

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or LC_{50} values for Corexit 9527 ranging between 1.6 and >1000 mg/L and for Corexit 9500 between 0.7 and >400 mg/L. Factors such as taxa, life stage, and water temperature affected the toxicity of Corexit. In general, crustaceans were more sensitive to dispersant toxicity than fish and toxicity decreased with temperature. Early life stages show varying toxicity depending on factors such as egg permeability and developmental stage. More recent studies, including those conducted by the EPA (Hemmer et al. 2010a), indicate that Corexit 9500 poses low to moderate risk to the inland silverside, *Menidia beryllina*, and the mysid shrimp, *Americamysis bahia*, two species commonly used in aquatic toxicity testing. In general, exposure to Corexit dispersants and dispersed oils poses less risk of toxicity than exposure to crude oil (i.e. Hemmer et al. 2010b). However, dispersants have the potential to increase uptake of polycyclic aromatic hydrocarbons by some organisms which is an additional consideration when evaluating dispersant risk (Ramachandran et al. 2004; Milinkovitch et al. 2011).

There is potential for aquatic mammals and birds to become exposed to Corexit during oil spill responses, particularly in application zones. There is, however, a significant data gap with respect to dispersant toxicity in these taxa. Birds in particular may experience changes in thermoregulatory capacity and plumage integrity as a result of dispersant exposure, an area in need of research identified by the National Research Council in both 1989 and 2005 (NRC 2005) as vital to dispersant risk assessment. However, no published studies since then have addressed Corexit effects in avian species. In addition to potential adult exposures during the Deepwater Horizon spill response, transfer of contaminants from adult birds to eggs during incubation (Rittinghaus 1956) may have resulted in embryotoxic effects in birds that were nesting during the period of oil spill response (for a list of species, see Finch et al. 2011). Albers (1979) previously examined the effects of crude oil, Corexit 9527, and crude oil-Corexit mixtures on the hatchability of mallard eggs, and reported that Corexit 9527 significantly decreased hatching success at doses as low as 5 μL. The reformulation of Corexit 9527 to 9500 removed a solvent, 2-butoxy ethanol, an ingredient with known toxic effects (NRC 2005). To our knowledge, no research examining avian embryotoxicity of Corexit 9500 has been published to date. Therefore, the aim of this study was to investigate the embryotoxicity of Corexit 9500 in mallard ducks.

Materials and methods

Prior to egg acquisition, the experimental protocol was approved by the Texas Tech University Institutional Animal Care and Use Committee (IACUC Approval No. 10042-07). Mallard duck eggs were purchased from Metzer Farms (Gonzales, CA) and delivered overnight via air freight to Lubbock, TX. Eggs were placed in a Profi-I forced air incubator (Lyon Electric Company, Inc.) on the same day as arrival; the time the last egg was placed in the incubator was set as the beginning of incubation day 0. The incubator automatically rotated eggs hourly, and controlled both temperature and relative humidity. Temperature and humidity were recorded once daily, and if necessary controls on the incubator were adjusted to reach the recommended values of 99.5°F and 57.7% humidity (Metzer Farms 2011). Light:dark cycle was maintained at 14:10 throughout the experiment.

On day 3 of incubation, eggs were randomly assigned to application groups and treated with Corexit 9500. Corexit 9500 or water (controls) was applied with a microliter pipette to an area on the surface of each egg, oriented vertically, below the air cell (Albers 1977). As application volume increased, the egg surface area used for dosing was increased to limit Corexit runoff from the egg surface. Corexit 9500 applications occurred at 10 μL incremental volumes between 10 and 100 μL ($n = 16$/treatment, 17 control), and control eggs were treated in a similar manner with 100 μL of DI water. All eggs were candled on incubation day 7; infertile and dead eggs were identified and removed. Removal of infertile eggs after dosing resulted in small differences in the number of eggs per treatment group. After incubation day 7, candling occurred every third day until incubation day 25 when eggs were transferred to a Profi-IH forced air hatcher (Lyon Electric Company, Inc.) until hatching. Hatcher temperature and humidity were recorded once daily, and adjusted if necessary to the recommended values of 98.5°F and 84.3% humidity (Metzer Farms 2011). Infertile or dead eggs were stored refrigerated, and examined later to confirm infertility or determine developmental stage at death. Developmental stage represented the degree of development expected on a given day of incubation (i.e. stage 8 corresponds to an embryo that had developed as much as an average embryo does by day 8), and was determined based on information presented in Caldwell and Snart (1974).

Ducklings were euthanized within 1.5 h of hatching. Body mass including yolk sac (±0.1 g) was obtained using an Avinet hanging balance and body organ masses were measured to the nearest 0.01 mg using a Metler Toledo analytical balance. Crown-rump and bill lengths (±0.1 mm) were measured with a Vernier (Type 6914) micrometer caliper. Hatchlings were examined for major anatomical defects and gender was determined. Eggs that failed to hatch by incubation day 33 were candled to confirm death and then staged as described above.

Statistical analyses were performed using R statistical software ($\alpha = 0.05$; R Core Development Team 2010). The

binomial test was used to compare hatching success among eggs from Corexit 9500 treatments and the control group. A control-corrected median lethal application (LA_{50}) was derived using a logistic regression. This value represents the dose at which 50% of control hatching success was observed. While this value can be compared to previously published LD_{50}s, lethal application more accurately reflects the way eggs were dosed in the current study. A one-way analysis of variance (ANOVA) was used for the analysis of morphological data as well as developmental stage at death. If the ANOVA indicated significant differences among treatments, Dunnett's test was used to compare treatments to controls. Frequency of deformities was evaluated using χ^2 analysis. To account for natural size variations between animals, organ masses were analyzed both as measured values and as relative values, the organ mass as a percentage of total body mass.

Results and discussion

Corexit 9500 was quite toxic to mallard embryos. The control-corrected LA_{50} of Corexit 9500 was 15.5 μL/egg. Of 159 fertile eggs at the outset of this experiment, only 17 successfully hatched, all at applications ≤30 μL. Hatching success was significantly decreased among eggs treated with ≥20 μL ($P \leq 0.047$; Table 1) compared to controls. Considerable mortality was observed during the first candling with the highest incidence occurring at developmental stage 4 (47 embryos), corresponding to the day after treatment with Corexit 9500. Control hatching success was unexpectedly low, with only seven eggs (43.75%) hatching, as compared to expected hatching values from Metzer Farms (70–75%; Metzer Farms 2011). There was no obvious damage or mishandling of the eggs that would explain low control hatching success. Temperature and humidity means (±standard error) for the incubator were 99.5 ± 0.05°F and 63.9 ± 0.54%, and for the hatcher were 97.3 ± 0.17°F and 68.3 ± 0.39%. Hatcher humidity was the only parameter that differed considerably from recommended values. Low humidity during hatching can cause embryos to stick to the eggshell, a phenomenon which was not observed in the present study. Small variations in humidity during the entirety of the incubation period have previously been demonstrated to lead to variable hatching success, though changes in humidity have less of an effect than changes in temperature (Prince et al. 1969; Stubblefield and Toll 1993). While the Prince et al. (1969) study examined multiple humidity levels at constant incubation temperature, unexpectedly low hatching success in some groups limited the authors' ability to correlate the changes in relative humidity with an expected percent decrease in hatching success. In addition, uncertainty exists as to the decrease in hatching success expected if lowered humidity was restricted to the hatching period rather than the entire incubation period, as observed in the present study.

Developmental stage at death decreased as application volume increased ($F = 9.1875$, $P < 0.000001$), with significant differences from the control in applications ≥40 μL (Fig. 1). Over half the treated embryos died by stage 8, much earlier than the mortality observed following applications of weathered crude oil, which occurred mostly after incubation day 25 (Finch et al. 2011). Early developmental stage mortalities observed in this study may have been due to treatment of eggs on incubation day 3, as mallards appear to be particularly susceptible to contaminants

Table 1 Hatching success of mallard embryos after topical egg application of Corexit 9500 on day 3 of incubation

Treatment (μL)	*n*	Hatching success (%)	*P* value (binomial test)
0 (control)	16	43.75	
10	14	35.71	0.60
20	16	18.75	0.047*
30	15	13.33	0.018*
40	14	0	<0.001*
50	15	0	<0.001*
60	13	0	<0.001*
70	14	0	<0.001*
80	15	0	<0.001*
90	12	0	0.0018*
100	15	0	<0.001*

* Significantly different from controls ($P < 0.05$)

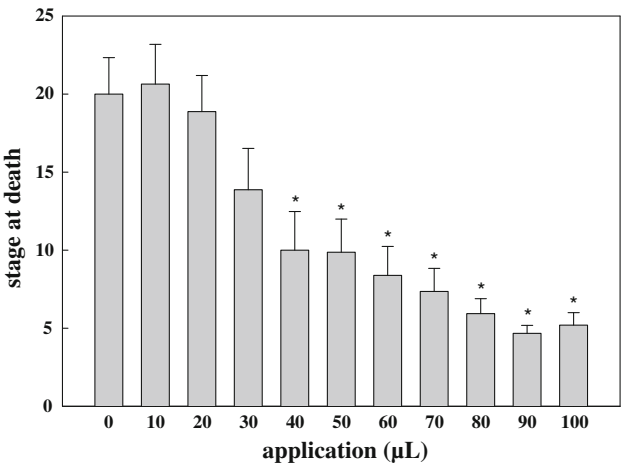

Fig. 1 Mean developmental stage at death of mallard embryos exposed to Corexit 9500 on day 3 of incubation. Developmental stage is approximately the same as day of incubation. *Significant difference ($P < 0.05$) from controls, Dunnett's test

Table 2 ANOVA results for post-hatch morphometrics of successfully hatched mallard embryos

Measure	*P* value*
Body mass[a]	0.21
Crown-rump length	0.92
Bill length	0.15
Liver mass	0.82
Relative liver mass[b]	0.59
Spleen mass	0.39
Relative spleen mass[b]	0.34

All successful embryos received Corexit 9500 applications of 0–30 μL

* No significant differences observed in ANOVA ($P > 0.05$); intergroup comparisons not performed

[a] Body mass includes remaining yolk sac

[b] Relative masses are organ masses as a percentage of body mass

during the first 8–9 days of development (Hoffman and Albers 1984). Morphometrics of Corexit 9500-treated hatchlings did not differ significantly from controls (Table 2). Anatomical defects were observed in all groups at similar frequencies ($\chi^2 = 0.8254$, d.f. $= 3$, $P = 0.8434$). The small number of hatchlings at all applications, in particular 20 and 30 μL, limited power to detect differences.

The National Response Team is an interagency group responsible for preparation and response to oil and other hazardous material spills, and regional subunits of the National Response Team maintain dispersant usage preapprovals which define areas where dispersants may be applied at the time of a spill event. For the contiguous United States, these regions are delineated in the same manner as the US EPA regions; however, Alaska, Hawaii, and other US territories occupy different regions under the National Response Team. In regions 4 and 6, those including the states around the Gulf of Mexico, preapprovals allow dispersant use at least 3 miles from shore at a minimum water depth of 10 m, not including marine sanctuaries (RRT-6 2001, RRT-4 1996). Based on usage criteria including distance from shore and water depth, Corexit 9500 presents a low risk of exposure to young birds in hatcheries, shorebirds, and wading birds that reside in shallow water within 3 miles of shore. A number of avian species in the Gulf of Mexico, however, are routinely present in Corexit use areas. Based on available US Fish and Wildlife Service data from the Deepwater Horizon spill and knowledge of avian habitat selection in the Gulf of Mexico, at least 18 of the 101 species identified by the Fish and Wildlife Service during the spill response are potentially at risk of exposure in application areas (Table 3). These 18 species represent a range of bird types, though many feed near schools of dolphins, whales, or tuna

Table 3 Gulf of Mexico bird species that spend a percentage of their time in potential dispersant application areas (water >10 m deep, >3 mi from shore)

Species	Breeding during DWH spill[c]
Black-crowned night-heron (*Nycticorax nycticorax*)[a]	
Brown pelican (*Pelecanus occidentalis*)[ab]	Yes
Cattle egret (*Bubulcus ibis*)[a]	Yes
Glossy ibis (*Plegadis falcinellus*)[a]	Yes
Herring gull (*Larus smithsonianus*)[b]	
Laughing gull (*Larus atricilla*)[a]	Yes
Least tern (*Sternula antillarum*)[b]	Yes
Lesser scaup (*Aythya affinis*)[b]	
Magnificent frigatebird (*Fregata magnificens*)[ab]	
Manx shearwater (*Puffinus puffinus*)[ab]	
Masked booby (*Sula dactylatra*)[b]	
Mottled duck (*Anas fulvigula*)[a]	Yes
Northern gannet (*Morus bassanus*)[ab]	
Osprey (*Pandion haliaetus*)[b]	Yes
Ring-billed gull (*Larus delawarensis*)[b]	
Royal tern (*Thalasseus maximus*)[ab]	Yes
Sandwich tern (*Thalasseus sandvicensis*)[b]	
Sooty tern (*Onychoprion fuscatus*)[b]	Yes

[a] Documented >3 mi from shore during Deepwater Horizon (DWH) spill response (USFWS 2011)

[b] Based on habitat preference or behavior, likely to be >3 mi from shore (Cornell Lab of Ornithology, American Ornithologist Union 2011)

[c] See Finch et al. (2011) for a full list of species nesting around the Gulf of Mexico during the DWH spill

in open water. Smaller birds such as laughing gulls are also known to feed with brown pelicans, feeding on fish that escape the pelicans, so finding them in the same areas is likely. Breeding and roosting will decrease the amount of time spent >3 mi from shore, with the amount of time spent on the open water foraging during these periods varying by species (Cornell Lab of Ornithology, American Ornithologist Union 2011).

Waterbirds may also be exposed to dispersant-oil mixtures, which should be a consideration in any risk assessment of birds associated with an oil spill. In studies with aquatic organisms, the toxicity of oil-dispersant mixtures is between individual values for oil and dispersant (NRC 2005). Extrapolating existing data to avian embryotoxicity, however, is complicated. In addition to potential taxa differences in toxicity, comparisons between topical doses in avian eggs, where the amount of chemical that penetrates the eggshell and enters the embryo is likely to be only a fraction of the external dose, and aquatic exposures with known concentrations are difficult. Oil, like Corexit 9500,

is composed of multiple components, with components varying according to source and weathering. Predicting the interactions between oil and dispersant components is difficult, therefore mixture studies are required to estimate risk to embryos. Mallard egg exposures to oil:Corexit 9527 mixtures, both directly applied to the egg surface (Albers 1979) and applied via transfer from the mother during incubation (Albers and Gay 1982), have been performed. The toxicity of the oil:Corexit 9527 mixture when transferred from maternal plumage was greater than Corexit 9527 alone, but less than crude oil alone. When eggs were dosed directly, toxicity was dependent on the ratio of oil:Corexit 9527. Similar hatching success was observed for the 5:1 mixture and Corexit 9527 alone, while the 30:1 mixture was less toxic than either crude oil or Corexit 9527 alone. It is unknown what changes in mixture toxicity may have resulted from the reformulation of Corexit 9527 to 9500. Future studies should focus on oil-dispersant mixtures, as well as exposures to embryos at different stages of development to provide a more complete assessment of avian embryo risk during an oil spill.

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