Mississippi Canyon 252

LABORATORY AVIAN TOXICOLOGY STUDIES TO DETERMINE THE EFFECTS OF THE DEEPWATER HORIZON/MC252 OIL SPILL ON BIRD VIABILITY (BIRD STUDY #20)

Approval of this Avian Toxicity Phase 2 Work Plan is for the purposes of obtaining data for the Natural Resource Damage Assessment. Each party reserves its right to produce its own independent interpretation and analysis of any data collected pursuant to this work plan.

This plan will be implemented consistent with existing trustee regulations and policies. All applicable state and federal permits must be obtained prior to conducting work.

The trustees have developed a preliminary conceptual model of the DWH release, potential pathways and routes of exposure, and potential receptors. This preliminary model has informed the trustees' decision to pursue the studies outlined in the work plan.

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Department of the Interior Trustee Representative Date

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Louisiana Trustee Representative Date

1. INTRODUCTION AND PURPOSE

This work plan for Phase 2 of the Avian Toxicity work for the *Deepwater Horizon* Mississippi Canyon 252 (MC 252) Oil Spill (hereafter referred to as "DWH") Natural Resource Damage Assessment (NRDA) describes in more specificity and detail the Avian Toxicity studies that are included as Section 5.13 in "U.S. Department of Interior Interim, Partial Claim for Assessment Costs", July 1, 2011, that was submitted to the U.S. Coast Guard Oil Spill Liability Trust Fund. The work described here will inform the U.S. Department of the Interior's NRDA injury determination and quantification for injuries to avian resources caused by the DWH spill. The July 1, 2011 Interim Partial Claim for Assessment Costs included two phases of NRDA avian toxicity work: Phase 1, in which the avian toxicity studies would be developed and planned, and Phase 2, in which the studies would be implemented. The Phase 1 review of existing literature, planning, organization, and identification of experts and facilities are completed. This Phase 2 work plan, as was described in the July 2011 Interim Partial Claim, builds on the results of Phase 1 and covers the implementation of the avian toxicity study.

The acutely lethal effects to birds of heavy oiling is well-known from many oil spills around the world (e.g., *Exxon Valdez*, *Nestucca, Apex Houston,* various spills in the North Sea) (Burger, 1993). As expected following the DWH spill, many oiled bird carcasses were recovered from shoreline areas along the Gulf of Mexico (GOM). However, in addition there were many more birds observed during and after the spill that were oiled and still alive. Preliminary estimates are that many tens of thousands of birds were oiled and not killed immediately by the oiling. This large number of oiled live birds stands in contrast to many other large oil spills, where oiled birds died relatively quickly through a combination of loss of feather buoyancy and thermoregulatory function, along with toxicological effects. However, these other large spills with high acute bird mortality have typically occurred in colder climates where the birds that became oiled were rafting seabirds for whom the loss of feather function and effects on metabolism have immediate dire consequences. In addition to climate differences, the birds exposed to MC 252 oil and dispersant (hereafter referred collectively to as "MC 252 oil") by the DWH spill were predominantly diving and wading birds (e.g., pelicans, terns, gulls, skimmers, shorebirds) that spend shorter periods of time on the water than do rafting sea birds.

For the NRDA, the fate of the tens of thousands of birds that were oiled during and after the spill is a very important component of the avian injury assessment. The avian toxicity studies proposed herein are focused on determining: (1) what the toxicity is of MC 252 oil to GOM birds; and (2) how that toxicity affects individual bird viability for the species and environmental conditions specific to the DWH oil spill.

An extensive literature survey conducted for the U.S. Fish and Wildlife Service (FWS) Natural Resource Damage Assessment (NRDA) has illustrated that while there is a large body of work on some specific effects of oil spills on birds, there is a distinct lack of information that provides for a systematic and integrated understanding of the relationship between oil exposure and the full extent of effects on avian physiology and behavior.

Observations from previous spills of heavily oiled birds show that they exhibit reduced buoyancy, reduced thermoregulatory ability, anemia, inability to fly, presence of oil and resulting pathology in the digestive system, and can suffer eventual starvation. While several thousand dead and oiled birds were recovered from the GOM in the months during and after the DWH oil spill, many more birds (many tens of thousands) were observed during DWH NRDA and response efforts that were more lightly oiled and still alive. A review of the current state of knowledge on the effects of oil exposure on birds shows that our ability to determine and quantify the injuries to the many thousands of oiled live birds from the available literature and base of knowledge may be limited.

One of the toxic effects of ingested oil to birds is hemolytic anemia (Hartung and Hunt, 1966; Eastin and Rattner, 1982; Pattee and Franson, 1982; Lee et al., 1986; Leighton et al., 1985; Leighton, 1986; Hughes et al., 1990; Yamato et al., 1996; Walton et al., 1997; Newman et al., 2000; Seiser et al., 2000; Troisi et al., 2007). Hemolytic anemia occurs by damage to the hemoglobin in red blood cells associated with the formation of epoxides of polycyclic aromatic hydrocarbons (PAHs) by the action of cytochrome P450 (CYP450) mono-oxygenases in the liver. These reactive oxygen species cause oxidative damage to exposed sulfhydryl groups on the hemoglobin. This results in alterations to the tertiary structure of hemoglobin and causes precipitates to form that can coalesce into Heinz bodies. This damage is irreversible and the body can only compensate by producing more red blood cells to account for the loss of hemoglobin. If compensation is incomplete, then the bird suffers from the potentially life-threatening effects of reduced oxygen carrying capacity. Hemolytic anemia may be particularly important for birds in migration, which is a time when metabolic oxygen demands are extremely high and hemoglobin concentrations are elevated (Piersma et al., 1996). While hemolytic anemia has been observed previously in environmentally oiled birds, there is a distinct paucity of laboratory studies that provide dosage information with respect to type of oil, degree of weathering, species sensitivity or difference between sexes and age classes. Controlled studies have shown that hatchling and nestling gulls can suffer from these effects within four days after oral dosing with 5ml/kg/day (Leighton et al., 1985; Leighton, 1986). While this provides some basic information on potential dosages, it represents only a small step in understanding the development and progression of hemolytic anemia in all of the birds exposed to MC 252 oil.

In the summer of 2010 as part of the NRDA preassessment, the USFWS conducted a field study[1] in the GOM in which black skimmers, brown pelicans, great egrets, and clapper rails were collected from oiled and unoiled areas and their blood was analyzed for signs of hemolytic anemia (as well as other parameters). Approximately 35 birds of each species were collected from oiled areas, and approximately 18 to 35 birds of each species were collected from unoiled areas. The data suggest that birds in the oiled areas were suffering from hemolytic anemia, and that the incidence of hemolytic anemia was higher in birds with more external oiling. These results strongly support the basis for the proposed avian toxicity studies, specifically that external oiling of birds following the DWH oil spill caused hemolytic anemia. However, the preassessment study on hemolytic anemia is limited in its scope. The additional studies proposed

[1] *Evaluating Blood Parameters as a Measure of Physiological Injury to Oiled Birds from the Deepwater Horizon (MC 252) Oil Spill*; also referred to as the *Blood Physiology Study* (DOI 2010)

here will add greatly to the information base established by the preassessment study, and may enable the Trustees to more accurately characterize the likely myriad injuries to an assortment of GOM birds.

Thermoregulation (including both overheating and hypothermia) is also of critical importance to homeothermic species with high energetic demands, such as migratory birds. Oil spills in colder climates have illustrated the detrimental effects of extensive oil coverage on bird survival (Perry et al., 1978). Not only does oil cause reduced buoyancy, which in turn increases the surface area of the bird exposed to cold water, but feather barbules become matted, further reducing the insulative properties of the feathers (Lambert et al., 1982; O'Hara and Morandin, 2010). In areas such as the sub-arctic, this results in rapid hypothermia and death. In more temperate regions, such as in the GOM, these changes in thermoregulatory ability have not been well studied. In areas such as the GOM, the changes in thermoregulatory ability are more likely to increase energetic demands and cause behavioral modifications (Jenssen, 1994). Birds exposed experimentally to oil for only a few hours showed increases in heat production and thermal conductance even at room temperature (McEwan and Koelink, 1973; Erasmus et al., 1981). Jackass penguins with environmental oil exposure covering up to 70% of their bodies had a 2.5^{o} C drop in body temperature after only 15 minutes in water at 19.5-20.5^{o}C. In water temperatures of 4.5 - 6^{o} C eiders exposed to 70ml of oil became hypothermic in only 70 minutes (Jenssen and Ekker, 1991). Sanderlings with 20% oil coverage of their feathers spent significantly more time preening, less time resting and were more aggressive (Burger and Tsipoura, 1998). Environmentally exposed kittiwakes had shorter, more frequent foraging trips following oiling (Walton et al., 1997). The consequences of increased energetic demands to maintain body temperature, combined with behavioral changes such as increased time spent preening and changes in foraging patterns, have the potential to cause weight loss, interfere with reproduction, affect the immune response and prevent optimal body condition for migration. However, currently available information may not be sufficient to assess these injuries for the thousands of live oiled birds exposed to MC 252 oil that were observed during and after the DWH oil spill.

Food intake and nutrient absorption are of critical importance in coping with changes in metabolic demand. Both increased and decreased food intake have been reported in experimentally oiled birds (Holmes et al., 1978a; Holmes et al., 1978b; Szaro et al., 1978; Szaro et al., 1981 1981; Gorsline et al., 1981; Harvey et al., 1982; Miller et al., 1982; Pattee and Franson, 1982; Lee et al., 1985; Hughes et al., 1990; Evans and Keijl, 1993; Burger and Tsipoura, 1998). A bird’s ability to absorb nutrients and produce sufficient fat stores can be affected by a combination of factors including impairments to gastro-intestinal function (Hartung and Hunt, 1966; Beer, 1968; Miller et al., 1982), inflammatory responses (Briggs et al., 1997; Newman et al., 2000) and increased metabolic rate (Butler et al., 1988). While loss of body condition is often reported in oiled birds, the extent of oiling, type of oil and length of exposure that cause these changes are not well understood. Effects on nutrient and fat utilization could be particularly important for the many birds oiled in the GOM during or prior to migration, as the ability of the birds to absorb energy from food is crucial during and immediately after the metabolically taxing process of migration.

Environmental exposure to oil has also been shown to disrupt homeostatic mechanisms. The hypothalamic-pituitary-adrenal (HPA) axis plays a critical role in maintaining homeostasis. Activation of the HPA axis results in secretion of corticosterone and aldosterone from the adrenal cortex. Corticosterone is typically described as being one of the 'stress' hormones, meaning that it coordinates physiological processes to allows the organism to respond to the stressful event. Corticosterone is responsible for re-directing resources from reproduction, reducing inflammatory responses and increasing catabolism of energetic reserves. Oil ingestion has a direct effect on the adrenal cortex that results in cellular damage and affects the body's ability to mount an appropriate stress response. In birds these effects include increased adrenal weight and hypertrophy (Hartung and Hunt, 1966; Miller et al., 1982; Holmes et al., 1979; Peakall et al., 1983; Leighton, 1986), changes in circulating corticosterone concentrations (Rattner and Eastin, 1981) and a blunted stress response (Gorsline and Holmes, 1982). However, it is less clear how this relates to other physiological changes following oil exposure, particularly metabolic and immune changes.

Immune and inflammatory responses are also upregulated in response to stresses such as exposure to oil (Briggs et al., 1996; Perez et al., 2010). Birds exposed to oil show increases in inflammatory responses, depressions in circulating lymphocyte numbers and immunosuppression that results in secondary infections (Fry and Lowenstine, 1985; Briggs et al., 1997; McOrist and Lenghuas, 1992; Newman et al., 2000). The irritant effects of oil on the gastro-intestinal tract, combined with observed decreases in adrenal function, make it unsurprising that inflammatory responses are upregulated. Decreased circulating lymphocyte numbers could be indicative of site-specific action; however, inflammation processes are not well understood in avian responses to oil. Further, the ability of a bird to fight off secondary infections is likely to be diminished given its immunocompromised state.

Conclusion

The information in the current literature helps to identify some of the effects of oil on individual systems or responses in birds. Literature studies show that when ingested by birds at levels less than acutely lethal, oil can cause a wide range of adverse effects, ranging from anemia (loss of oxygen-carrying capacity of blood), decreased nutrient absorption and energy absorption from food, altered stress responses, and decreased immune function. External exposure reduces a bird's thermoregulatory ability and can affect flight performance. For the DWH oil spill, these longer-term injuries to birds may be of particular importance for the tens of thousands of birds that were exposed to MC 252 oil but did not suffer immediate mortality. A limited NRDA preassessment study confirmed that oiled birds were suffering from hemolytic anemia consistent with oil toxicity. However, currently available information from the literature and the field is not sufficient to fully characterize the nature and extent of the injuries to the tens of thousands of oiled birds, or to quantify those injuries in terms of effects on bird viability. The studies proposed here will address these uncertainties and allow us to interpret the observations of the many tens of thousands of live oiled birds in terms of injury and to evaluate causal connections between oiling and observed effects in the field, including mortality.

2. OBJECTIVES

The overall objectives of the proposed Plan are to:

(1) Determine the relationship between internal and external exposure to oil and adverse toxicological effects for GOM-relevant bird species using MC 252 oil and using dosing techniques that can be related directly to the observations of the tens of thousands of birds exposed to MC 252 oil from the spill, including the dead oiled birds recovered during and after the spill.

(2) Assess the acute, subacute and subchronic sublethal impacts, including effects on migratory capability and survival, thermoregulatory capacity, and disease resistance, of exposure to MC 252 oil to the long-term viability of birds.

By addressing these two objectives, the proposed Plan will thus provide information and data that will be used directly in the determination and quantification of injuries to birds from the DWH oil spill.

3. LABORATORY AVIAN TOXICOLOGY STUDIES – GENERAL APPROACH

The general approach of the proposed work is to conduct a series of studies on the toxicology, exposure, and longer-term effects of DWH oil on GOM-relevant bird species to help inform avian injury determination and quantification. There are four distinct but related components to the proposed work (Figure 1), each comprised of a specific study, and the four studies are described in more detail in the next section. The four studies are:

1. **Oral dose-response study.** In this study, GOM-relevant bird species will be used in controlled dose-response studies where MC 252 oil is used to dose birds orally at levels that will be less than acutely lethal. A comprehensive suite of toxicological endpoints will be measured, and quantitative relationships between oral dosing and toxicological response will be developed. This Oral Dosing study will produce comprehensive oral dose-response relationships that will be directly relevant to the DWH spill in terms of bird species, life stages, oil, environmental conditions, and toxicological endpoints, and it will serve as the foundation for the other studies.

2. **External oiling dose-response study**. In this Study, oil will be applied externally to GOM-relevant bird species to mimic the oiling observed in the field. Through preening, the birds will ingest some of the oil, and the oil dose ingested will be determined through the use of the quantitative dose-response relationships developed in the Oral Dosing Study (and perhaps through other means as well, such as indicator compounds). Changes in the detectability of the externally-applied oil by visual

observations and ultraviolet (UV) light will also be measured to provide information for interpreting the magnitude and duration of exposure experienced by birds in the field. The results of this External Dosing Study will be used to relate the field observations of oiled birds post-spill to the toxicological effects determined in the Oral Dosing Study.

3. **Metabolic, thermoregulatory, and flight performance effects study.** In this study, the effects of the toxicological responses caused by MC 252 oil ingestion and the physical effects of oiled feathers on bird metabolism and thermoregulatory capacity will be determined. This Metabolic Effects Study will serve as a surrogate to investigate effects of exposure to MC 252 oil on avian migration, when birds require maximum metabolic output and experience increased stress. Western sandpipers will be dosed orally and externally with MC 252 oil, and their flight performance, metabolism, and refueling capacity will be measured using a laboratory hypobaric climatic wind tunnel for bird flight and quantitative magnetic resonance technique. Other metabolic performance measurements will be made on birds while under other environmental metabolic challenges, such as cold temperatures or moisture. The result of this work will be a quantitative description of how exposure to MC 252 oil (internally and externally) affects bird viability during and immediately after the crucial period of migration and during other periods of metabolic stress.

4. **Field-based flight effects study.** This study will extend the results of the metabolic and thermoregulatory effects study to field conditions. Homing pigeons, which have been used extensively in toxicology tests using toxicants other than oil (Brasel et al. 2006; Brasel et al. 2007; Moye and Pritsos 2010), will be dosed internally and externally with MC 252 oil, and the effects of the oil exposure on their viability will be measured through field flight challenges. Homing pigeon tests have been shown to be very useful in documenting the toxic effects of various chemicals that can affect flight performance, and in combination with the experimental flight chamber work will be used to determine and quantify the effects of exposure to MC 252 oil on the ability of birds to survive migration and migrate successfully.

Under Phase 1 of the NRDA Avian Toxicity Study, these four studies have been carefully planned over the course of many months using input from expert researchers in the fields of avian toxicology, avian metabolism and nutrition, avian flight performance and aerodynamics, immunology, endocrinology, and experimental design. Together, the four studies will produce information that will be used directly in the avian injury assessment of the NRDA.

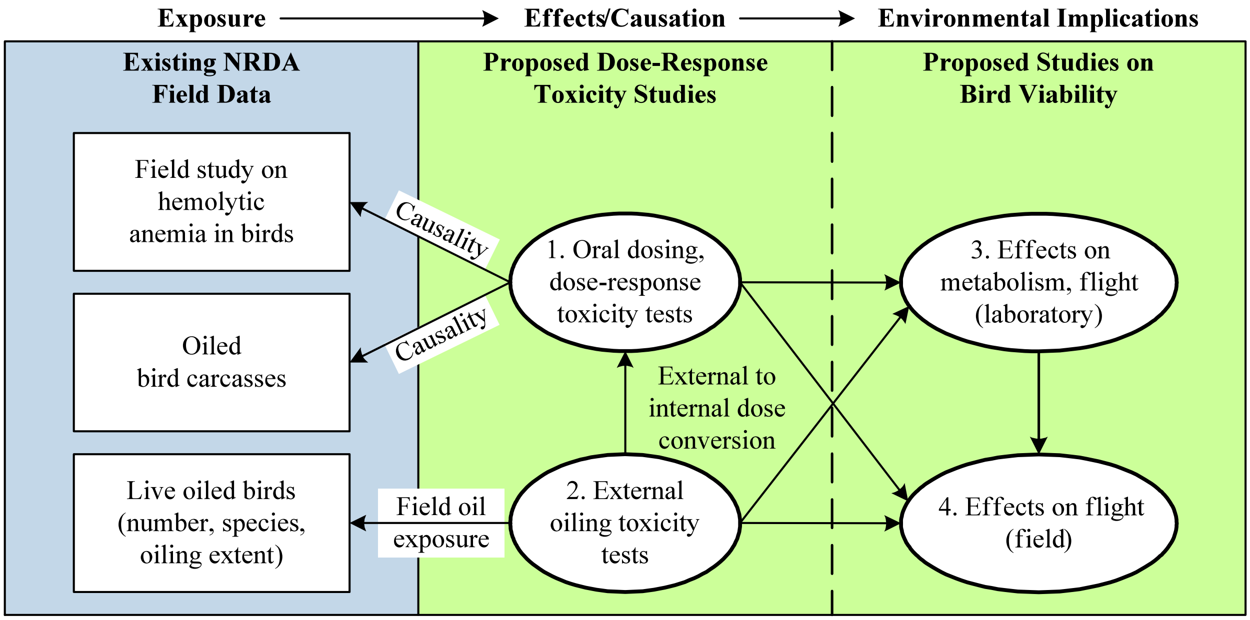

Figure 1. Conceptual model demonstrating linkages among four avian toxicity studies and existing NRDA field data collected during preassessment studies.

4. STUDY DESCRIPTIONS

4.1 Oral Dosing Study

The Oral Dosing Study consists of multiple tests that all investigate the toxicity of ingested MC 252 oil to birds. The measured endpoints will focus on indicators of hemolytic anemia as well as other known and potential toxic effects of oil on birds.

4.1.1 Objectives

The objective of the Oral Dosing Study is to:

- Develop oral dose-response relationships that can be used to predict the toxicity of MC 252 oil ingested by birds following the DWH oil spill (in combination with estimates of oil ingestion by birds exposed in the GOM).

4.1.2 Individual Tests

The Oral Dosing Study will include the following individual tests:

- A preliminary scoping test in which the biochemical and physiological responses of the primary test species (laughing gull (*Leucophaeus atricilla*) and potentially other species, such as double-crested cormorant (*Phalacrocorax auritus*)) (depending on bird availability at the times of the test) to ingested MC 252 oil will be confirmed.

- A comprehensive dose-response test in which the primary species (laughing gull) will be dosed orally with up to five graded doses of MC 252 oil, for durations ranging from one day to approximately 30 days, and a full range of toxicological responses will be measured.

- A test that determines the toxicokinetics of the toxic responses (and recovery, as appropriate) caused by ingestion of MC 252 oil.

The oral dosing studies will be conducted at USDA National Wildlife Research Center (NWRC) avian testing facilities in Fort Collins, CO, and in Starkville, MS.

4.1.3 Species

After investigating species availability, amenability to testing, and test facility capabilities, we anticipate that the following species will be used in the Oral Dosing Study:

- The central, comprehensive oral dose-response test and the toxicokinetics test will be conducted with laughing gull (an omnivorous species). This species was oiled by MC 252 oil, and it represents the tens of thousands of birds that were oiled during the DWH spill.

- Other species that may be preliminarily tested for indications of relative sensitivity to MC 252 oil include (as available): brown pelican (*Pelecanus occidentalis*), black skimmer (*Rynchops niger*), western sandpiper (*Calidris mauri*), royal tern (*Thalasseus maximus*), great egret (*Ardea alba*), and domestic rock pigeon (*Columbia livia*).

All birds, with the possible exceptions of western sandpiper and rock dove, will be collected from the field by NWRC staff who are highly experienced and trained in capturing these species. Western sandpiper and rock dove may be collected by the Principal Investigators responsible for the metabolic effects and field flight effects studies, respectively, as described below. The NWRC bird testing facilities in CO and MS are well-equipped and personnel are highly experienced in housing and maintaining birds collected from the wild and using them as subjects in tests.

4.1.4 Toxicant

Oil that is or represents the MC 252 oil to which birds in the GOM were exposed will be used in dosing studies. This oil may be field-weathered MC 252 oil collected during the spill, artificially weathered (i.e., weathered in the laboratory) MC 252 oil collected from the riser during the spill, or a surrogate, such as an artificially weathered Louisiana sweet crude oil. Pending any chemical analyses of bird carcasses collected during the spill, birds may also be exposed to dispersant along with oil.

4.1.5 Exposure/dosing

Birds will be exposed to MC 252 oil via oral ingestion. Tests will be conducted using single oral doses and daily doses for 5, 15, and approximately 30 days to evaluate the effects of exposure duration on oil toxicity.

4.1.6 Endpoints

The test endpoints will focus on measures of hemolytic anemia, primarily including:

- Heinz bodies
- Haptoglobin and ferritin content in blood
- Complete blood count, which includes red and white blood cell count, hemoglobin, reticulocyte count, platelet count, and packed cell volume

The hemolytic anemia endpoints will be measured at regular intervals, starting before oral dosing and continuing for several days, in order to provide a timeline of hemolytic anemia response. Other measurements or endpoints that will be made may include:

- Plasma clinical chemistry panel
- Cytochrome P450 (CYP450) 1A activity and concentrations
- Immune system measurements, including structural attributes, such as thymus and bursa weight and histology, and functional attributes, such as aspergillus killing assay, which uses a small volume of blood; phytohemagluttinin skin test (a common immune function test in birds); and lipopolysaccharide (LPS) challenge and/or pro-inflammatory cytokines
- Full tissue gross pathology, measurements (e.g., weight) and histopathology
- Oxidative stress markers associated with low hemoglobin and high ferritin, including: glutathione peroxidase in whole blood, superoxidase dismutase in serum, total antioxidant capacity, and lipid peroxidation products (e.g., red blood cell malondialdehyde)
- Corticosteroids and adrenal weight, which can respond to oxidative and metabolic stresses
- Thyroid hormones, which can change in response to hypoxia and thermoregulatory capability

- Body temperature and body weight, which can be indicators of thermoregulatory function and food consumption
- Food nutrient and energy assimilation, which can be adversely affected by the presence of oil in the bird gut

4.2 External Dosing Study

In this Study, MC 252 oil will be applied to bird feathers. Measurements of internal oil dose (e.g., hemolytic anemia, PAH concentrations in blood, or other parameters) from the Oral Dosing Study (Section 4.1) will then be taken to develop quantitative relationships between external oiling and internal dose. The amount of external oil that can be detected visually and under UV light will also be measured over time.

4.2.1 Objectives

The objectives of the external dosing study are to:

- Develop quantitative relationships between external oiling of birds (percent cover, amount, location of oil) and internal dose from the Oral Dosing Study.
- Determine how the ability to detect externally-applied oil visually and with UV light changes over time.
- Identify any other apparent adverse effects from external oiling.

This information will be used to estimate the oral doses of MC 252 oil that GOM birds ingested based on the degree of external oiling observed on birds in the field.

The External Dosing Study will be conducted at the USDA National Wildlife Research Center avian testing facility in Fort Collins, CO.

4.2.2 Species

This Study will be conducted with laughing gull, and potentially other avian species, to provide a direct comparison to the results of the oral dosing tests.

4.2.3 Toxicant

The same MC 252 oil used in the Oral Dosing Study will be used in the External Dosing Study.

4.2.4 Exposure/dosing

Oil will be applied to bird feathers at set percent coverages and volumes that correspond to oiling categories (e.g., trace, light, moderate, or heavy) observed in the field following the DWH oil spill. Exposure variables that will be incorporated into the study include percent body coverage, density of the oiling (i.e., volume of oil/area covered), and location of the oiling. Oil will be applied to areas most commonly observed as oiled or expected to be oiled, such as bills, wings, tail feathers, abdomen, and legs, as well as areas that are more difficult to groom, such as the back of the head.

If a feasible compound is identified, a marker compound could also be mixed with MC 252 oil prior to external application. The marker compound must be soluble in crude oil, resistant to gastrointestinal uptake and metabolism, relatively non-toxic in birds, and readily measurable in feces.

In addition to determining the internal doses that birds ingest from preening oiled feathers, a separate test will be conducted that will test the effects of externally-applied oil on metabolic and thermoregulatory capacity under environmental conditions representative of the GOM. Oil will be applied to the feathers of gulls, and they will be housed in environmental chambers set to mimic GOM conditions. Their metabolic and thermoregulatory status will be closely monitored.

4.2.5 Endpoints

The endpoints of the Study will include both measures of external oiling and measures of internal dose (from the Oral Dosing Study) over time as follows:

- Measurements of internal dose. The exact measurements and their frequency and duration will be determined following the Oral Dosing Study (Section 4.1) and the presence/duration of remaining surface oil on externally oiled birds. The measurements may include:

    - Heinz bodies
    - Haptoglobin and ferritin content in blood
    - Complete blood count, which includes red and white blood cell count, hemoglobin, reticulocyte count, platelet count, and packed cell volume
    - Clinical chemistry
    - PAH concentrations in blood
    - PAH metabolite concentrations in bile and feces
    - CYP450 1A enzymatic activity

- If a suitable marker compound is identified, the concentration or mass of the marker compound in bird feces would be measured as an indicator of the dose of MC 252 oil ingested by birds from preening feathers.

- Regular and frequent visual and UV characterization of oil coverage to establish both level and duration of surface oil on oiled birds. Cages or enclosures will also be inspected under UV light in order to determine whether some oil is being lost to the enclosure environment rather than through ingestion.

- Time budgets with respect to grooming and feeding. Regular assessment of feather damage caused by preening and/or oil/dispersants. Feather damage assessment could be conducted both visually and microscopically.

4.3 Metabolic, Thermoregulatory, and Flight Performance Effects Study

In the Metabolic Effects Study, western sandpipers will be dosed orally, and the metabolic costs and effects of ingestion of MC 252 oil, including the effects of hemolytic anemia on flight performance and thermoregulation and of other toxicological responses on over-wintering or migratory birds exposed to MC 252 oil, will be measured. Additionally, the effects of physical damage (e.g., from preening) or feather-oiling on flight performance will be assessed. The flight performance of the sandpipers will be tested in a laboratory hypobaric climatic wind tunnel for bird flight, where detailed measurements of aerodynamics, flight speed, and flight duration can be made.

4.3.1 Objectives

The objectives of the metabolic, thermoregulatory, and flight performance effects study are to:

- Determine the adverse effects of oral oil dosing on bird energetics and metabolism, including flight performance, thermoregulation, food/energy assimilation, and body composition

- Measure the effects of feather-oiling and feather damage from excessive preening on flight performance

The results will be used to interpret the results of the Oral Dosing and External Dosing Studies (Section 4.1 and 4.2) in terms of bird viability, particularly for oiled birds that are further stressed by the demands of migration or thermoregulatory maintenance.

The Study will be conducted at the Advanced Facility for Avian Research at the University of Western Ontario. Researchers there have been using an experimental wind tunnel for bird flight and environmental chambers to study bird flight mechanics, metabolism, and the effects of toxicants (other than oil) on flight performance for many years. The methods that the researchers use have been well established and published in the peer review literature (Engel et al., 2010; Gerson and Guglielmo 2011; Guglielmo et al. 2011; Seewagen and Guglielmo 2011).

4.3.2 Species

This test will be conducted using western sandpipers as representatives of shorebirds and other similar species exposed to MC 252 oil. Other species of similar or smaller size and relevance to the DWH oil spill may be tested if test methods are applicable. Many species of birds, such as western sandpipers, migrate long distances, and because of their relatively small size (compared to other birds exposed to MC 252 oil) and applicability to methods used in metabolism and flight performance studies, are useful test species for the type of study proposed here. Western sandpiper is also an appropriate species for this study because researchers at the University of Western Ontario have successfully used this species for their laboratory metabolism and flight studies in the past.

4.3.3 Toxicant

The same MC 252 oil used in the Oral Dosing Study will be used in this Study.

4.3.4 Exposure/dosing

Birds will be dosed orally using methods (feed, gavage) and doses based on the results of the Oral Dosing Study. Tests may also be run on birds exposed to phenylhydrazine (a positive control for hemolytic anemia) or on non-exposed birds after removal of red blood cells in order to evaluate the effects of anemia alone. Single or multiple oil/dispersant dosing regimens will be used based on results from the Oral and External Dosing Studies.

Tests will also be conducted using birds with oil applied to their feathers. These tests will provide information on the specific effects of oiling on feather flight performance, which are expected to be substantial and have high metabolic costs for birds in flight (Lambert et al., 1982; Alerstam 1991; Landys et al., 2005; Vaillancourt et al., 2005; Lyons et al., 2008; O'Hara and Morandin, 2010; Eder et al., 2011). Tests will also be run in which birds are exposed both orally and externally to MC 252 oil to evaluate the combined effects from oral toxicity and decreased feather flight performance on the birds’ overall flight performance.

The testing regimen may also include various scenarios of metabolic stress, recovery, and refueling in terms of timing and duration. For example, birds that are orally dosed with MC 252 oil at levels that induce hemolytic anemia would be tested for flight performance or thermoregulatory function. Their ability to refuel and their subsequent response to additional metabolic and immunologic stress would then be tested.

4.3.5 Endpoints

The test endpoints in the flight performance study could include (depending on the size and other characteristics of the test species):

- Flight performance
- Rate of energy expenditure during flight
- Metabolic rates, including basal metabolism and metabolism in response to a shivering challenge, following LPS challenge or ACTH (adrenocorticotropic hormone) stress test

- Body temperature

- Body composition

- Refueling ability after prolonged flight
  - Food restriction/refeeding
  - Digestibility of formulated diet with marker
  - Rate of refueling and rehydration following flight
  - Digestive physiology

- Western sandpiper range-finding dosing study – see Study 4.1, Oral Dosing Study, for list of endpoints. Endpoints chosen will be based on Study 4.1 endpoints considered relevant for avian studies. This could include:
  - Heinz bodies
  - Haptoglobin and ferritin content in blood
  - Complete blood count, which includes red and white blood cell count, hemoglobin, reticulocyte count, platelet count, and packed cell volume
  - Complete blood count
  - CYP450 1A enzymatic activity
  - Clinical chemistry

- Immune system measurements (e.g., aspergillus killing assay, phytohemagluttinin skin test, LPS challenge, pro-inflammatory cytokines)

- Hormone measurements: corticosteroids and thyroid hormones (both of these are directly involved in metabolism and will increase with energetic demands on the system).

4.4 Field Flight Performance Study

In this Study, flight performance and accuracy in free-flying homing pigeons dosed with MC 252 oil orally or externally on their feathers will be determined. The results of the Study will be used in conjunction with the laboratory Oral and External Studies (Sections 4.1 and 4.2) and the Metabolic Effects Study (Section 4.3) to evaluate the effects of internal and external oiling on the viability of birds in the field under high flight performance demands.

4.4.1 Objective

The objective of the Field-Based Flight Performance Study is to determine how oral and external doses of MC 252 oil affect performance in homing pigeons trained for long-distance free flights between release sites and their loft.

The Field-Based Flight Performance Study will be conducted at the homing pigeon research facility at the University of Nevada at Reno.

4.4.2 Test species

Test species will be homing pigeons trained for long-distance flight. This species provides a unique model that allows measurement of flight accuracy and performance of oil-affected birds under unrestrained field conditions. Homing pigeons have been used extensively in toxicity studies, including environmental monitoring of PAHs (Sicolo et al., 2009; Liu et al., 2010).

4.4.3 Toxicant

The same MC 252 oil used in Studies 1 through 3 will be used in this Study.

4.4.4 Exposure/dosing

A single oral dose or, if necessary, multiple doses over a few number of days will be used. The dosing levels will be determined based on the Oral Dosing Study and a preliminary scoping study with pigeons to determine the dose leading to a hemolytic anemia and toxicity response similar to the results in the Preassessment field work (See Footnote 1). External dosing of birds (as described in Section 4.2) will also be employed to assess the aerodynamic and performance effects of MC 252 oil-fouling of feathers, only.

4.4.5 Endpoints

Study endpoints will include those developed to calibrate pigeon dose-response relationships to the Oral Dosing Study (Section 4.1) (including hematological and other analyses to characterize hemolytic anemia and other established toxic effects) and the Metabolic Effects Study (Section 4.3) (at doses bracketing those leading to metabolic and flight impairment, orally and on feathers, respectively) in addition to the following return to loft flight assessments:

- Pigeon range-finding dosing study – see Section 4.1, Oral Dosing Study, for list of endpoints. Endpoints chosen will be based on Oral Dosing Study endpoints considered relevant for avian studies. These could include:
    - Heinz bodies
    - Haptoglobin and ferritin content in blood
    - Complete blood count, which includes red and white blood cell count, hemoglobin, reticulocyte count, platelet count, and packed cell volume
    - Complete blood count
    - CYP450 1A enzymatic activity
    - Clinical chemistry

- Pigeon oral exposure challenge flying study

    - Baseline and post-dosing return flight times following oral dosing in control and MC 252 oil-dosed pigeons as a function of days post dose and degree of hemolytic anemia expressed.

  - Flight trajectory, accuracy and performance in control and MC 252 oil-dosed pigeons returning to the loft using GPS data loggers in backpack mounts with point per second accuracy.

  - Post-flight food and water consumption in control and MC 252 oil-dosed pigeons

  - Pre- and post-flight body condition estimates in control and MC 252 oil-dosed pigeons.

- Pigeon oil and immune challenge flying study – will be performed similarly to the oral dose oil challenge flying study but will add the additional stressor of an immune challenge to groups of control and oil-dosed pigeons.

- Pigeon external feather oiling study - will be performed similarly to the oil challenge flying study but will involve only external oiling of feathers applied immediately prior to flight challenge to avoid preening and oral exposure. Focus will be on flight assessment parameters, only, and will not assess hemolytic anemia endpoints.

5. QUALITY ASSURANCE/QUALITY CONTROL

All studies will be conducted under strict Quality Assurance/Quality Control (QA/QC) requirements that will ensure that the studies produce data of known and acceptable quality. It is anticipated that the studies will be conducted under a single Quality Assurance Project Plan that will address overall QA/QC aspects. In addition, each study will be conducted according to study-specific plans that are developed prior to study initiation, including:

- Study protocols that define all aspects of study design and execution, such as experimental design, dosing regimes, endpoint measurements, and all study methods.

- Standard operating procedures for standard specific study methods such as animal care and feeding, blood withdrawals, biological tissue preparation and preservation, chemistry analysis, data recording, and sampling handling, transport, and chain of custody.

- All written policies and procedures for compliance with each test facility's Institutional Animal Care and Use Committee requirements.

The Quality Assurance Project Plan and other study plan documents will incorporate and adhere to all applicable QA/QC documents for the DWH spill NRDA, such as the "Analytical Quality Assurance Plan, Mississippi Canyon 252 (Deepwater Horizon) Natural Resource Damage Assessment" (version 3.0, December 2011) (AQAP).

The USDA NWRC routinely conducts studies in accordance with Good Laboratory Practice (GLP) requirements, and their GLP policies and procedures will be incorporated into the Quality Assurance Project Plan and specific study protocols and standard operating procedures, as appropriate. In addition, the researchers at NWRC, the University of Nevada at Reno, and Michigan State University are all intimately familiar with the strict QA/QC requirements of conducting avian toxicity studies for litigation. In addition, prior to study initiation all study team members will receive training in QA/QC procedures, adherence to the Quality Assurance Project Plan, and handling and storing environmental samples as evidence.

6. STUDY LOGISTICS

6.1 Facilities

6.1.1 Avian Holding and Testing Facilities

The avian toxicity testing studies will be conducted at the following facilities:

- The USDA NWRC avian research facilities in Fort Collins, CO and Starkville, MS (Oral and External Dosing Studies)
- The Advanced Facility for Avian Research at the University of Western Ontario (Metabolic Effects Study)
- The University of Nevada at Reno (Field Flight Study).

Researchers from all of these institutions have been deeply involved in all aspects of study planning, design, and logistics to date. All of the facilities' personnel are highly experienced in the kind of studies that are being proposed here, and are widely recognized as being top-level institutions for avian research.

6.1.2 Laboratory Facilities

The study will include the analysis of many biological tissue samples for both biological and chemistry analytical endpoints. The specific laboratories that will conduct the biological analyses have not yet been identified. Once study funding is received, a formal process will be initiated to enter into agreements with laboratories that can conduct the analyses. In the process of study planning we have contacted several laboratories that routinely conduct the tests listed under the "endpoints" sections of the study descriptions provided above, and laboratories that have the expertise, facilities, and capacity to conduct the required biological analyses are available.

The analytical chemistry laboratory has also not been identified. Columbia Analytical Services of Kelso, WA, which is currently conducting similar analytical chemistry analyses for the DWH spill, anticipates having capacity and will be considered for the analytical chemistry work. Only laboratories operating in a manner consistent with the AQAP guidelines will be used.

6.2 Bird Capture, Transport, and Maintenance

For the studies conducted at the NWRC facilities, NWRC field staff will conduct all bird capture, transport, and maintenance. NWRC field staff is highly experienced in capturing, transporting, and maintaining wild birds. Based on prior experience, NWRC anticipates collecting double-crested cormorants from the Great Lakes area and southeastern U.S. and all other wild test species, which may include laughing gull, brown pelican, royal tern, great egret, and black skimmer, from the southeastern U.S. NWRC has established policies, procedures, and protocols that comply with all relevant animal care requirements.

Western sandpipers for the Metabolic Effects Study at the University of Western Ontario will be collected from the Pacific coast of Canada during bird migration and transported to the University of Western Ontario for maintenance and testing. The researchers at the University of Western Ontario have prior experience in collecting western sandpipers from the same area and in transporting the birds back to their research facility.

Homing pigeons for the field flight effects study at the University of Nevada-Reno will be taken from the University's flock of trained homing pigeons. Procedures and practices for maintaining the birds are well established.

6.2.1 Bird Collection, Transport, and Land Access Permits

All necessary permits or permissions for handling wild birds (e.g., Scientific Collecting Permit), transporting birds across state or province borders, and accessing public or private lands will be obtained from the appropriate federal, province, or state authority, or other entities. Field staff will carry these permits with them at all times during the collection and transport. In addition, copies of the permits will be kept in any field vehicles involved in collection or transport. While not anticipated, any necessary aerial operations will abide by airspace policies and regulations applicable to the sites being overflown.

6.3 Schedule

The tentative schedule for the proposed work is shown in the table below. The schedule shown assumes that this first phase of funding is available no later than late May, 2012.

SCHEDULE FOR PHASE 2 AVIAN TOXICITY STUDY	2012										2013						
	M	A	M	J	J	A	S	O	N	D	J	F	M	A	M	J	J
Prepare study protocols, QAPP	■	■	■	■													
Capture wild birds			■	■	■	■			■								
Preliminary oral dosing scoping study: Gulls and other species (80 necropsies)																	
Study execution					■												
Sample/data analysis					■	■											
Reporting						■	■										
Oral dosing study: Gulls - Comprehensive																	
Study execution							■	■	■	■	■						
Sample/data analysis									■	■	■	■					
Reporting											■	■	■				
Oral dosing study: Gulls - Toxicokinetics																	
Study execution											■	■	■				
Sample/data analysis													■	■			
Reporting														■	■		
External dosing study: Gulls																	
Study execution													■	■	■		
Sample analysis															■	■	
Data analysis/reporting																■	■
Preliminary oral dosing study: Western sandpipers																	
Study execution				■													
Sample/data analysis					■												
Reporting						■											
Flight metabolism study: Western sandpipers																	
Study execution						■	■	■									
Sample/data analysis									■								
Reporting								■	■	■							
Thermoregulation study: Western sandpipers																	
Study execution											■	■	■				
Sample/data analysis														■			
Reporting															■	■	
Field flight performance: Rock doves - External dosing study																	
Study execution (3 study components)					■	■											
Sample/data analysis						■	■										
Reporting								■	■								
Field flight performance: Rock doves - Oral dosing study																	
Study execution (3 study components)								■	■								
Sample/data analysis										■	■						
Reporting												■	■				
Field flight performance: Rock doves - Oral + external dosing study																	
Study execution (3 study components)													■	■			
Sample/data analysis														■	■		
Reporting																■	

7. PUBLIC OUTREACH AND INVOLVEMENT

Individuals not affiliated with the implementation of this Plan may encounter federal or university personnel in the field during wild bird capturing events. To help prevent third parties from accidentally interfering with the capture of birds, it may be beneficial to conduct public outreach regarding the nature of the field work. The Trustees, in consultation and coordination with USDA and universities conducting this work, will cooperatively determine the extent of advanced notice or other mid-Plan public outreach that should be provided within the capture areas. At a minimum, USDA personnel will identify themselves and vehicles with federal license plates for vehicles and federal identification badges for each employee in the field. Generally, USDA field personnel will also wear USDA labeled clothing and or hats. University field personnel will identify themselves according their institutional requirements.

8. DATA RECORDING AND HANDLING

The data generated during the Plan will include the following:

- Field records (e.g., notes, photographs, video) from the capture and transport of wild birds for use in the testing
- Records generated at the study facilities during animal care and maintenance and study execution
- Sample analysis data, including both biological analyses and chemical analyses.

All data and records will be managed in strict accordance with the data recording and management requirements in the QAPP and in GLP or other similar procedures followed at each facility. Data and records will be generated according to strict QAPP procedures (e.g., data validation of analytical chemistry data, QA/QC controls on all data entry, routine checking of all data records, audits of data recording and maintenance procedures). The USFWS will maintain ultimate control of all data and records. All specific requirements for data recording and handling will be detailed in the project QAPP and other similar documents that apply to each study facility.

The Trustee representative for avian toxicity studies will retain custody of all completed records until they are transferred to the USFWS NRDA Office in Fairhope, Alabama, at the end of the Plan for archiving after implementation of all avian toxicity studies. Camera memory cards containing photos of animals, facilities, procedures, and photo documentation of study activities will remain in the custody of the Trustee representative until the conclusion of the avian toxicity studies and will be archived at the USFWS NRDA Office. Carcasses generated from the avian toxicity studies will be maintained under chain-of-custody at USDA and university facilities until ultimately being transferred to the USFWS NRDA Office or a facility designated by the USFWS NRDA Office, after conclusion of the studies.

All materials associated with the collection or analysis of samples under these protocols or pursuant to any approved work plan, including any remains of samples and, including remains of extracts created during or remaining after analytical testing, must be preserved and disposed of in accordance with the preservation and disposal requirements set forth in Pretrial Orders ("PTOs") # 1, # 30, #35, # 37, #39 and #43 and any other applicable Court Orders governing tangible items that are or may be issued in MDL No. 2179 IN RE: Oil Spill by the Oil Rig "DEEPWATER HORIZON" (E.D. LA 2010). Destructive analytical testing of oil, dispersant or sediment samples may only be conducted in accordance with PTO # 37, paragraph 11, and PTO # 39, paragraph 11. Circumstances and procedures governing preservation and disposal of sample materials by the trustees must be set forth in a written protocol that is approved by the state or federal agency whose employees or contractors are in possession or control of such materials and must comply with the provisions of PTOs # 1, # 30, # 35, 37, #39 and #43.

Laboratories used to implement the four studies described herein shall deliver raw data, including all necessary metadata, generated as part of this work plan as a Laboratory Analytical Data Package (LADP) to the Trustee Data Management Team (DMT), including U.S. Fish and Wildlife Service (USFWS) – Deepwater Horizon NRDAR Field Office, U.S. Army Corps of Engineers Engineer Research and Development Center, and the Louisiana Oil Spill Coordinator's Office (LOSCO) on behalf of the State of Louisiana. All data (including electronically archived data), and copies of original data sheets or electronic files, must be promptly transferred to LOSCO. Originals will be conveyed to USFWS. The electronic data deliverable spreadsheet with pre-validated analytical results, which is a component of the complete LADP, will also be delivered to the secure FTP drop box maintained by the Trustee DMT. Any preliminary data distributed to the Trustee DMT shall also be distributed to LOSCO. Thereafter, the Trustee DMT will validate and perform quality assurance/quality control procedures on the LADP consistent with the authorized Analytical Quality Assurance Plan, after which time the validated data shall be made available simultaneously to all Trustees. Any questions raised on the validated results shall be handled per the procedures in the Analytical Quality Assurance Plan and the issue and results shall be distributed to all parties. In the interest of maintaining one consistent data set for use by all parties, only the validated data set released by the Trustee DMT shall be considered the consensus data set. In order to assure reliability of the consensus data and full review by the parties, no party shall publish consensus data until 7 days after such data has been made available to the parties. Also, the LADP shall not be released by the Trustee DMT or LOSCO, prior to validation absent a showing of critical operational need. Should any party show a critical operational need for data prior to validation, any released data will be clearly marked "preliminary/unvalidated" and will be made available equally to all Trustees.

9. BUDGET

Detailed cost estimate for the work proposed herein has been developed over the last several months. The estimates are based on a detailed level of study design that addresses all aspects of the proposed work to be conducted by the researchers at the facilities where the studies will be conducted.

A summary of the Phase 2 costs are presented in the table below. As shown in the table, the total cost of the Phase 2 Plan is $4,501,989.

Avian Toxicity Plan Phase 2 Cost Estimate	
Study Component	**Cost**
Finalizing study QA/QC and protocol documents	$391,194
Oral dosing study: Preliminary scoping	$338,447
Oral dosing study: Comprehensive study with laughing gulls	$955,428
Oral dosing study: toxicokinetics study	$282,123
External dosing study	$616,134
Metabolic effects study	$824,951
Field flight effects study	$671,898
Data analysis	$421,814
Total	**$4,501,989**

10. PERSONNEL

Project Managers

Doug Beltman (303-381-8000; dbeltman@stratusconsulting.com), Stratus Consulting, Inc.; Role: Project management and primary contact for Stratus Consulting, Inc.

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