Marine Pollution Bulletin 127 (2018) 170–174

Contents lists available at ScienceDirect

Marine Pollution Bulletin

journal homepage: www.elsevier.com/locate/marpolbul

Effects of glyphosate herbicide on the gastrointestinal microflora of Hawaiian green turtles (*Chelonia mydas*) Linnaeus☆

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ARTICLE INFO

Keywords:
Bacteria
Digestion
Glyphosate toxicity
Green turtle
Hawaii
Microbial communities

ABSTRACT

In Hawaii, glyphosate-based herbicides frequently sprayed near shorelines may be affecting non-target marine species. Glyphosate inhibits aromatic amino acid biosynthesis (shikimate pathway), and is toxic to beneficial gut bacteria in cattle and chickens. Effects of glyphosate on gut bacteria in marine herbivorous turtles were assessed *in vitro*. When cultures of mixed bacterial communities from gastrointestinal tracts of freshly euthanized green turtles (*Chelonia mydas*), were exposed for 24 h to six glyphosate concentrations (plus deionized water control), bacterial density was significantly lower at glyphosate concentrations $\geq 2.2 \times 10^{-4}$ g L^{-1} (absorbance measured at 600 nm wavelength). Using a modified Kirby-Bauer disk diffusion assay, the growth of four bacterial isolates (*Pantoea*, *Proteus*, *Shigella*, and *Staphylococcus*) was significantly inhibited by glyphosate concentrations $\geq 1.76 \times 10^{-3}$ g L^{-1}. Reduced growth or lower survival of gut bacteria in green turtles exposed to glyphosate could have adverse effects on turtle digestion and overall health.

1. Introduction

Gastrointestinal (GI) microbes are critical to green turtles (*Chelonia mydas* Linnaeus) for the digestion of the complex carbohydrates in their diet of marine macroalgae and seagrasses (Bjorndal, 1979; Bjorndal, 1980; Bjorndal, 1985; Bjorndal et al., 1991). Microbial populations in green turtle GI tracts are dependent on a number of factors, including turtle diet and habitat (Bjorndal, 1985). Glyphosate or *N*-(phosphonomethyl) glycine, the world's most commonly used herbicide (Baylis, 2000) for weed control in agricultural, urban, conservation, and aquatic areas is advertised for its reputed low toxicity to animals (Dill et al., 2010). However, glyphosate has been shown to be toxic to some bovine gut microflora, to increase short-chain fatty acid production, to decrease NH3-N concentrations in the rumen, and to cause less efficient digestion in cattle (Krüger et al., 2013; Samsel and Seneff, 2013; Riede et al., 2016). In poultry, Shehata et al. (2013) reported that many beneficial gut bacteria, e.g. *Bifidobacterium adolescentis*, *Bacillus* sp., *Enterococcus* spp., and *Lactobacillus* sp., some of which also occur in green turtle cloacal fluid (Aguirre et al., 1994, Santoro et al., 2006, Keene et al., 2014, Price et al., 2017), were moderately to highly susceptible to glyphosate.

Glyphosate inhibits enzyme activity, Fe^{+2} transport, as well as, respiration and growth in some bacteria (Roisch and Lingens, 1980, Barton et al., 1982, Chan and Leung, 1986). Most notably, glyphosate inhibits a key enzyme, 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase, in the shikimate pathway (or shikimic acid pathway), a metabolic pathway in plants, as well as fungi and bacteria, for the biosynthesis of aromatic amino acids (phenylalanine, tyrosine and tryptophan) that are essential for protein synthesis (Amrhein et al., 1980; Franz et al., 1997). Glyphosate adheres strongly to particles, which facilitates long distance transport and persistence in the environment, because binding may help protect glyphosate from degradation (Solomon and Thompson, 2003) by microbes into aminomethylphosphate (AMPA) and CO_2 (Balthazor and Hallas, 1986; Franz et al., 1997; Schuette, 1998). Stormwater and wastewater from urban areas contaminated by herbicides can transport glyphosate to receiving waters (Kolpin et al., 2006; Botta et al., 2009; Zgheib et al., 2012). The coastal areas near Hilo, Hawaii, receive a mean annual rainfall of 3303 mm, experience approximately 275 days $year^{-1}$ of precipitation (Giambelluca et al., 2013), and are subject to seasonal flash flooding and run-off, which could convey glyphosate to coastal waters. In flask experiments, the half-life for glyphosate in seawater with marine bacterial populations was 47 days (25 °C in low-light) up to 310 days (31 °C in the dark) (Mercurio et al., 2014), leading to the conclusion that

☆ This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.
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https://doi.org/10.1016/j.marpolbul.2017.11.030
Received 10 August 2017; Received in revised form 13 November 2017; Accepted 13 November 2017

"little degradation would be expected during flood plumes in the tropics, which could potentially deliver dissolved and sediment-bound glyphosate" into the marine environment. Low levels of glyphosate were detected in Marennes–Oléron Bay along the Atlantic coast of France, during an 11-day period (maximum concentration = 1.2×10^{-6} g L^{-1}) of a run-off event in Spring 2004 (Burgeot et al., 2007). Skeff et al. (2015) observed glyphosate concentrations ranging from 2.8×10^{-8} to 1.69×10^{-6} g $L^{-1}$and AMPA concentrations ranging from 4.5×10^{-8} to 4.16×10^{-6} g L^{-1} in water samples from ten estuaries in the Baltic Sea in 2012. These studies show that measurable amounts of glyphosate and its primary metabolite can be transported to coastal environments where marine species may be exposed to contaminants.

In the Hawaiian Islands, glyphosate-based herbicides (e.g. Roundup® and Rodeo®) are frequently sprayed by the public and private sector to combat unwanted or invasive vegetation along roadways, in yards, on golf courses, in fields, and near freshwater, marine, and anchialine shorelines. Although, to our knowledge, no coastal waters in the Hawaiian Islands have been tested for glyphosate, this compound may be reaching coastal waters and affecting non-target marine species, such as green turtles, their food plants, and/or their GI bacterial communities. Green turtles in the Hawaiian Islands comprise a genetically and geographically discrete subpopulation that has rebounded in abundance over the past four decades (Balazs et al., 2015), yet shows reduced somatic growth in some nearshore foraging areas (Balazs and Chaloupka, 2004). Only a handful of studies have documented effects of glyphosate on reptiles, such as skinks, lizards, and freshwater turtles (Sparling et al., 2006; Carpenter et al., 2016; Schaumburg et al., 2016), and all involved direct, topical application of glyphosate to eggs or skin. Douros et al. (2015) found measurable amounts of glyphosate in snapping turtle tissue that were lower than glyphosate concentrations in the water column. None of these studies examined glyphosate effects on reptile gut bacteria. No studies have assessed the effect of glyphosate on marine turtles or their GI bacterial communities.

The objective of this study was to quantify the sensitivity of microbes in the GI tract of green turtles to a glyphosate-based herbicide. The hypotheses were: 1) bacterial communities will be negatively affected by exposure to glyphosate; 2) different taxa of bacteria from green turtles will demonstrate different sensitivities to glyphosate; and 3) inhibition of bacterial growth will be dependent on glyphosate concentration.

2. Methods

Eight green turtles that had required euthanization after a collaborative assessment by Dr. Thierry Work, Wildlife Pathologist of the USGS, and the National Marine Fisheries Service of NOAA, due to mortal injury or terminal illness, served as donors immediately post-mortem for this project (Work, 2014). No attempts were made to capture wild, free-ranging turtles from the sea for euthanasia because of stringent protections under the US Endangered Species Act. All turtles sampled had seaweed in the gut without any indication of rotting digesta (H_2S smell). Turtles were collected from several locations in the main Hawaiian Islands, and included males and females with straight carapace lengths ranging from 49 to 74 cm. Microbial samples were taken at five locations within the gastrointestinal tract (crop, stomach, small intestine, caecum, and large intestine) by wiping two sterile cotton swabs on the interior of each GI tract area. One swab was placed inside a BD BBL Vacutainer™ anaerobic specimen collector (Becton, Dickinson and Company), a second swab was put on nutrient agar medium for transport, and both were re-streaked within 4 h on nutrient agar. Cultures were grown under aerobic and anaerobic conditions at 30 °C.

The mixed bacterial community from the caecum of a 14.7 kg male turtle (case #25338) obtained from Haleiwa, Oahu was cultured in nutrient broth at 30 °C on May 10, 2016. Aliquots (1.0 mL) of this mixed bacterial community culture were inoculated in triplicate into 50 mL centrifuge tubes containing 40 mL of nutrient broth plus glyphosate concentrations representative of low (2.2×10^{-4} and 4.4×10^{-4} g L^{-1}), medium (5.6×10^{-2} and 0.1125 g L^{-1}), and high (1.8 and 3.6 g L^{-1}) concentrations of glyphosate. The source of the glyphosate in our experiments was Rodeo®, the brand name for a glyphosate-based herbicide manufactured by Dow AgroSciences that contains 53.8% isopropylamine salt of glyphosate as its active ingredient. Rodeo® Herbicide was chosen because it contains no surfactant, and is recommended for use in and around aquatic sites and wetlands. The glyphosate concentrations were selected to approach amounts reported from European coastal environments (Burgeot et al., 2007; Skeff et al., 2015), and to include the manufacturer's recommended concentration of 0.75% Rodeo® (= glyphosate concentration 3.6 g L^{-1}) (Rodeo® label). A control was maintained with glyphosate concentration of 0 g L^{-1}. Initial mixed bacterial community densities were assessed by measuring the absorbance of the samples at 600 nm wavelength using a Beckman DU-600 spectrophotometer, similar to the method described by Moneke et al. (2010). The wavelength of 600 nm was chosen because the least amount of interference from nutrient broth occurred at 600 nm. After 24 h, the bacterial communities incubated with and without glyphosate were re-assessed by determining the optical density of the samples at 600 nm. Glyphosate treatment effects on the survival and growth of the bacterial community at different concentrations were arcSine transformed to normalize data, and analyzed using a one-way ANOVA and post-hoc Tukey test.

Individual bacterial colonies were isolated from the original samples using MacConkey agar (for the isolation of Enterobacteriaceae), mannitol salt agar (for the isolation of *Staphylococcus* species), and xylose-lysine deoxychloate (XLD) agar (for the isolation of *Salmonella* and *Shigella* species). Plates were incubated at 30 °C in a 12-140E incubator (Quincy Lab) aerobically. Isolated colonies were identified by Gram-staining and the following biochemical reactions: indole production (BD Cat. no. 261185), oxidase production (BD Cat. no. 261181), and Voges-Proskauer A & B reagents (BD Cat. no. 261192 and 261193). Identification of Gram-negative organisms was confirmed by Enteropluri Test Kit (Becton, Dickinson and Company, Cat. no. L010570), a 12-sector system containing special culture media that permits identification of Enterobacteriaceae and other Gram-negative, oxidase-negative bacteria. Cultures were incubated at 36 °C and examined after 18 h, according to manufacturer instructions.

The Kirby-Bauer disk diffusion assay method (Bauer et al., 1966) was used to determine the effects of different concentrations of glyphosate on four bacterial taxa isolated from the original GI tract areas (crop, stomach, small intestine, caecum, or large intestine) from turtles euthanized on May 13, 2015, September 29, 2015 and November 1, 2015 (case # 25196, 25197, 25245, 25246 and 25254), as described in the preceding paragraph. The four taxa included *Pantoea* sp., a member of the Family Enterobacteriaceae involved in biodegradation and fermentation in host animals i.e. fish, insects, crabs, crustaceans, chickens, cows, cattle, and panda (Walterson and Stavrinides, 2015); *Proteus* sp., a Gram-negative, heterotrophic, facultative anaerobe, and symbiotic or neutral commensal in normal intestinal microflora. (Drzewiecka, 2016); *Shigella* sp., a common, Gram-negative, non-motile, GI tract bacterium; and *Staphylococcus* sp., a Gram-positive, non-pathogenic or pathogenic bacterium involved in carbohydrate breakdown (Strasters and Winkler, 1963). Fifteen serial dilutions of glyphosate-based herbicide (Rodeo®) resulting in glyphosate concentrations from 2.2×10^{-4} to 3.6 g L^{-1} were prepared in a 500 mL flask using sterile deionized (DI) water. These glyphosate concentrations were chosen to approach the maximum amounts reported from coastal waters in France and the Baltic Sea (Burgeot et al., 2007; Skeff et al., 2015), and to include the manufacturer's recommended concentration of 0.75% Rodeo® (= glyphosate concentration 3.6 g L^{-1}) (Rodeo® label). Isolates of each bacterial taxon were streaked in a "lawn" to cover the nutrient agar in the petri dish, and immediately, three filter paper disks that had been soaked for

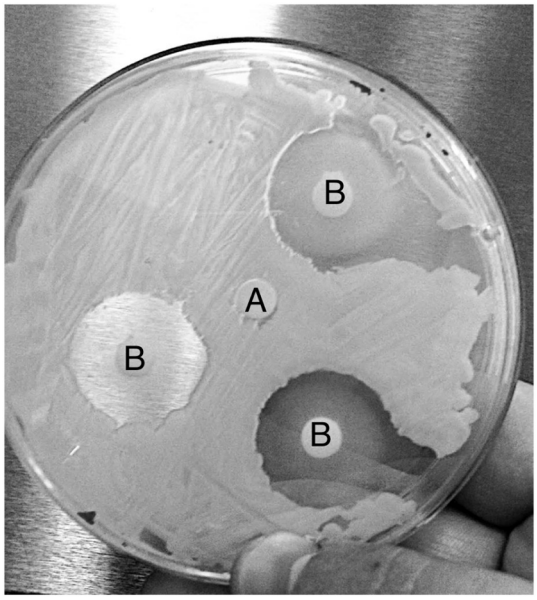

Fig. 1. Kirby-Bauer modified disk diffusion assay used for glyphosate sensitivity of *Shigella* sp. from green turtle GI tract. Center disk (A) is a deionized water control. Disks labeled B were soaked for 10 s in a glyphosate solution with concentration 0.45 g L^{-1}.

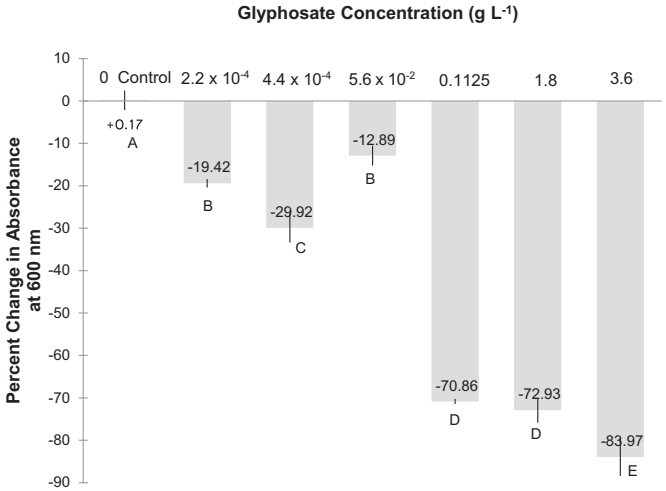

Fig. 2. Percent change (values stated inside the bars) in optical density of nutrient broth cultures of green turtle gastrointestinal bacterial communities incubated with seven different concentrations of glyphosate (0 to 3.6 g L^{-1}). Absorbance was measured spectrophotometrically at 600 nm wavelength at start of experiment and 24 h later. One-way ANOVA was used to detect differences in percent change of absorbance among concentrations with a post-hoc Tukey test (df = 20, F = 29.213, $p < 0.001$). Error bars denote standard deviation. Shared letters denote no significant difference.

10 s in a specific concentration of glyphosate were firmly placed on the streaked surface of the petri dishes. An additional filter paper disk soaked in sterile DI water, to serve as a control, was firmly placed in the center of each streaked petri dish. Six replicate petri dishes were used for each concentration for each of the four bacterial taxa (total of 360 petri dishes). Cultures were incubated for 18 h at 30 °C and any zones of inhibition were measured (Fig. 1). The mean zone of inhibition for each concentration for each bacterium was determined by averaging the diameters of the "halo" of no growth across the six petri dishes (n = 18 disks/concentration). One-way ANOVA was used to detect differences in zone of growth inhibition among the concentrations plus a post-hoc Tukey test.

3. Results

In the bacterial community experiment, high absorbance in a sample indicates high bacterial density in the nutrient broth, and all samples had an initial absorbance of 0.614 or greater. After 24 h, all glyphosate doses showed significantly negative changes in absorbance (= lowered density of bacterial community) compared to the control (Fig. 2), which increased in absorbance (mean = + 0.17 ± 2.32). The lowest observed effect level (LOEL) was 2.2×10^{-4} g L^{-1} and a no observed effect level (NOEL) was not determined. Bacterial communities incubated with glyphosate concentrations ≥ 0.1125 g L^{-1}, showed 70% or more reduction in absorbance.

From the Kirby-Bauer assay, zones of growth inhibition increased in a dose-dependent manner for all four bacterial taxa tested (Fig. 3A–D). The LOELs were 1.76×10^{-3}, 0.225, 2.81×10^{-2}, and 7.03×10^{-3} g L^{-1} for *Pantoea*, *Proteus*, *Shigella*, and *Staphylococcus*, respectively. The respective NOELs were 8.8×10^{-4}, 0.1125, 1.4×10^{-2}, and 3.52×10^{-3} g L^{-1}.

4. Discussion

Glyphosate had significant effects on the mixed microbial community of the green turtle microflora and the four individual aerobic GI tract microbial taxa tested in this study. Kirby-Bauer disk diffusion assays and spectrophometric optical density assessments can be utilized as complementary techniques to understand the effects of glyphosate on GI tract microflora in green turtles. In experimental freshwater pools and lab cultures, glyphosate modified the growth, respiration, and enzyme activity of aquatic bacteria (Chan and Leung, 1986). In natural marine planktonic microbial communities, glyphosate concentrations as low as 1.0×10^{-6} L L^{-1} reduced species richness, lowered species diversity, and significantly changed species composition (Stachowski-Haberkorn et al., 2008). In comparison, our LOEL was a glyphosate concentration 2.2×10^{-4} g L^{-1} that caused reduced density of the turtle GI tract bacterial community. Changes in species composition and lowered species diversity may also occur in the microbial communities in the GI tract of green turtles exposed to glyphosate, but remain to be tested.

A common intertidal red macroalga, *Pterocladiella capillacea*, which is a predominant component of green turtle diets at many foraging locations throughout the Hawaiian Islands (Russell and Balazs, 2009), showed significantly lower photosynthetic efficiency, reduced chlorophyll content, and lower survival after experimental glyphosate exposure (Kittle and McDermid, 2016). Glyphosate residue, although not quantified in seaweeds and seagrasses, has been shown to reduce nutrient quality in terrestrial plants (Jolley et al., 2004; Gordon, 2007; Cakmak et al., 2009). When macroalgae and seagrasses are exposed to glyphosate, their nutritional quality may be reduced for grazing green turtles.

Information on the direct effects of glyphosate on reptiles that in nature are exposed to glyphosate-based herbicides is sparse. Sparling et al. (2006) found a reduction in body weight of red-eared slider turtles and lowered hatching success in eggs exposed to high, but not to low, concentrations of glyphosate. Common snapping turtles (*Chelydra serpentina*) in the Embarras River in Illinois, USA showed measurable amounts of glyphosate in tail-snip tissue samples, leading Douros et al. (2015) to conclude that snapping turtles would be a valuable indicator species in long-term herbicide monitoring. Diurnal skinks (*Oligosoma polychroma*) in New Zealand altered their thermoregulatory behavior and selected significantly higher temperatures for three weeks following experimental dermal exposure to glyphosate (Carpenter et al., 2016), but their body mass was unaffected. Topical exposure of endemic Argentinian tegu lizard eggs (*Salvator merianae*) to Roundup® under laboratory conditions caused DNA damage, but did not affect lizard size at birth or at six months post-exposure (Schaumburg et al., 2016). The direct effects of glyphosate on green turtles (*Chelonia mydas*) in the marine environment are unknown.

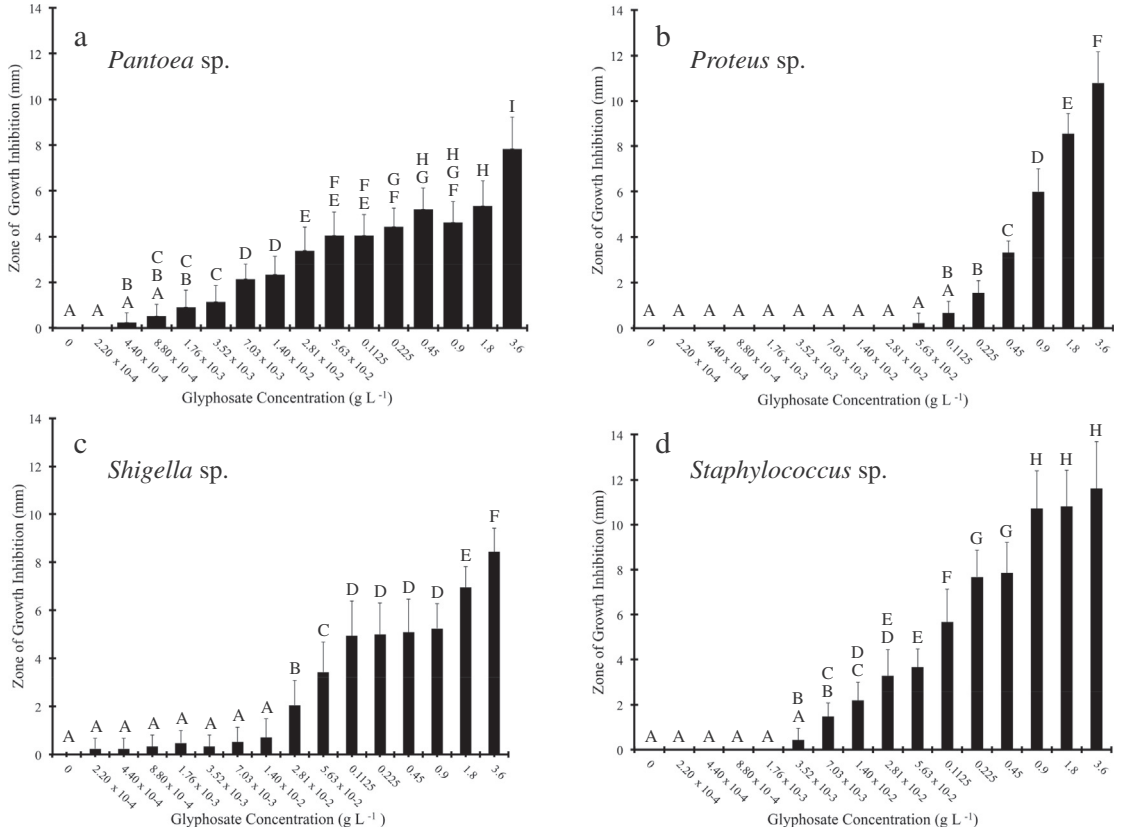

Fig. 3. Mean diameter (mm) of zone of growth inhibition in four bacterial taxa isolated from turtle GI tracts and incubated with disks soaked in 15 different concentrations of glyphosate with a DI water control. One-way ANOVA was used to detect differences in zone of inhibition among concentrations plus a post-hoc Tukey test. Error bars denote standard deviation. Shared letters denote no significant differences.
a. *Pantoea* sp. (df = 15, F = 151.76, $p < 0.001$).
b. *Proteus* sp. (df = 15, F = 362.37, $p < 0.001$).
c. *Shigella* sp. (df = 15, F = 187.69, $p < 0.001$).
d. *Staphylococcus* sp. (df = 15, F = 332.47, $p < 0.001$).

This is the first study assessing the effect of glyphosate-herbicide on the GI tract microflora of any reptile. Significant effects were observed and future studies should test even lower concentrations in order to determine the NOEL for glyphosate. Use of glyphosate in coastal environments might be expected to have detrimental ramifications for green turtles via dermal exposure, changes in marine plant communities, and/or alterations of GI tract microflora. Green turtles and their GI tract bacteria could be exposed to glyphosate via ingestion of glyphosate-contaminated water during foraging or consumption of glyphosate-exposed foods. Inhibition of gut microflora by glyphosate may have adverse effects on digestion efficiency and overall health. A change or reduction of critical aerobic and anaerobic bacteria in the green turtle could negatively impact digestion of seaweeds and seagrasses. Thus, if green turtles are exposed to glyphosate via their food or environment, their GI microflora may be altered, and digestion efficiency could be compromised. The consequences of long-term exposure to glyphosate, even at low levels, to green turtle gut microflora are unknown.

Acknowledgements

We are grateful to Dr. Jon Awaya of the University of Hawaii at Hilo for the use of his microbiology lab, to Dr. Thierry Work, Renee Breden, Bob Rameyer and others in the USGS Lab, and to T. Todd Jones and Shandell Brunson of the NOAA Marine Turtle Biology and Assessment Program. We appreciated the careful reading of our manuscript by an anonymous reviewer!

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