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Results of Laboratory Testing for Diphacinone in Seawater, Fish, Invertebrates, and Soil Following Aerial Application of Rodenticide on Lehua Island, Kauai County, Hawaii, January 2009

Open-File Report 2009–1142

U.S. Department of the Interior
U.S. Geological Survey

Cover. Black-footed albatross on Lehua Island, Kauai County, Hawaii (photograph courtesy of Eric VanderWerf, Pacific Rim Conservation).

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U.S. Department of the Interior
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U.S. Department of the Interior
KEN SALAZAR, Secretary

U.S. Geological Survey
Suzette M. Kimball, Acting Director

U.S. Geological Survey, Reston, Virginia: 2009

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Conversion Factors

Multiply	By	To obtain
	Length	
meter (m)	0.3048	feet (ft)
centimeter (cm)	2.54	inch (in)
kilometer (km)	0.621388	mile (mi)
nanometer (nm)	=	10^{-9} meter
	Volume	
microliter (µL)	0.00003382	ounce, fluid (fl. oz)
milliliter (mL)	0.03382	ounce, fluid (fl. oz)
liter (L)	33.82	ounce, fluid (fl. oz)
	Mass	
kilograms (kg)	2.204	pounds (lbs)
gram (g)	0.03527	ounce, avoirdupois (oz)
microgram (µg)	=	1 x 10^{-6} grams
nanogram (ng)	=	1 x 10^{-9} grams
	Concentration	
molar (M)	=	moles per liter
millimolar (mM)	=	millimoles per liter (10^{-3} M)
nanograms per milliter (ng/mL)	=	part per billion (ppb; 10^{-9}))
microgram per gram (µg/g)	=	parts per million (ppm: 10^{-6})
microgram per milliliter (µg/mL)	=	parts per million (ppm: 10^{-6})
nanogram per gram (ng/g)	=	parts per billion (ppb: 10^{-9})
microgram per liter (µg/L)	=	parts per billion (ppb: 10^{-9})
nanogram per liter (ng/L)	=	parts per trillion (ppb: 10^{-12})
kilograms per hectare (kg/ha)	0.8888	pounds per acre (lb/acre)
	Area	
hectare (ha)	2.47	acre

Temperature in degrees Celsius (°C) may be converted to degrees Fahrenheit (°F) as follows:

$$°F = (1.8 \times °C) + 32$$

Concentrations of chemical constituents in solid materials (tissues) are given in nanogram per gram (ng/g, or parts per billion, ppb) wet weight. Concentrations of chemical constituents in calibration standard solutions and in liquid samples (seawater) are given in nanograms per liter (ng/mL, or parts per billion, ppb).

Results of Laboratory Testing for Diphacinone in Seawater, Fish, Invertebrates, and Soil Following Aerial Application of Rodenticide on Lehua Island, Kauai County, Hawaii, January 2009

By Carl E. Orazio[1], Michael J. Tanner[1], Chris Swenson[2], Jeffrey Herod[2], Peter Dunlevy[3], and Robert W. Gale[1]

Abstract

In January 2009, rodenticide bait (Ramik Green® pellets) containing the active ingredient diphacinone was aerially applied to Lehua Island. Reported herein are the results of laboratory analyses to determine diphacinone concentrations in samples of seawater, fillet of fish, soft tissue of limpets (opihi), whole-body crabs, and soil collected from Lehua Island, Kauai County, Hawaii, after aerial application of the rodenticide bait. Diphacinone was specifically chosen because of its low toxicity to nontarget organisms. Its use on Lehua Island is the second time it has ever been used for an aerial application to eradicate rodents. Testing of the Lehua Island samples for diphacinone utilized high-performance liquid chromatography with photodiode array detection. No detectable concentrations of diphacinone were found in any of the samples from Lehua Island. The limits of detection for diphacinone were 0.4 nanograms per milliliter (parts per billion) seawater, 15 nanograms per gram (dry weight) soil, 20 nanograms per gram (parts per billion) fish fillet, 13 nanograms per gram whole crab, and 34 nanograms per gram soft tissue limpet.

Introduction

Oceanic islands contain a disproportionate share of the world's unique terrestrial species and are especially vulnerable to the impacts of invasions by nonnative species, including rats. More than 80 percent of all oceanic islands worldwide have been infested by some species of invasive rodent. The ecosystems on oceanic islands are extremely susceptible to disturbances caused by infestations of invasive species because of their limited habitat coverage and the close integration of niche species. Most species extinction events that have occurred or are occurring in these isolated ecosystems are caused by invasive species. Many island rodent eradication projects have been successfully conducted worldwide using anticoagulant rodenticides.

Lehua Island is an uninhabited 312-acre crescent-shaped volcanic cone located about three-fourths of a mile north of Niihau and 20 miles (mi) west of Kauai (fig. 1). Lehua Island is a State designated seabird sanctuary that supports colonies of seabirds such as Laysan (*Phoebastria immutabilis)* and black-footed albatross (*Phoebastria nigripes)*, red-footed (*Sula sula*) and brown boobies (*Sula leucogaster)*, black noddies (*Anous minutus)*, Newell's (*Puffinus newelli*) and wedge-tailed shearwaters (*Puffinus pacificus)*, red-tailed tropicbirds (*Phaethon rubricauda)*, and band-rumped storm petrels (*Oceanodroma castro)*. During the first biological surveys of Lehua in 1931, Polynesian rats (*Rattus exulans*) and European rabbits (*Oryctolagus cuniculus*) were found. Both of these species are alien to Hawaii and had been present on Lehua for an unknown period of time. Rabbits were eradicated from Lehua in 2006. Other restoration efforts include restoring native plant communities and managing the barn owl, an introduced seabird predator species. A coalition of conservation agencies have developed a comprehensive 10-year restoration plan that includes managing the owls, eradicating rats, and transplanting native birds, land snails, terrestrial arthropods and plants to the island.

For the Lehua Island rat eradication, the U.S. Fish and Wildlife Service (USFWS), the Hawaii Department of Fish and Wildlife (DOFAW), and the U.S. Department of Agriculture, Animal and Plant Health Inspection Service, Wildlife Services (USDA–APHIS–WS) jointly proposed aerial application of diphacinone (0.005% active ingredient). Diphacinone is a chronic anticoagulant rodenticide that acts by disrupting the normal blood clotting mechanisms of vertebrates, competing at receptor sites in the liver with vitamin K, a necessary chemical for blood clotting. Diphacinone has been shown to

[1]U.S. Geological Survey.

[2]U.S. Fish and Wildlife Service.

[3]U.S. Department of Agriculture.

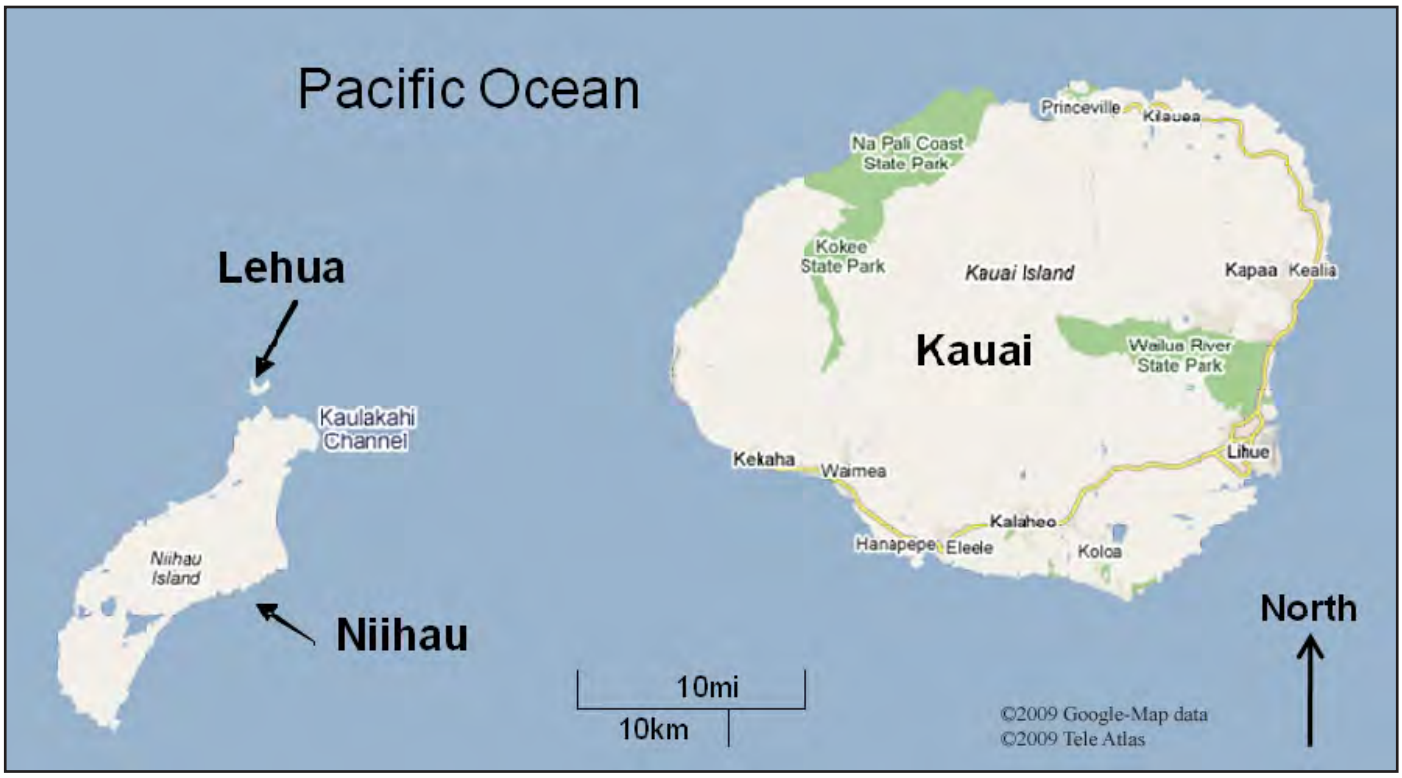

Figure 1. Lehua and Niihau Islands, Hawaii (Created from GoogleEarth© image).

be an effective toxicant for rats in Hawaii and elsewhere. It is efficacious, yet has relatively low risk of impacts to nontarget species through consumption of prey that have consumed the bait pellets (secondary impacts). Diphacinone was specifically chosen for use because of its low toxicity to nontarget organisms, its relatively short half-life in the environment, and its low biomagnifications potential (U.S. Environmental Protection Agency, 1998). Its use on Lehua Island is the second time it has ever been used for an aerial application to eradicate rodents. The first such use was on Mokapu Island, Hawaii, near Molokai in winter 2008. Early wintertime was chosen for the bait application time when alternate rat foods and rat populations are lowest and migratory native nontarget species are not present or are only present in low numbers.

Previous to the Lehua aerial applications, staff from various Federal and State agencies camped on the island and marked transects. Albatross (Laysan, black-footed) nests were mapped so pellets could be removed that fell near nests with chicks the day of application. Populations of desired nontarget species, including nesting seabirds and protected plants, were monitored actively for approximately 2 years prior to the 2009 application to produce reliable population estimates of adverse impacts before and after rodenticide treatments.

On January 6 and 12, 2009, the USDA–APHIS–WS Hawaii State office, in cooperation with USFWS Pacific Islands Wildlife Office and the State of Hawaii, applied a total of 7,800 pounds (lbs) of rodenticide bait (Green Ramik® pellets) containing the active ingredient diphacinone on Lehua Island. Bait pellet count within plots confirmed the targeted distribution of bait across the island. The January 12 application was conducted to ensure bait was available to every rat on the island for a lethal exposure period. During operations, four people worked on a 240-foot (ft) long barge staged near Lehua Island that served as a helicopter landing zone for loading bait, refueling, and a Geographical Information Station (GIS) workstation for downloading Global Positioning System (GPS) data during application to track and document bait distribution. As many as six people also worked on Lehua Island during bait applications to monitor bait distribution and to remove pellets around albatross chick nests. Two different helicopter companies were used for transport, one to ferry people to the island and another to transport gear and water to the Lehua Camp. A third helicopter and pilot were used for bait application. The bait was applied by a USDA–APHIS–WS and local pilot contractor team that had experience aerially applied rodenticide 11 months earlier on Mokapu Island. A fourth helicopter, operated by the U.S. Coast Guard, patrolled the area around Lehua Island and was on standby in the unlikely event of an emergency. Biologists were limited in their stay on Lehua Island during the project because of offshore military operations in the area.

Monitoring for primary and secondary adverse impacts of diphacinone on nontarget species was one of the foremost concerns during this rodent eradication project. Pre- and post-application samples of crabs, limpets (opihi), fish, and soil were collected from three sites on the south side of the

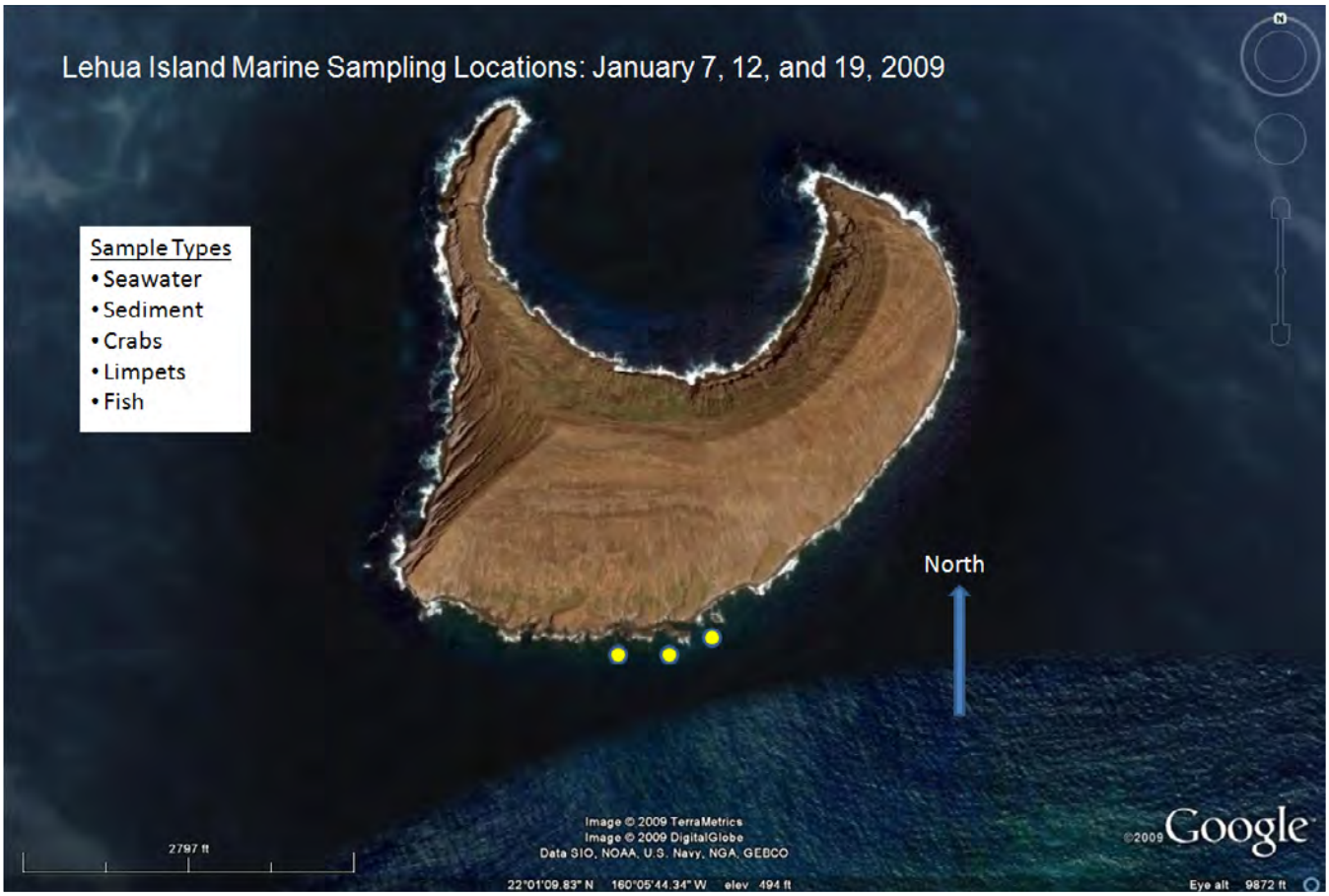

Figure 2. Lehua Island, Niihau, Hawaii (Created from GoogleEarth© image) showing the three sampling sites (yellow dots) on the south of the island. Indicated sample types were collected at each sampling location.

island (fig. 2). Pre-application specimens were collected on December 30 and 31, 2008, and January 2, 2009. Post-application samples were collected on January 7, 12, and 19, 2009, at 1-day and 1-week periods after each bait application. The location of the sampling sites was determined in part by human safety and logistical concerns. There was very high winter surf during most of this period coming primarily from the north and wrapping around the island. These three locations were the only places where the shore-based sampling crews could safely access the shoreline and where there would be potential use by people. In addition, the sites chosen represent the most conservative monitoring result and, thus, provide for the highest possible confidence that the results were indicative of actual circumstances for future analysis of risk of nontarget organism exposure. This is due to the fact that Lehua Island's largest gulch drainages extend to the ocean along the south shore where runoff enters the sea near the three sampling sites. Thus, the biota and soils sampled in the area were a good representation of the higher end diphacinone exposure potential. However, it should be noted that the automated weather station on the island recorded no significant rainfall events following the Lehua bait application that would have washed pellets into the water. Plots across the island were monitored to measure the disappearance of diphacinone rodenticide and ensure enough bait pellets remained on the ground for all rats to consume a lethal amount. Transects were searched multiple times for rat carcasses and to document any nontarget mortality, if it occurred. More than 20 dead rats were found within transects on the island; no dead nontarget species were found.

The objective of this study was to assess the potential presence of diphacinone in seawater, biota, and soil as a consequence of aerial application of rodenticide to eradicate invasive, alien rats from Lehua Island. The study consisted of three sampling periods, each addressing the post-application diphacinone exposure levels in the immediate environment.

Methods

Sample Collection and Handling

Personnel from the USFWS, DOFAW, and USDA–APHIS–WS collected specimens from three sites for

determination of potential diphacinone exposure. Pre-application and post-application samples that were collected at each site include seawater, soil, limpet (*Cellana exarata*), aama crab (*Grapsus tenuicrustatus*), taape (*Lutjanus kasmira*), stocky hawkfish (*Cirrhitus pinnulatus*), nenue (*Kyphosus biggibus*), toau (*Lutjanus fulvus*), Christmas wrasse (*Thalassoma trilobatum*), and hogfish (*Bodianus bilunulatus*). Limpets are intertidal grazers and the crabs are omnivorous, intertidal scavengers, and were collected from the splash zone. For each site, samples were collected a distance of 25 to 50 meters (m) along the shoreline. The GPS locations of the sites were recorded. Site #1 was about 100 m from Site #2, and Site #2 was 500 m from Site #3. Surface soils were collected 20 to 30 m from the water's edge at a depth of 2 to 5 inches (in.), at the base of gulches that drain into the ocean. Seawater and soil samples were contained in clean glass 250-milliliter (mL) jars with Teflon-lined lids. Limpets and aama crabs were collected in near-shore habitats and weighed whole. At each site, three to five fish were caught by hook and line; the six species of fish collected feed on a variety of organisms, including algae, invertebrates, and other fish. Weights of the fish fillet samples were recorded. The biological samples were wrapped in aluminum foil, placed in a plastic bag and labeled with the sample type, weight, site, date, and field number information. Tissues were stored frozen, whereas, soils and waters were stored cool.

Samples were shipped to the U.S. Geological Survey (USGS) Columbia Environmental Research Center (CERC) under chain of custody; a total of 18 fish samples, 9 soil composites, 9 seawaters, 9 composites of 5 to 8 whole limpet carcasses, and 7 composites of 2 whole crabs were shipped. (A similar set of samples was sent to the USDA–APHIS laboratory in Fort Collins, Colordao, for inter-laboratory comparison.) The pre-application samples arrived at CERC on January 6 and the post-application samples arrived on January 23, 2009. Upon receipt at CERC, the samples were logged into the sample database system and assigned a unique identification number. Fish fillets were analyzed as individuals; limpets were analyzed as composites of five to eight individuals; and crabs were analyzed as composites of two individuals, except for when only a single sample was available. Seawater and soil samples were refrigerated in the dark at 4 degrees Celsius (°C) and vertebrate and invertebrate tissue samples were stored in the dark at -20ºC until analysis.

Diphacinone Laboratory Analysis

The analytical method consisted of extraction of diphacinone from the sample and subsequent concentration and purification by solid-phase extraction (SPE) or low pressure size-exclusion chromatography (LP-SEC). The detailed diphacinone laboratory analytical method is provided in appendix 1. Seawater (200 mL) was extracted with a solid phase cartridge; biological tissues (5 g aliquots) were column extracted; soil samples (15–20 g dry weight) were refluxed. The resulting extracts were then cleaned with C-18 SPE and size exclusion chromatography as needed. Sample preparation methods increased in their complexity as the richness of the sample matrices increased, generally in the order: seawater < soil < fish fillet < crab < limpet. Analytical separation of diphacinone was performed by high performance liquid chromatography (HPLC) followed by ultraviolet-visible photodiode array absorbance (PDA) detection and quantification following the methods of Yang and others (2001). Coumarin was the instrumental internal standard. Quality-control samples included diphacinone-fortified pre-application samples (matrix spikes) triplicate analysis, procedural blanks of sodium sulfate, and matrix blanks (pre-application samples). The Limits of Detection (LOD) for diphacinone were 0.4 nanograms per milliliter (ng/mL, parts per billion, ppb) for seawater, 15 nanograms per gram (ng/g, ppb) for dry weight soil, 20 ng/g for fish fillet, 13 ng/g for whole crab, and 34 ng/g for soft tissue of limpet. Detection limits for the diphacinone analysis method were calculated from the responses of the multiple matrix blanks (Keith and others, 1983, 1991).

Results

Seawater

No diphacinone was detected in the Lehua Island seawater (table 1, at the back of the report). The LOD for diphacinone in the Lehua Island seawater samples was 0.4 ng/mL. The quality-control data indicated that the analysis of the seawater is of high quality. Extraction efficiency from diphacinone fortified seawater ranged from 80 to100 percent; the seawater spike analyzed with this study resulted in 87 percent recovery. The negative control seawater sample did not have any detectable concentrations of diphacinone.

Soil

No diphacinone was detected in the soil samples (table 2, at the back of the report). The LOD for diphacinone in the Lehua Island soil samples was 15 ng/g (dry weight). The quality-control data indicated that the analysis of the soil was of high quality. Extraction efficiency of diphacinone from fortified pre-application soil sample was 101 percent and the negative control soil sample did not have any detectable concentrations of diphacinone.

Fish Fillets

No diphacinone was detected in the fish samples (table 3, at the back of the report). The LOD for diphacinone in the Lehua Island fish samples was 20 ng/g (wet weight). The quality-control data indicated that the analysis of the fish fillets was of high quality. For biological samples including fish,

extraction efficiency of diphacinone for this method ranged from 80 to 100 percent; recoveries from two fortified pre-application fish samples analyzed with this study were 85 and 94 percent. The negative control fish sample did not have any detectable concentrations of diphacinone.

Whole Crabs

No diphacinone was detected in the crab samples (table 4, at the back of the report). The LOD for diphacinone in the Lehua Island crab samples was 13 ng/g (wet weight). The quality-control data indicated that the analysis of the crabs was of high quality. Extraction efficiency of diphacinone from fortified pre-application crab samples was 101 percent and the negative control crab sample did not have any detectable concentrations of diphacinone.

Limpets

No diphacinone was detected in the limpets (table 5, at the back of the report). The LOD for diphacinone in the Lehua Island limpet samples was 34 ng/g (wet weight). The quality-control data indicated that the analysis of the limpets was of high quality. For biological samples including limpets, extraction efficiency is 80 to 100 percent; recovery of diphacinone from the fortified pre-application limpet sample was 99 percent. The negative control limpet samples (pre-application samples) did not have any detectable concentrations of diphacinone.

Conclusions

Diphacinone was not detected in any of the Lehua Island environmental samples that were collected after the aerial application of the rodenticide bait. The lack of detectable diphacinone residues in Lehua Island marine habitats mirrors the test results from Mokapu Island eradication in 2008 (Gale and others, 2008). Inter-laboratory analysis of duplicate Lehua Island samples corroborates the finding that diphacinone was not detected in the Lehua samples (T.M. Primus, written commun., 2009). No live rats have been detected on Lehua Island since the January 2009 bait application. Monitoring will continue for 2 years before the eradication is declared a success. No nontarget mortalities were documented on Lehua Island. The Lehua monitoring represents the largest marine sampling effort to date associated with any aerial rodenticide application.

Acknowledgments

The authors thank staff of USDA–APHIS–WS, Catherine Swift and others of USFWS for their contributions in this effort. They made the primary selection of the study area and continue to work on follow-up monitoring and other aspects of this study.

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Tables

Table 1. Results of laboratory testing for diphacinone in Lehua Island, Hawaii, seawater samples.

[USGS, U.S. Geological Survey; ID, identification; ng/mL, nanograms per milliliter (parts per billion, ppb); <, less than; LOD, limits of detection; hr, hour; --, not applicable; %, percent]

USGS identification	Sample identification	Field ID	pH	Diphacinone (ng/mL)
45045	Site 1: baseline (1/2/09)	JJH09-ST001	8.10	<LOD [0.4]
45046	Site 2: baseline (1/2/09)	JJH09-ST002	8.13	<LOD [0.4]
45047	Site 3: baseline (1/2/09)	JJH09-ST003	8.20	<LOD [0.4]
45176	Site 1: 24hr post (1/7/09)	JJH09-006	8.10	<LOD [0.4]
45177	Site 2: 24hr post (1/7/09)	JJH09-005	8.14	<LOD [0.4]
45178	Site 3: 24hr post (1/7/09)	JJH09-004	8.01	<LOD [0.4]
45179	Site 1: 24hr/7 day (1/13/09)	JJH09-009	8.22	<LOD [0.4]
45180	Site 2: 24hr/7 day (1/13/09)	JJH09-008	8.23	<LOD [0.4]
45181	Site 3: 24hr/7 day (1/13/09)	JJH09-007	8.10	<LOD [0.4]
45182	Site 1: post 7 day (1/19/09)	JJH09-12	8.10	<LOD [0.4]
45183	Site 2: post 7 day (1/19/09)	JJH09-11	8.12	<LOD [0.4]
45184	Site 3: post 7 day (1/19/09)	JJH09-10	8.18	<LOD [0.4]
	Quality-control samples Expressed as 200 mL equivalent sample volume basis			
Mock (3/16/09)	Diphacinone standard	--	--	0.76
PB031609	Procedural blank	--	--	<LOD [0.4]
MB031609	Matrix blank (composite)	--	--	<LOD [0.4]
MS031609	Matrix spike (composite)	--	--	0.66 (87%)

Table 2. Results of laboratory testing for diphacinone in Lehua Island, Hawaii, soil samples.

[USGS, U.S. Geological Survey; ID, identification; ng/g, nanograms per gram (parts per billion, ppb); values reported on a dry-sediment weight (dw) basis; <, less than; LOD, limits of detection; hr, hour; --, not applicable; %, percent]

USGS identification	Sample identification	Field ID	Diphacinone (ng/g) dw
45048	Site 1: baseline (1/2/09)	JJH09-ST001	<LOD [15]
45049	Site 2: baseline (1/2/09)	JJH09-ST002	<LOD [15]
45050	Site 3: baseline (1/2/09)	JJH09-ST003	<LOD [15]
45185	Site 1: 24hr post (1/7/09)	JJH09-006	<LOD [15]
45186	Site 2: 24hr post (1/7/09)	JJH09-005	<LOD [15]
45187	Site 3: 24hr post (1/7/09)	JJH09-004	<LOD [15]
45188	Site 1: 24hr/7 day (1/13/09)	JJH09-009	<LOD [15]
45189	Site 2: 24hr/7 day (1/13/09)	JJH09-008	<LOD [15]
45190	Site 3: 24hr/7 day (1/13/09)	JJH09-007	<LOD [15]
45191-1	Site 1: post 7 day (1/19/09)	JJH09-12	<LOD [15]
45191-2	Replicate #2	JJH09-12	<LOD [15]
45191-3	Replicate #3	JJH09-12	<LOD [15]
45192	Site 2: post 7 day (1/19/09)	JJH09-11	<LOD [15]
45193	Site 3: post 7 day (1/19/09)	JJH09-10	<LOD [15]
Quality-control samples Expressed as 20-g equivalent sample dry mass basis			
Mock (3/18/09)	Diphacinone standard	--	34
PB031809	Procedural blank	--	<LOD [15]
MB031809	Matrix blank (45048)	--	<LOD [15]
MS031809	Matrix spike (45048)	--	34.5 (101%)
SPE mock			33
SPE recovery: soil			31.5 (97%)

Table 3. Results of laboratory testing for diphacinone in Lehua Island, Hawaii, fish fillet samples.

[Concentrations given in micrograms of diphacinone per gram (ug/g) wet weight (ww) of fish sample, USGS, U.S. Geological Survey; ID, identification; ng/g, nanograms per gram (parts per billion, ppb); values reported on a wet-tissue weight basis; <, less than; LOD, limits of detection; --, not applicable; %, percent; QAQC, quality assurance/quality control]

CERC identification	Sample identification	Fish	Field ID	Diphacinone (ng/g fish) ww
45054	Site 1: baseline (1/2/09)	Not identified	JJH09-ST001	<LOD [20]
45055	Site 2: baseline (12/30/08)	Hawiian hogfish	JJH08-ST002	<LOD [20]
45056	Site 3: baseline (12/30/08)	Blueline snapper	JJH08-ST003	<LOD [20]
45201-1	Site 1: 24hr post (1/7/09)	Toau	JJH09-006 St001	<LOD [20]
45201-2	Replicate #2	Toau	JJH09-006 St001	<LOD [20]
45201-3	Replicate #3	Toau	JJH09-006 St001	<LOD [20]
45202	Site 1: 24hr post (1/7/09)	Hogfish	JJH09-006 St001	<LOD [20]
45203	Site 1: 24hr post (1/7/09)	Stocky hawkfish	JJH009-06 St001	<LOD [20]
45204	Site 2: 24hr post (1/7/09)	Stocky hawkfish	JJH09-005 St002	<LOD [20]
45205	Site 2: 24hr post (1/7/09)	Stocky hawkfish	JJH09-005 St002	<LOD [20]
45206	Site 3: 24hr post (1/7/09)	Hinalea	JJH09-004 St003	<LOD [20]
45207	Site 3: 24hr post (1/7/09)	Taape	JJH09-004 St003	<LOD [20]
45208	Site 3: 24hr post (1/7/09)	Stocky hawkfish	JJH09-004 St003	<LOD [20]
45209	Site 3: 24hr post (1/7/09)	Stocky hawkfish	JJH09-004 St003	<LOD [20]
45210	Site 1: 24hr/7 day (1/12/09)	Stocky hawkfish	JJH09-009 St001	<LOD [20]
45211	Site 1: 24hr/7 day (1/12/09)	Hawkfish #2	JJH09-009 St001	<LOD [20]
45212	Site 2: 24hr/7 day (1/12/09)	Nenue	JJH09-008 St002	<LOD [20]
45213-1	Site 3: 24hr/7 day (1/12/09)	Nenue	JJH09-007 St003	<LOD [20]
45213-2	Replicate #2	Nenue	JJH09-007 St003	<LOD [20]
45213-3	Replicate #3	Nenue	JJH09-007 St003	<LOD [20]
45214	Site 3: 24hr/7 day (1/12/09)	Stocky hawkfish	JJH09-007 St003	<LOD [20]
45215	Site 3: 24hr/7 day (1/12/09)	Stocky hawkfish #2	JJH09-007 St003	<LOD [20]
45216	Site 1: post 7 day (1/19/09)	Surge wrasse	JJH09-012 St001	<LOD [20]
45217	Site 2: post 7 day (1/19/09)	Stocky hawkfish	JJH09-011 St002	<LOD [20]
45218	Site 2: post 7 day (1/19/09)	Stocky hawkfish	JJH09-011 St002	<LOD [20]
	Quality-control samples Expressed as 5-g equivalent sample mass basis			
Mock (2/19/09)	Mock: diphacinone standard	--	--	873
PB021909	Procedural blank	--	--	<LOD [20]
MB021909	Matrix blank (45055)	--	--	<LOD [20]
MS021909	Matrix diphacinone spike (45055)	--	--	738 (85%)
QAQC SPE mock (2/25/09)	Mock SPE: diphacinone standard	--	--	975
QAQC SPE recovery (2/25/09)	Recovery SPE: diphacinone standard	--	--	970 (100%)
Mock (2/23/09)	Mock: diphacinone standard	--	--	455
PB022309	Procedural blank	--	--	<LOD [20]
MB022309	Matrix blank (45055)	--	--	<LOD [20]
MS022309	Matrix diphacinone spike (45055)	--	--	429 (94%)
QAQC SPE mock (2/26/09)	Mock SPE: diphacinone standard	--	--	1,000
QAQC SPE recovery (2/26/09)	Recovery SPE: diphacinone standard	--	--	1,000

Table 4. Results of laboratory testing for diphacinone in Lehua Island, Hawaii, whole crab samples.

[USGS, U.S. Geological Survey; ID, identification; ng/g, nanograms per gram (parts per billion, ppb); values reported on a wet-tissue weight basis; <, less than; LOD, limits of detection; --, not applicable; %, percent]

USGS identification	Sample identification	Field ID	Diphacinone (ng/g)
45051	Site 1: baseline (12/31/08)	JJH08-St001 (n=2)	<LOD [13]
45052	Site 2: baseline (12/31/08)	JJH08-St002 (n=2)	<LOD [13]
45053	Site 3: baseline (12/31/08)	JJH08-St003 (n=1)	<LOD [13]
45194	Site 1: 24hr post (1/7/09)	JJH09-006 (n=2)	<LOD [13]
45195	Site 2: 24hr post (1/7/09)	JJH09-005 (n=2)	<LOD [13]
45196	Site 3: 24hr post (1/7/09)	JJH09-004 (n=2)	<LOD [13]
45197	Site 1: 24hr/7 day (1/12/09)	JJH09-009 (n=1)	<LOD [13]
45198	Site 1: post 7 day (1/20/09)	JJH09-12 (n=2)	<LOD [13]
45199	Site 2: post 7 day (1/19/09)	JJH09-11 (n=2)	<LOD [13]
45200-1	Site 3: post 7 day (1/19/09)	JJH09-10 (n=2)	<LOD [13]
45200-2	Replicate #2	JJH09-10 (n=2)	<LOD [13]
45200-3	Replicate #3	JJH09-10 (n=2)	<LOD [13]
	Quality-control samples Expressed as 5-g equivalent sample mass basis		
Mock (4/1/09)	Diphacinone standard	--	107
PB040109	Procedural blank	--	<LOD [13]
MB040109	Matrix blank (45057)	--	<LOD [13]
MS040109	Matrix spike (45058)	--	108 (101%)
SPE mock	--	--	105
SPE recovery: crabs	Recovery SPE	--	102 (99%)

Table 5. Results of laboratory testing for diphacinone in Lehua Island, Hawaii, soft tissue limpet samples.

[USGS, U.S. Geological Survey; ID, identification; ng/g, nanograms per gram (parts per billion, ppb); values reported on a wet-tissue weight basis; <, less than; LOD, limits of detection; --, not applicable; %, percent]

USGS identification	Sample identification	Field ID	Diphacinone (ng/g)
45057	Site 1: baseline (12/30/08)	JJH08-001 (n=5)	<LOD [34]
45058	Site 2: baseline (12/30/08)	JJH08-002 (n=8)	<LOD [34]
45059	Site 3: baseline (12/30/08)	JJH08-003 (n=6)	<LOD [34]
45219-1	Site 1: 24hr post (1/7/09)	JJH09-006 (n=5)	<LOD [34]
45219-2	Replicate #2	JJH09-006 (n=5)	<LOD [34]
45219-3	Replicate #3	JJH09-006 (n=5)	<LOD [34]
45220	Site 2: 24hr post (1/7/09)	JJH09-005 (n=5)	<LOD [34]
45221	Site 3: 24hr post (1/7/09)	JJH09-004 (n=5)	<LOD [34]
45222	Site 1: 24hr/7 day (1/13/09)	JJH09-009 (n=5)	<LOD [34]
45223	Site 2: 24hr/7 day (1/13/09)	JJH09-008 (n=5)	<LOD [34]
45224	Site 3: 24hr/7 day (1/13/09)	JJH09-007 (n=6)	<LOD [34]
45225	Site 1: post 7 day (1/19/09)	JJH09-12 (n=5)	<LOD [34]
45226	Site 2: post 7 day (1/19/09)	JJH09-11 (n=6)	<LOD [34]
45227	Site 3: post 7 day (1/19/09)	JJH09-10 (n=5)	<LOD [34]
Quality-control samples Expressed as 5-g equivalent sample mass basis			
SPE mock/5-g equiv	Diphacinone standard	--	125
SPE recovery: limpet	Recovery SPE	--	124 (99%)
GPC mock	Diphacinone standard	--	116
GPC recovery: limpet	Recovery LP-GPC	--	114 (98%)
Mock (3/23/09)	Mock	--	140
PB032309	Procedural blank	--	<LOD [34]
MB032309	Matrix blank (45057)	--	<LOD [34]
MS032309	Matrix spike (45058)	--	138 (99%)

Appendix 1: Analytical Methods for Diphacinone

Materials

Solid-phase extraction cartridges (Oasis-HLB (hydrophilic/lipophilic balance) 6 milliliters (mL) x 500 milligrams (mg)) were purchased from Waters Corp., Milford, Massachusetts. Octadecyl SPE cartridges (Isolute C18 (EC) 6 mL x 1 gram (g)) were purchased from International Sorbent Technology, Mid Glamorgan, United Kingdom. The size exclusion material (SX-3 biobeads 200–400 mesh) was purchased from Bio-Rad Co., Richmond, California. Acetone, acetonitrile, dichloromethane, methanol (OPTIMA grade), anhydrous sodium sulfate, and 2 N *o*-phosphoric acid (ACS grade) were purchased from Fisher Scientific, Fair Lawn, New Jersey. Whatman 0.45-micrometer (µm) polytetrafluoroethylene (PTFE) syringe filters were purchased from Whatman, Inc., Sanford, Maine. A 1-molar (M) solution of tetrabutylammonium hydroxide (TBAH) was purchased from Sigma-Aldrich, St. Louis, Missouri. Diphacinone and coumarin standard solutions (in methanol) were purchased from AccuStandard, New Haven, Connecticut. Milli-Q water (18mΩ, Millipore Synergy UV, Millipore Corp., Bedford, Massachusetts) was used throughout the analytical process. Aqueous tetrabutylammonium hydroxide ion pair (TBAH-IP) reagents used for high performance liquid chromatography (HPLC) and for SPE were prepared at 0.03 M in water and pH adjusted to 6.0 with 2N *o*-phosphoric acid.

Sample Preparation and Quantification of Diphacinone in Seawater Samples

The seawater samples (200 mL) were extracted by HLB-SPE cartridge to adsorb diphacinone that was then recovered by acetonitrile elution of the SPE and quantified by HPLC with PDA detection; the pH of the water was measured using a Mettler-Toledo Seven Easy pH meter (Schwerzenbach, Inc., Switzerland) (Gale and others, 2008). The final extract was prepared in 700 microliters (µL) methanol with 300 µL of the aqueous TBAH-IP reagent added to match the liquid chromatography mobile phase, and 1 microgram (µg) coumarin (instrumental internal standard) added.

Sample Preparation and Quantification of Diphacinone in Biological Tissues and Soil

Fish Samples

Homogenized fillet tissue samples (5 g of skinless fillets) were dehydrated for 2 hours with 25 g anhydrous sodium sulfate. The mixture was then blended into a free-flowing powder with a stainless steel-bladed commercial blender. It has been previously established that ascorbic acid treatment, needed for some types of vertebrate samples, was not required for fish, crab, and limpet matrices (Hunter and Sharp, 1988). The dehydrated sample was loaded into an extraction column with a total of about 20 mL of acetonitrile rinses of the sample container and then saturated with acetonitrile. The acetonitrile saturated dehydrated matrix was allowed to interact approximately 1 hour before extraction. Additional acetonitrile (150 mL) was added to the column, and the sample extracted at a flow rate of approximately 2 milliliters per minute (mL/min) until flow ceased; additional acetonitrile (100 mL) was added to the column, the extraction continued, and the eluant collected with the original extract. The extracts were rotary evaporated under vacuum (<50 degrees Celsius (°C)) to approximately 3 mL, and quantitatively transferred to 15-mL culture tubes with three sequential 2 mL rinses with acetonitrile. The sample extracts were evaporated by nitrogen stream to 2 mL and mixed thoroughly. Some coextracted interferences (mainly lipid material) were removed by C18 SPE-(EC). The cartridges were conditioned with 10 mL methanol followed by 10 mL 0.03 M TBAH-IP, dried under vacuum for about 1 minute with 10 mL acetonitrile (the cartridge remained wet). The 2 mL sample extracts were applied to the cartridge at about 2 mL/min (with three 1 mL acetonitrile rinses). Diphacinone was recovered from the cartridge with 7 mL acetonitrile (total acetonitrile 10 mL: three 1 mL rinses plus 7 mL for elution). The eluant was collected in a 15-mL amber culture tube. The final sample extracts were prepared for analysis by first evaporating the solvent to dryness with nitrogen. The residues were dissolved in 700 µL methanol, 300 µL of the aqueous TBAH-IP reagent was added to match the liquid chromatography mobile phase, and 1 µg coumarin (instrumental internal standard) was added. The final samples were filtered through 0.45-µm PTFE syringe filters directly into 1-mL amber autosampler vials.

Limpet

Limpet (opihi) (*Cellana exarata*) (soft tissues) sample homogenates (5 g) were accurately weighed and then dehydrated with 25 g anhydrous sodium sulfate and prepared with the similar procedure used for the fish samples with the addition of a couple of steps: Additional lipids and other biogenic material were removed by low-performance size-exclusion chromatography (SX-3 biobeads, dichloromethane mobile phase at a flow of 3.5 mL/min). The diphacinone fractions (LP-SEC collection window from 40 to 60 minutes) were rotary evaporated under vacuum (<50°C) to approximately 3 mL, and quantitatively transferred to 15-mL culture tubes with three sequential 2 mL rinses with dichloromethane. The extract was then run through C18 SPE-(EC). The final 1 mL sample extracts were prepared for analysis in methanol and aqueous TBAH-IP in the same manner described above.

Whole Crab

Whole crabs (*Grapsus tenuicrustatus*) were rinsed with distilled water and blended (shell included). Sample homogenates (5 g) were accurately weighed and then dehydrated with 25 g anhydrous sodium sulfate and extracted with the procedure used for the fish samples. The dichloromethane sample extracts were prepared for analysis in the same manner as the fish and limpets. The deep red color of the crab extract required dual C18 SPE-(EC). The final 1 mL sample extracts were prepared for analysis in methanol and aqueous TBAH-IP in the same manner described above.

Soil

Moistures of the soil samples were determined by oven drying on a heating block at 120°C and measuring moisture loss. Wet portions of soil samples equivalent to 20 g dry mass were extracted by refluxing with methanol for 8 hours. The extracts were cleaned up and prepared for analysis using the C18 SPE-(EC) procedure described above. The final 1 mL sample extracts were prepared for analysis in the same manner described above.

Instrumental Analysis and Data Reduction

HPLC-PDA Analysis

Quantification of diphacinone was performed with a Surveyor® HPLC system (Thermo-Fisher, Inc., San Jose, California) consisting of an autosampler, gradient pump, photodiode array (PDA) detector, and XCalibur® chromatography data collection and processing software. A Luna® C18(2) 100 Å, 150 x 2 millimeter (mm) x 3-µm analytical column with a SecurityGuard® C18 guard column cartridge (Phenomenex, Torrance, California) was used for the separation of diphacinone. The ion-pair reagent for the mobile phase was 0.03 M TBAH-IP in water adjusted to a pH of 6.0 using 2 N *o*-phosphoric acid. The mobile phase, methanol/0.03 M TBAH-IP (70:30 volume:volume) was delivered isocratically at 0.8 mL/min. The sample was applied onto the column via 20 µL full-loop injections. Diphacinone was detected by wavelength scanning from 230 to 400 nanometers (nm) with quantification at the primary wavelength (286 nm) and confirmation at the two secondary wavelengths (314 and 326 nm). The crab samples were quantified using the secondary wavelengths because of primary wavelength interference from the extract's color. The primary wavelength for the instrumental internal standard coumarin was 276 nm with a secondary wavelength of 312 nm. Calibration of the instrument was achieved through a range of standards from 20 to 5,000 ng/mL diphacinone with coumarin used as the instrumental internal standard. The instrumental limit of detection was 3 ng/mL, which equates to 0.6 ng/g for a 5 g sample analysis. The method LOD were based upon repeat analysis of each of the different matrices.

Quality Assurance and Quality Control

The quality-assurance plan for this study was to ensure that the analyses provided accurate and precise measurements of the samples collected in this study. The general scheme included replication of various stages (table A), comparison and calibration against known standards, proper maintenance and calibration of equipment, accurate sample tracking and custody, proper documentation at all steps of sample processing, and other considerations of good laboratory practice.

The accuracy and precision of analytical methods for this study were assessed by the following monitors of sample preparation and instrumental analysis: individual method stage recoveries, replicated sample or reference matrices, procedural blanks, negative control (reference) matrix blanks, and

Table A. Quality-control sample types for analysis of environmental samples.

Sample type	Quality-control function
Procedural blank	Determines analyte laboratory background levels or background interferences with analyte signal.
Negative control material (matrix blank)	Determines background interferences with analyte signal related to a representative and controllable sample matrix.
Fortified negative control material (matrix spike)	Determines analyte recovery and assesses potential signal enhancement or suppression from a representative and controllable sample matrix.
Replicate sample	
within set	Determines repeatability analyte signal associated with a specific environmental matrix.
between sets	Determines the reproducibility of analyte signal associated with a specific environmental matrix.

fortified negative control matrix samples. The fortified matrix samples were amended with native analyte during sample preparation. The numbers of quality-control samples of each type are presented in table B.

Criteria for Quantification of HPLC-PDA Data

Method limits of detection and limits of quantification (LOQ) were estimated from low-level standards and determined by the signal-to-noise ratio generated by a matrix blank sample. Keith and others (1983, 1991) established the LOD as 3 times the matrix blank background signal, and the LOQ as 10 times background signal. For the positive identification and quantification of each analyte, the following criteria were established:

1. The analyte peak area must be greater than 10 times matrix blank background signal (LOQ) for quantification, or 3 times background signal (LOD) to be considered detected, but <LOQ. If a peak is not present, or is less than three times background signal, it will be considered "Not Detected".
2. The analyte elution must occur at retention times that are equivalent to those for the corresponding calibration standards (within ± 3 seconds or < 1 percent difference, as established by the method validation).
3. The spectrum of an unknown analyte must be comparable to the spectrum of a corresponding calibration standard (within purity factors established by the method validation and the expertise of the analyst).

Acceptance or Rejection Criteria for Results

Background responses from procedural and matrix blanks were quantified and used to estimate method limits of detection and quantification.

Table B. Quality-control sample types and levels selected for study.

Sample type	Quality-control level (number of samples)				
	Seawater	Fish fillet	Whole limpet	Crab	Soil
Mock fortification solutions	1	2	1	1	1
Procedural blanks	1	2	1	1	1
Fortified procedural blanks	0	2	1	1	1
Negative control (reference) matrices	1	2	1	1	1
Fortified negative control (reference) matrices	1	2	1	1	1
Replicates within set	0	1	1	1	1

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