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Potential interactions among disease, pesticides, water quality and adjacent land cover in amphibian habitats in the United States

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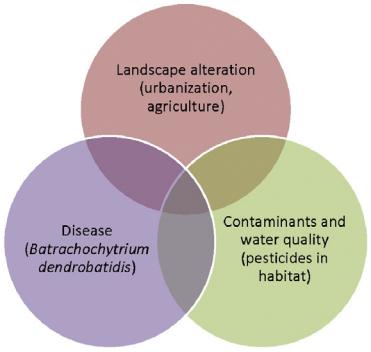
HIGHLIGHTS

- Disease, pesticides, and landscape alteration can cause amphibian decline.
- We assessed Bd and pesticides in frog tissue and their aquatic habitats.
- Pesticide concentrations in tissue were correlated to land cover around the sites.
- Bd correlated positively with DOC, total nitrogen and total phosphorous in water.
- Bd in water was not associated with differences in land use around sites.
- Fungicides occurred more frequently than expected in frog habitats and tissue.

GRAPHICAL ABSTRACT



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ABSTRACT

To investigate interactions among disease, pesticides, water quality, and adjacent land cover, we collected samples of water, sediment, and frog tissue from 21 sites in 7 States in the United States (US) representing a variety of amphibian habitats. All samples were analyzed for >90 pesticides and pesticide degradates, and water and frogs were screened for the amphibian chytrid fungus *Batrachochytrium dendrobatidis* (Bd) using molecular methods. Pesticides and pesticide degradates were detected frequently in frog breeding habitats (water and sediment) as well as in frog tissue. Fungicides occurred more frequently in water, sediment, and tissue than was expected based upon their limited use relative to herbicides or insecticides. Pesticide occurrence in water or sediment was not a strong predictor of occurrence in tissue, but pesticide concentrations in tissue were correlated positively to agricultural and urban land, and negatively to forested land in 2-km buffers around the sites. Bd was detected in water at 45% of sites, and on 34% of swabbed frogs. Bd detections in water were not associated with differences in land use around sites, but sites with detections had colder water. Frogs that tested positive for Bd were associated with sites that had higher total fungicide concentrations in water and sediment, but lower insecticide concentrations in sediments relative to frogs that were Bd negative. Bd concentrations on frog swabs were positively correlated to dissolved organic carbon, and total nitrogen and phosphorus, and negatively correlated to pH and water temperature.

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Data were collected from a range of locations and amphibian habitats and represent some of the first field-collected information aimed at understanding the interactions between pesticides, land use, and amphibian disease. These interactions are of particular interest to conservation efforts as many amphibians live in altered habitats and may depend on wetlands embedded in these landscapes to survive.

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1. Introduction

For a variety of reasons, many of the world's amphibian species face extinction or decreasing populations (Adams et al., 2013; Lannoo et al., 2011; Sodhi et al., 2008). Numerous studies have demonstrated that exposure to agricultural chemicals can have adverse effects on amphibians (Bruhl et al., 2013; Hayes et al., 2006; Mann et al., 2009; Relyea and Diecks, 2008; Rohr et al., 2003; Sparling and Fellers, 2009). For example, fungicides occur in water and sediment in agricultural, urban, and remote locations (Battaglin et al., 2011; Reilly et al., 2012; Smalling et al., 2012, 2013a) and recent studies have indicated that fungicides may be of particular concern to amphibians, freshwater fish, and invertebrates (Belden et al., 2010; Hooser et al., 2012; Junges et al., 2012; Li et al., 2016; McMahon et al., 2011; Morrison et al., 2013). In amphibians, the presence of fungicides has been linked to alteration of predator–prey interactions (Junges et al., 2012; Teplitsky et al., 2005), endocrine disruption (McMahon et al., 2011; Poulsen et al., 2015), and mortality (Belden et al., 2010; Hooser et al., 2012). Glyphosate-based herbicides also are of concern as they occur widely in the environment (Battaglin et al., 2014; Coupe et al., 2012) and have been shown to cause poor survivorship and mortality of larval and adult amphibians (Moore et al., 2012; Relyea, 2005a, 2005b; Wagner et al., 2013).

Pesticides found in frogs and their habitats can have effects beyond the scale of individual animals or populations. Similar to results reported for metamorphosing insects and the movement of contaminants from aquatic to terrestrial systems (Kraus et al., 2014), amphibians accumulate pesticides (Smalling et al., 2015) and may potentially concentrate some of them during metamorphosis. This transfer of contaminants across ecological boundaries (i.e., water and sediment to plants and animals) is relevant to subsequent offspring (contaminants transferred to eggs) (Metts et al., 2013) and to the animals that prey on juveniles or adults (Wu et al., 2009). Amphibians can represent a substantial source of biomass in a pond (Gibbons et al., 2006) such that the implications of this transfer may be important.

Despite a large body of literature on contaminants in the laboratory (Mann et al., 2009; Sparling and Fellers, 2009), there are few field studies that assess the effects of pesticides, or the potential interactions between disease and pesticides, on individual amphibians or populations of amphibians persisting in contaminated environments (Egea-Serrano et al., 2012; Smalling et al., 2015). A focus on amphibians and the effects of pesticides is warranted because, as a taxonomic group, they are disappearing at a rate far greater than the expected background rate of extinction (McCallum, 2007), representing a likely mass extinction event (Wake and Vredenburg, 2008).

Land-use change resulting in habitat destruction or alteration remains a leading cause of amphibian decline (Lannoo et al., 2011; Sodhi et al., 2008). Since the mid-1700s, humans have transformed landscapes across the US from forests, grasslands, and wetlands to farmland, rangeland, and urban land (U.S. Department of Agriculture, 2013). A notable result of the increase in developed land was the loss of natural wetland landscapes, an estimated 53% of which were removed between 1780s and 1980s (Dahl, 1990). More recently, various conservation programs have started to reverse this trend by encouraging the construction of new wetlands and restoration of natural wetlands (Dahl, 2011; Sucik and Marks, 2014). Some amphibians persist in altered habitats and some may benefit from practices associated with agriculture (e.g., farm ponds; Knutson et al., 2004). There is also evidence that amphibians may develop some tolerance to pesticide exposure in agricultural settings (Cothran et al., 2013; Hua et al., 2013, 2015). In contrast, some such situations may represent environmental traps or "sinks" (Pulliam, 1988) where amphibians may survive but are unable to successfully reproduce. Such scenarios may represent an opportunity to manage wetlands for the benefit of amphibians. However, a better understanding of the biotic and abiotic interactions occurring within these systems is needed before management actions can be formulated.

Disease is another contributor to amphibian decline that may exacerbate the effects of other stressors (Puglis and Boone, 2007; Hayes et al., 2010). Chytridiomycosis, caused by the amphibian chytrid fungus *Batrachochytrium dendrobatidis* (Bd; Longcore et al., 1999; Van Rooij et al., 2015) is one such disease. Amphibian responses to Bd vary widely by species and individuals (Retallick and Miera, 2007). Chytridiomycosis caused by Bd exposure is implicated in local declines and extinctions of amphibians worldwide (Bosch et al., 2001; Lips et al., 2006), including North America (Muths et al., 2008; Vredenburg et al., 2010). Despite considerable attention to the effects of chytridiomycosis on individuals and populations, the effect of stressors such as pesticides on Bd occurrence is unknown, and the effect of Bd on amphibians when they are already challenged by pesticide exposure is equivocal. Exposure to pesticides can exacerbate the effect of Bd on host animals (Hayes et al., 2010), decrease negative effects (Brown et al., 2013; Gahl et al., 2011; Hanlon and Parris, 2012; Rumschlag et al., 2014), or have no effect (Edge et al., 2013).

Three stressors - landscape alteration, contaminants, and disease - are critical factors in the persistence of amphibians on the landscape. Efforts to address multiple stressors simultaneously can be fraught with difficulty, especially in observational studies from the field. However, these real-world situations and resulting data are vital to provide context for results from controlled laboratory and mesocosm efforts. Specifically, few field studies have measured current-use pesticides in key habitat components (water or sediment) in conjunction with their occurrence in tissue or blood of wildlife (Grant et al., 2013; Masia et al., 2013; Nilsen et al., 2015; Smalling et al., 2013b, 2015). Amphibians, especially those in altered habitats, offer a system with the potential to provide a better understanding of the interactions among pesticides, disease and the movement of contaminants from aquatic to terrestrial landscapes (Reeves et al., 2016). We combined analyses of pesticide concentrations in sediment, water, and frog tissue with concurrent analysis of Bd in water and on frogs at sites representing a range of amphibian habitats surrounded by a variety of land uses. We hypothesize that the area of agricultural and urban land surrounding a site is positively correlated with the pesticide content of the amphibians' food source or potential for direct dermal exposure in cropped fields or other pesticide treated landscapes. Our objectives were to (1) characterize pesticide occurrence and concentrations in water, sediment, and frog tissue and evaluate potential site differences; (2) identify interactions between pesticide concentrations in water, sediment, frog tissue and the surrounding land use; and (3) identify interactions between pesticide concentrations in water, sediment, frog tissue, land use, and the occurrence of Bd.

2. Materials and methods

2.1. Sampling sites

We investigated the potential for exposure of frogs to pesticides at 21 sites from 7 States (CA, CO, GA, ID, LA, ME, and OR) (Table 1, Fig. 1) representing a range of land-use settings from highly developed to largely undeveloped (Table S1), by analyzing 33 water, 26 bed sediment, and 138 frog tissue samples for >90 pesticides and pesticide degradates. Eighty-six water samples were screened (Kirshtein et al., 2007) for Bd and 192 frogs were swabbed for Bd. At seven sites we did not catch enough frogs for tissue analyses, and at two sites we did not catch any frogs to assess for the presence of Bd. At one site we did not collect any Bd water filter samples, and at one site we did not collect a sediment sample (Table 1).

The four sites in California fall along an east-west transect from the eastern edge of the Central Valley to the Pacific Coast, representing a range of land uses (Fig. 1, Table 1) (Smalling et al., 2012). The five sites in Colorado are in the San Luis Valley, bordered to the west by an area of intensive irrigated agriculture with the primary crops of potatoes, alfalfa, native hay, barley, and wheat, and to the east by Great Sand Dunes National Park. The two sites in Georgia are on streams in the Piedmont province in predominantly forested and rural land (White Oak Creek) and agricultural land (Lime Creek). The two sites in Idaho are in the Boise-Snake River Valley and are adjacent to irrigated agricultural land with crops of potatoes, peas, barley, and onions. The two sites in Louisiana are at low elevation and are surrounded predominantly by wetlands. The two sites in Maine are surrounded by a mixture of forested and agricultural land with crops of potatoes. Two of four sites in Oregon are at low-elevation in the Willamette Valley and are surrounded by urban land or grasslands. The other two Oregon sites are at higher elevation in the Klamath Basin and are surrounded by grasslands. Detailed land cover information in a 2-km (km) diameter circular buffer around each sampling site was extracted from the 2010 CropScape dataset (Table S1) (Boryan et al., 2011; Han et al., 2012).

2.2. Sample Collection

Water, bed sediment, Bd, and frog samples were collected concurrently from 21 sites in 2009 and/or 2010 (Table 1). Approximately 1-L of water was collected from each site in a pre-cleaned (baked at 450 °C for 2 h) glass bottle. At the laboratory, each water sample was filtered through a glass fiber filter (Whatman, Piscataway, NJ) into a pre-cleaned glass bottle to be analyzed for 98 pesticides or pesticide degradates (Smalling et al., 2012). Bed sediment samples were collected adjacent to water sampling locations, using a stainless steel scoop to transfer recently deposited sediment into pre-cleaned glass jars to be analyzed for 94 pesticides or pesticide degradates (Smalling et al., 2012, 2013c). Water filters were collected from three locations at each site and tested for the presence of Bd using molecular methods: these samples were collected initially in a 60-mL syringe then filtered through a 0.2-μm Sterivex capsule filter until the filter reached capacity, generally 100–300 mL (Chestnut et al., 2014; Kirshtein et al., 2007). One to 15 adult frogs were collected at each site by hand or net and swabbed for Bd (Hyatt et al., 2007) (Table 1). Most of these frogs were euthanized using a 0.2% benzocaine solution (Fellers and Freel, 1995), wrapped in aluminum foil, and frozen for whole body analysis for 98 pesticides or pesticide degradates (Smalling et al., 2013a).

2.3. Water extraction and analysis for pesticides

Filtered water samples were analyzed for a suite of 98 pesticides and pesticide degradates by gas chromatography mass spectrometry (Hladik et al., 2008; Reilly et al., 2012). Briefly, 1-L of sample water

Table 1
Sampling site names, locations, map numbers, pond type, and the number of each sample type.
[Horizontal datum: North American Datum 83 (NAD 83); vertical datum: North American Vertical Datum of 1988 (NAVD 88).
Abbreviations: USGS, U.S. Geological Survey].

USGS station name	USGS station number	Map number (Fig. 1)	Latitude	Longitude	Elevation (meters)	Habitat type	Pesticide samples in			Bd samples	
							Water	Bed sediment	Frog tissue	Water filters	Swabs
Pond next to Jordan Creek nr Groveland, CA	374,611,120,045,301	1	37.770	−120.081	808	Seasonal	2	2	12	6	12
Brushy Pond nr Livermore, CA	374,630,121,414,701	2	37.775	−121.696	280	Seasonal	1	1	14	3	15
Pond nr Woodward Reservoir nr Oakdale, CA	375,019,120,520,201	3	37.839	−120.867	58	Permanent	2	2	15	6	15
Pond nr Olema, CA	380,141,122,465,601	4	38.028	−122.782	40	Seasonal	2	2	15	3	15
North Pond at Blanca Wetlands, CO	374,352,105,431,200	5	37.731	−105.720	2299	Permanent	1	1	8	3	11
Sand Creek nr Dollar Lake, CO	374,319,105,424,200	6	37.722	−105.712	2306	Modified stream	1	1	5	3	5
Wetlands at Cochran Ln at Rio Grande SWR, CO	373,404,106,034,201	7	37.568	−106.062	2319	Seasonal	2	1	5	6	5
Wetlands at Mumm Lateral at Alamosa NWR, CO	372,449,105,451,601	8	37.414	−105.754	2292	Permanent	2	0	5	6	5
Wetlands at Stanley Rd. at Rio Grande SWR, CO	373,433,106,030,701	9	37.576	−106.052	2316	Permanent	2	1	5	6	5
Lime Crk nr Cobb, GA	02,350,080	10	32.034	−83.996	76	Lotic stream	2	2	10	3	25
White Oak Crk nr Raymond, GA	02,344,797	11	33.305	−84.704	232	Lotic stream	2	2	13	3	14
Claytonia Pond nr Marsing, ID	433,403,116,493,500	12	43.568	−116.826	685	Permanent	2	2	5	6	5
Wetlands at Bar Diamond Ln at Ft Boise SWMA, ID	434,814,116,591,200	13	43.804	−116.987	670	Permanent	1	1	0	3	1
Bayou Amy near Henderson, LA	302,344,091,482,600	14	30.396	−91.807	0.2	Canal	2	1	10	5	10
Iron Gate Road nr Krotz Springs, LA	303,207,091,421,700	15	30.535	−91.705	5.2	Permanent	1	1	0	3	10
Aroostook Pond at Aroostook Farm nr Presque Isle, ME	463,902,068,011,201	16	46.651	−68.020	144	Permanent	1	1	0	3	7
Glidden Brook nr Caribou, ME	01,017,058	17	46.780	−67.995	158	Lotic stream	1	1	0	3	5
Barnes Road Mitigation Pond, OR	453,108,122,475,200	18	45.519	−122.799	63	Permanent	1	1	0	3	0
Pond nr Miller Island Boat Ramp at	420,850,121,504,700	19	42.147	−121.846	1248	Permanent	2	1	0	6	0

Table 1 (*continued*)

USGS station name	USGS station number	Map number (Fig. 1)	Latitude	Longitude	Elevation (meters)	Habitat type	Pesticide samples in			Bd samples	
							Water	Bed sediment	Frog tissue	Water filters	Swabs
Miller Island, OR											
Pond at Miller Island Rd. nr E Miller Island, OR	420,920,121,481,900	20	42.156	−121.808	1248	Canal	2	1	16	6	16
Turtle Flats, OR	442,523,123,185,400	21	44.423	−123.315	78	Seasonal	1	1	0	0	11

was extracted onto an Oasis HLB solid phase extraction (SPE) cartridge (6 cm^3, 500 mg, 60 μm, Waters Corporation, Milford, MA). All samples were spiked with $^{13}C_3$-atrazine (Cambridge Isotope, Andover, MA) as a recovery surrogate. SPE cartridges were eluted with ethyl acetate. The empty bottle was rinsed with dichloromethane (DCM) to remove any pyrethroids that may have sorbed to the bottle during sample processing; this fraction was added to the SPE eluent. Prior to analysis, samples were reduced to 200 μL and a deuterated internal standard was added.

All sample extracts were analyzed on an Agilent (Santa Clara, CA) 7890 gas chromatograph coupled to an Agilent 5975 (Folsom, CA) mass spectrometer (GC–MS) operating in electron ionization (EI) mode. Data were collected in selective ion monitoring (SIM) mode with each compound having one quantifier ion and one to two qualifier ions. Method detection limits (MDLs) for all compounds ranged from 1 to 10 ng/L (Reilly et al., 2012).

Water samples also were analyzed for glyphosate, aminomethylphosphonic acid (AMPA), and glufosinate by liquid chromatograph (LC) tandem mass spectrometry (MS/MS). The MS/MS was operated under electrospray ionization (ESI) in negative-ion mode and data were collected in multiple reaction monitoring (MRM) modes. Filtered water samples were stored at 4 °C then derivatized within 5 days after collection using a 5% borate buffer to adjust the pH to 9.0, followed by the addition of 2.5 millimolar (mM) 9 fluorenylmethylchloroformate in acetonitrile. Derivatization was carried out in the dark in a water bath at 40 °C for approximately 24 h. Following derivatization, the samples were extracted onto SPE cartridges, and the SPE cartridges were rinsed with 500 μL of deionized water. MDLs for AMPA, glyphosate, and glufosinate in surface water were 20 ng/L (Meyer et al., 2009).

2.4. Sediment Extraction and Analysis for Pesticides

Bed sediment samples were extracted and analyzed for 94 pesticides and pesticide degradates (Smalling et al., 2012, 2013c). Wet sediments (10 g) were spiked with trifluralin-d10, ring-13C-p,p′-DDE, and phenoxy-13C-*cis*-permethrin (Cambridge Isotopes, Andover, MA) as recovery surrogates, homogenized with sodium sulfate, and extracted using pressurized liquid extraction. Following extraction, extracts were dried over sodium sulfate, reduced, and sulfur was removed by gel permeation chromatography. Samples were again reduced to 0.5 mL, split in half and subjected to two different clean-up methods: (1) 6% deactivated Florisil for all fungicides and (2) carbon/alumina stacked SPE cartridges for all herbicides and insecticides. Prior to analysis, samples were reduced to 200 μL, and a deuterated internal standard was added to each extract. Sample extracts were analyzed on an Agilent

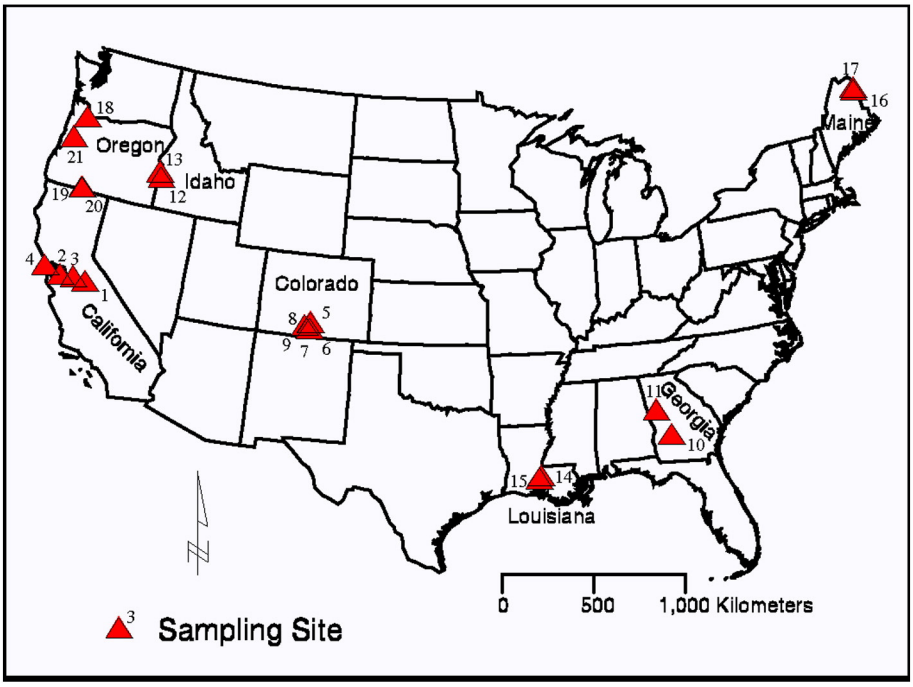

Fig. 1. Location of sampling sites in California, Colorado, Georgia, Idaho, Louisiana, Maine, and Oregon, 2009–10 (Map numbers are referenced in Tables 1, 2, and S3).

5975 GC/MS (Santa Clara, CA) in EI mode. Data were collected in SIM mode with each compound having one quantifier ion and one to two qualifier ions. MDLs for all compounds ranged from 0.6 to 3.8 μg/kg sediment dry weight (Smalling et al., 2013c).

2.5. Tissue Extraction and Analysis for Pesticides

Frog tissue samples were extracted and analyzed for 98 pesticides and pesticide degradates (Smalling et al., 2013a). Individual whole frogs (3–5 g) were thawed and homogenized with sodium sulfate (Na_2SO_4) using a clean, solvent-rinsed mortar and pestle. Samples were spiked with trifluralin-d_{10}, ring-13C-*p*,*p*′-DDE, and phenoxy-13C-*cis* permethrin as recovery surrogates and extracted three times with dichloromethane using pressurized liquid extraction. Following extraction, sample extracts were dried over Na_2SO_4 and reduced to 1 mL. Ten percent by volume of each raw extract was allowed to evaporate to a constant weight in a fume hood for gravimetric lipid determination to the nearest 0.001 g using a microbalance. A majority of the lipid was removed using gel permeation chromatography followed by 6% deactivated Florisil previously activated at 550 °C for 16 h. Prior to analysis, samples were reduced to 200 μL, and a deuterated internal standard containing [$^2H_{10}$] acenaphthene was added to each extract.

Sample extracts were analyzed on an Agilent 6890 gas chromatograph coupled to an Agilent 5975 mass spectrometer (GC–MS) operating in electron ionization mode. Data for all pesticides were collected in selective ion monitoring mode with each compound having one quantifier ion and one to two qualifier ions. MDLs for all compounds ranged from 0.5 to 4.2 μg/kg wet weight (Smalling et al., 2013a).

2.6. Water filter and swab extraction and analysis for Bd

Dry swab samples were processed by adding the swab tip to a sterile microfuge tube, adding 100 μg/L Prepman Ultra and 30 mg of zirconia beads, and centrifuging at full speed for 30 s. Then sample tubes were boiled for 10 min, cooled for 2 min, centrifuged for 3 min, and supernatant was transferred to a fresh tube and stored frozen prior to extraction. Deoxyribonucleic acid (DNA) was extracted from the capsule water filter and swab extracts for Bd analysis using a Puregene kit for tissue (Gentra Systems, Valencia, CA), and amplified and analyzed by quantitative polymerase chain reaction (qPCR) technique (Kirshtein et al., 2007). Purified DNA was analyzed by qPCR on a MX4000 QPCR system (Statagene®, La Jolla, CA) that used a Taq polymerase assay with a TaqMan MGB probe with ITS1-3chytr and 5.8Schytr primers. Bd results are expressed as zoospore equivalents per liter (Ze/L) for water filters and zoospore equivalents (Ze) for swabs.

2.7. Nutrients and other water-quality properties

Several basic water-quality properties were measured on site in association with each sample. Water temperature, pH, and specific conductance were measured using a multi-parameter meter (for example, YSI model 63). Water samples were collected and analyzed for nutrients including total nitrogen (TN), nitrate/nitrite, total phosphorus (TP), and orthophosphate at the U.S. Geological (USGS) National Water Quality Laboratory in Denver, CO, using standard methods (Fishman, 1993; Patton and Kryskalla, 2003). Filtered water samples were analyzed for dissolved organic carbon (DOC) by high-temperature catalytic combustion (Smalling et al., 2012).

2.8. Quality-assurance samples

All sample glassware was hand washed and rinsed with tap water followed by acetone and hexane prior to use. All solvents and other reagents used were American Chemical Society (ACS) grade or better (Thermo Fisher Scientific, Waltham, MA). Pesticide standard materials were purchased from Chem Service (West Chester, PA), Riedel-de Haën (Seelze, Germany), Supelco (Bellefonte, PA) and Ultra Scientific (North Kingstown, RI) or were provided by the U.S. Environmental Protection Agency (EPA) National Pesticide Repository (Ft. Meade, MD). Purities ranged from 95 to 99%. Internal standards ([$^2H_{10}$] acenaphthene) and surrogates (trifluralin-d_{10}, ring-13C-*p*,*p*′-DDE, and phenoxy-13C-*cis*-permethrin) were purchased from Cambridge Isotope Labs (Andover, MA). Neat pesticides were dissolved in acetone or methanol for an initial concentration of 1 mg/mL.

A performance-based quality assurance and quality control (QA/QC) program, which included the parallel analysis of procedural blanks, matrix spikes, and replicates, was implemented to ensure high-quality data. Procedural blanks consisting of 10 to 20 g of baked Na_2SO_4 run with every batch of 17 samples did not contain detectable levels of pesticides. Mean (± standard deviation) recoveries of trifluralin-d_{10}, ring-13C-*p*,*p*′-DDE, and phenoxy-13C-*cis*-permethrin added prior to sample extraction as recovery surrogates were 87 ± 15, 90 ± 16, and 95 ± 16, respectively. Ten matrix spikes were analyzed and the percent recovery ranged from 75 to 120% with a median of 90%. For detailed information on QA/QC for water, bed sediment, and Bd analysis see Smalling et al. (2012).

Analytes can be identified at concentrations less than the MDL with lower confidence in the actual value and are reported as estimates. Limits of detection (LOD) for all pesticides measured were also calculated and were defined as the amount of analyte in the spiked sample that produced a signal greater than three times the background signal and ranged from 0.5 to 1 μg/kg wet weight.

2.9. Statistical Methods

When pesticide concentrations were reported as "less than the MDL," those concentrations were set to zero for the purposes of calculating detection frequencies, the total concentrations, and all other statistics. In some samples a pesticide concentration value was reported that was less than the MDL but greater than the LOD. Those concentrations were used as reported, and are marked as estimates in Tables 2 and S2. Statistical tests were used to identify potential interactions among disease, pesticides, water quality, and adjacent land cover at both the site level and the individual animal level. For site level tests, sites were grouped as either Bd positive or Bd negative based separately on the results of Bd water filters and Bd swabbed frogs from each site. For individual level tests, frogs were grouped as either Bd positive or Bd negative based on the results of the Bd swab for each frog. The Kruskal-Wallis test (Helsel and Hirsch, 2002) was used to determine if there were significant differences in pesticide concentrations in water or sediment, other water-quality parameters, or adjacent land use between groups of Bd positive versus Bd negative sites or frogs (for example, pH in water from sites where Bd was detected on a water filter versus pH in water from sites where Bd was not detected on a water filter). Spearman's rho (Helsel and Hirsch, 2002) was used to determine the strengths of associations among pesticides, water quality, and adjacent land cover with disease at the individual animal level; and for associations among pesticide occurrence in tissue, water, and sediment with adjacent land cover at the site level.

To determine if immediate proximity to agriculture or other land cover type was indicative of pesticide or Bd occurrence, Kruskal-Wallis and Spearman's rho tests were used to compare water, sediment, and frog tissue pesticide concentrations and Bd occurrence on water filters and frog swabs with land cover in 2-km circular buffers around each site (Table S1). The land cover in these buffers is intended to be a surrogate for pesticide exposure from the amphibians' food source and from direct dermal exposure in pesticide treated landscapes. Correlations between a response variable and land use with circular buffers can be influenced by closure (Barringer et al., 1990), however, the use of six land-use categories reduces these variable dependencies. We used pesticide physical properties data from Norman et al. (2012) including the

Table 2
Pesticide type, Log K_{oc}, Log K_{ow}, detection frequency (%), and maximum concentration for all pesticide detected in either water, sediment, or tissue samples in 2009 and 2010. Values in parentheses are below method detection limits and are estimates. n represents the total number of samples collected during the study.

Pesticide	Type	Log K_{oc}	Log K_{ow}	Detection frequency (%)			Maximum concentration		
				Water	Sediment	Tissue	Water	Sediment	Tissue
				($n = 33$)	($n = 26$)	($n = 138$)	ng/L	μg/kg dry weight	μg/kg wet weight
3,4-DCA	D	2.74	2.69	nd	nd	2.9	nd	nd	75.5
3,5-DCA	D	2.49	2.90	nd	nd	2.2	nd	nd	16.5
AMPA	D	3.90	−1.63	33	na	na	750	na	na
Atrazine	H	2.00	2.70	18	nd	nd	183	nd	nd
Azoxystrobin	F	2.63	2.50	15	8	2.2	159	12.6	45.9
Bifenthrin	I	5.38	7.30	nd	38	3.6	nd	17.5	90.1
Boscalid	F	2.91	2.96	nd	8	nd	nd	9.4	nd
Carbofuran	I	2.02	1.80	nd	nd	2.9	nd	nd	21
Chlorothalonil	F	3.20	2.94	nd	23	nd	nd	9	nd
Chlorpyrifos	I	3.78	4.70	6	8	nd	30.4	444	nd
Clomazone	H	2.46	2.60	15	nd	nd	2880	nd	nd
Cyprodinil	F	3.23	4.00	nd	4	nd	nd	(2.3)	nd
EPTC	H	2.30	3.20	12	nd	nd	40.2	nd	nd
Esfenvalerate	I	3.72	6.24	nd	4	nd	nd	16.4	nd
Fenhexamid	F	2.68	3.51	nd	12	nd	nd	4.2	nd
Fludioxonil	F	4.88	4.12	3	nd	nd	(4.1)	nd	nd
Flusilazole	F	3.22	3.87	nd	nd	5.1	nd	nd	1.7
Glyphosate	H	3.56	−3.20	21	na	na	1630	na	na
Hexazinone	H	1.58	1.17	3	nd	nd	25.4	nd	nd
Imazalil	F	3.68	2.56	3	nd	nd	213	nd	nd
Malathion	I	3.26	2.75	nd	nd	1.4	nd	nd	264
Methidathion	I	2.60	2.57	nd	nd	2.2	nd	nd	131
Methoprene	I	3.40	5.00	nd	nd	23.9	nd	nd	720
Metolachlor	H	2.26	3.40	15	4	nd	389	(1.1)	nd
Myclobutanil	F	2.70	2.89	nd	nd	5.8	nd	nd	272
Napropamide	H	2.66	3.30	nd	4	nd	nd	7.6	nd
p,p'-DDD	D	5.00	6.02	nd	1	nd	nd	54.0	nd
p,p'-DDE	D	5.00	6.51	nd	35	29.7	nd	128	916
p,p'-DDT	I	5.40	6.91	nd	4	5.1	nd	75	92.6
Pendimethalin	H	4.13	5.20	nd	8	nd	nd	13.2	nd
Permethrin	I	4.80	6.10	nd	4	2.9	nd	1.1	93.9
Prometryn	H	2.60	3.34	nd	12	nd	nd	14.8	nd
Propiconazole	F	2.81	3.72	6	nd	nd	141	nd	nd
Pyraclostrobin	F	4.04	3.99	3	38	39.9	18.1	87.4	66
Pyrimethanil	F	2.48	2.84	nd	8	nd	nd	7.0	nd
Simazine	H	2.11	2.30	9	nd	34.8	263	nd	619
Tebuconazole	F	2.89	3.70	3	19	10.9	7.6	219	26.3
Trifluralin	H	4.14	5.27	3	4	nd	(0.8)	7.6	nd
Zoxamide	F	3.09	3.76	nd	8	nd	nd	7.0	nd

D = Degradate; F = fungicide; H = herbicide; I = insecticide.
na = not applicable, nd = not detected, DCA = dichloroaniline, AMPA = aminomethylphophonic acid, EPTC = S-Ethyl dipropylthiocarbamate.

octanol–water partition coefficient (K_{ow}) and soil organic carbon-water partitioning coefficient (K_{oc}) in several figures and tables.

We designed this study to be correlative and exploratory with a goal of identifying interactions and associations at the landscape scale. Given the amount of data and multiple tests for associations among variables it is possible that some reported significant differences or correlations are spurious, arising from random chance.

3. Results and discussion

3.1. Pesticide occurrence

Thirty-nine pesticides and pesticide degradates were detected in one or more of the samples collected during the study including 14 fungicides, 11 herbicides, 9 insecticides, and 5 degradates (Fig. 2, Table 2). Fifteen pesticides and one pesticide degradate were detected in the 33 water samples collected from 21 sites sampled in 2009 and 2010 with concentrations ranging from 4.1 ng/L for fludioxonil (a fungicide) to 2880 ng/L for clomazone (a herbicide) (Table 2). AMPA (glyphosate's primary degradate), glyphosate (herbicide), and atrazine (herbicide) were the most frequently detected compounds in water, occurring in 33%, 21%, and 18% of samples (Table 2), and at 62%, 24%, and 19% of sites, respectively. These herbicides are used in agricultural, urban, and some forestry settings and have historically been among the most frequently detected pesticides in surface and groundwaters in the US (Kolpin et al., 2000; Battaglin et al., 2014). The pesticides that were detected in water spanned a wide range of log K_{ow} values from −3.2 for glyphosate to 4.7 for chlorpyrifos (Table 2). Pesticides with of log K_{ow} values <2.5 would be expected to occur primarily in water, those with values between 2.5 and 4.0 would be expected to occur both in water and sediment, and those with values >4.0 would be expected to occur primarily on sediments (Rogers, 1996).

Nineteen pesticides and two pesticide degradates were detected in the 26 sediment samples collected from 20 sites sampled in 2009 and 2010 with concentrations ranging from 1.1 μg/kg (dry weight) for metolachlor (herbicide) to 444 μg/kg for chlorpyrifos (insecticide) (Table 2). Bifenthrin (insecticide), pyraclostrobin (fungicide), and *p,p'*-DDE (insecticide degradate) were the most frequently detected compound in sediment, occurring in 38%, 38%, and 35% of samples (Table 2), and at 40%, 45%, and 35% of sites, respectively. The pesticides that were detected in sediment spanned a range of log K_{ow} values from 2.5 for azoxystrobin to 7.3 for bifenthrin (Table 2). Glyphosate and AMPA

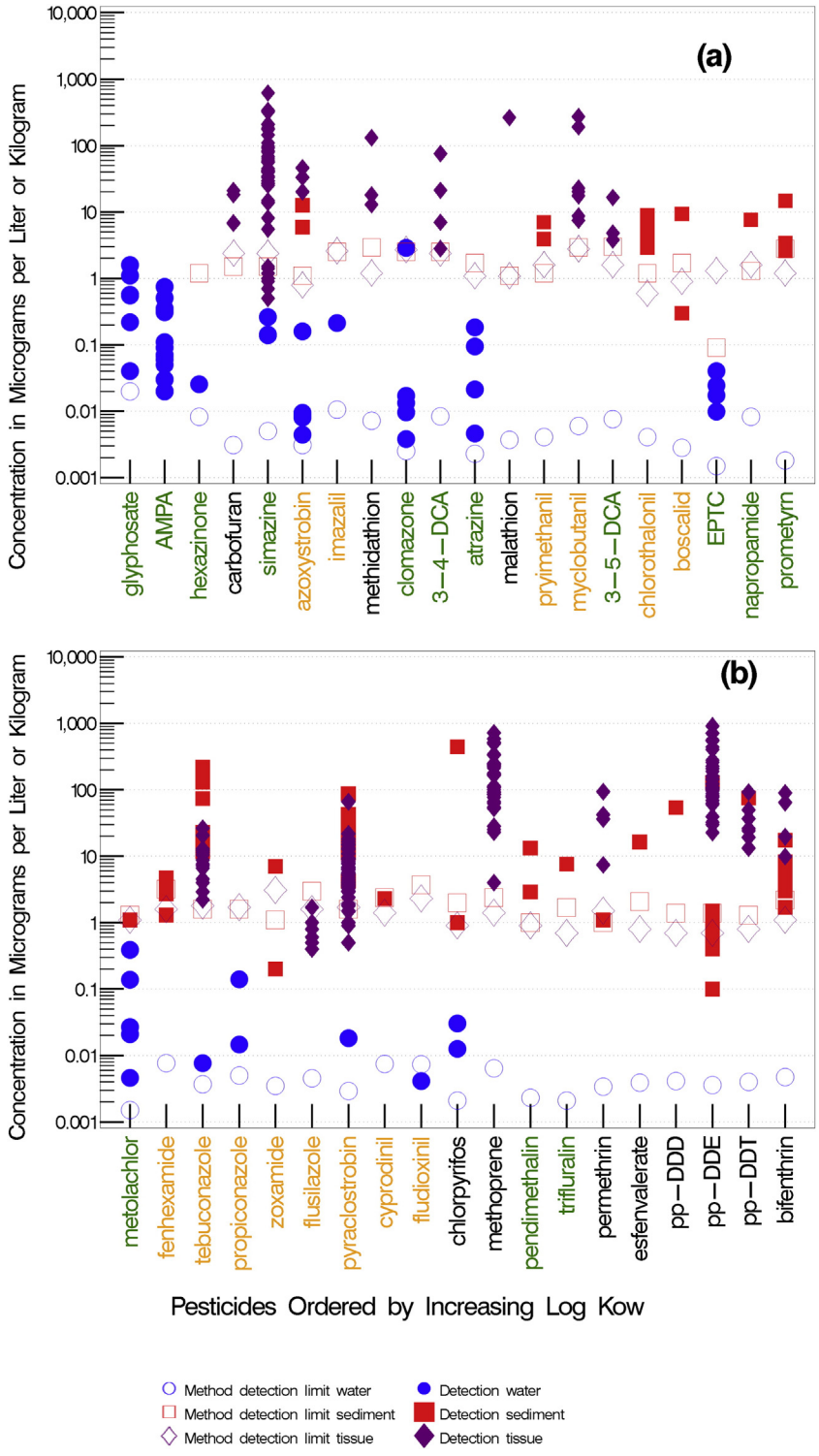

Fig. 2. Concentrations in µg/L (for water) or µg/kg (for sediment and tissue) (all equivalent to parts per billion) in 33 water, 26 sediment, and 138 tissue samples from all sites in 2009–10, of (a) 11 herbicides or herbicide degradates (green), three insecticides (black), and six fungicides (orange), ranging in log K_{ow} values from −3.2 to 3.34; and (b) three herbicides (green), eight insecticides or insecticide degradates (black), and eight fungicides (orange), ranging in log Kow values from 3.4 to 7.3.

were not measured in sediment due to limitations with analytical methods; however, previous studies suggest that both would occur commonly in sediment (Battaglin et al., 2014).

Thirteen pesticides and three pesticide degradates were detected in 138 adult frog tissue samples collected from 14 sites sampled in 2009 and 2010 with concentrations ranging from 1.7 µg/kg (wet weight) for flusilazole (fungicide) to 916 µg/kg for *p,p′*-DDE (Tables 2, S2). Pyraclostrobin, simazine (herbicide), and *p,p′*-DDE (DDT degradate) were the most frequently detected compounds in tissue, occurring in 40%, 35%, and 30% of samples (Table 2), and at 86%, 57%, and 57% of sites, respectively. The pesticides that were detected in tissue spanned a range of log K_{ow} values from 1.8 for carbofuran (insecticide) to 7.3

for bifenthrin (Table 2). Glyphosate and AMPA were not measured in tissue due to a lack of analytical methods, however, they could possibly occur (Kruger et al., 2014).

In the US, pesticide use is dominated by herbicides. For example, in 2007, approximately 250 million kilograms of conventional pesticides were used by agriculture of which 80% were herbicides, 12% were insecticides, and 8% were fungicides (Grube et al., 2011). However, our results indicate that fungicides occur frequently in water in a wide range of hydrologic settings, which corroborate the results reported in Battaglin et al. (2011), Reilly et al. (2012), and Smalling et al. (2013a, 2013b, 2013c, 2015). Similar to other studies (Smalling et al., 2013a, 2015), we also show that fungicides can accumulate in sediments and the tissue of non-target organisms including frogs, presumably via exposure from their habitats or food sources. This is of particular concern for aquatic ecosystems due to the toxicity of some fungicide formulations and their potential for disrupting ecosystem processes (Belden et al., 2010; McMahon et al., 2011; Morrison et al., 2013; Rodrigues et al., 2013; Zubrod et al., 2015).

Although banned in the US in 1972, DDT and the DDT degradates DDD and DDE persist in the environment and are biologically available for uptake by wildlife (Pereira et al., 1996). Maximum concentrations of DDT and DDE in our frog tissue samples were 92.6 and 916 μg/kg wet weight, respectively, and were similar to those observed in agricultural areas in California (Datta et al., 1998). Prior research suggests that DDT and its degradates can bioaccumulate in amphibians (Sparling, 2000). Our results support observations that amphibian populations persist in highly altered habitats, but are challenged by multiple sources of pesticide exposure (Hayes et al., 2010; Reeves et al., 2016; Relyea, 2009; Smalling et al., 2015).

In this study, it was uncommon for a particular pesticide to be detected in more than one media at a given site and time. Three fungicides (azoxystrobin, pyraclostrobin, and tebuconazole) were detected in all three media; however, no pesticides were detected in water, sediment, and tissue at the same site and time. In three instances, a pesticide was detected in both water and sediment samples from the same site and time, and in these cases sediment concentrations were 2 to 4 orders of

Table 3
Results of Kruskal–Wallis tests for significance of differences in pesticide concentrations in frog tissues, water, and sediment; and land use within a 2-km circular buffers around sites; at sites or associated with frogs with positive and negative Bd results. Statistically significant results ($p \leq 0.05$) are indicated by bold text and are followed by the median values of the explanatory variable for Bd-negative and Bd-positive sites or frogs [NA — not analyzed].

Explanatory variable	Bd from water filters — site level (8 Bd-negative and 6 Bd-positive sites for tissue comparisons; 11 Bd-negative and 9 Bd-positive sites for all other comparisons)		Bd from swabs — site level (5 Bd-negative and 11 Bd-positive sites for tissue comparisons; 5 Bd-negative and 14 Bd-positive sites for all other comparisons)		Bd from swabs — individual level (84 Bd-negative and 54 Bd-positive swabs for tissue comparisons; 126 Bd-negative and 66 Bd-positive swabs for all other comparisons)	
	p	Kruskal-Wallis test statistic	p	Kruskal-Wallis test statistic	p	Kruskal–Wallis test statistic
Pesticides in tissue (μg/kg)						
All pesticides	**0.028 (214.17.3)**	**4.82**	0.484	0.49	0.526	0.4
Insecticides	0.103	2.66	0.637	0.22	0.97	<0.01
Herbicides	0.086	2.95	0.691	0.16	0.276	1.19
Fungicides	0.196	1.67	0.533	0.39	0.95	<0.01
Pesticides in water (μg/L)						
All pesticides	0.298	1.08	0.849	0.036	0.591	0.29
Insecticides	0.827	0.05	0.385	0.75	0.779	0.08
Herbicides	0.724	0.12	0.769	0.09	0.173	1.85
Fungicides	0.356	0.85	0.243	1.36	**0.027 (0, 0)**	**4.89**
Pesticides in sediment (μg/kg)						
All pesticides	0.683	0.17	0.46	0.55	0.47	0.52
Insecticides	0.619	0.25	0.145	2.11	**0.001 (3.16, 0)**	**10.9**
Herbicides	**0.039 (0, 0)**	**4.24**	0.735	0.11	0.897	0.02
Fungicides	0.595	0.28	0.961	<0.01	**0.007 (5.9, 19.1)**	**7.29**
Land use (percent of 2-km buffers)						
Cropland	0.79	0.071	0.711	0.137	0.266	1.24
Grassland or pasture	0.518	0.417	0.579	0.309	**0.015 (13, 45.8)**	**5.98**
Forest	0.183	1.78	0.057	3.62	0.283	1.15
Wetlands or water	0.087	2.93	0.195	1.68	0.213	1.55
Urban land	0.759	0.094	0.575	0.315	0.177	1.82
Barren or shrubland	0.594	0.284	0.926	0.009	<**0.001 (0.4, 6.4)**	**16.62**
Other variables (various units)						
PH	0.159	1.99	0.178	1.81	<**0.001 (7.1, 6.6)**	**28.51**
Specific conductance (μS/cm)	0.849	0.03	0.195	1.68	0.744	0.11
Water temperature (degrees C)	**0.007 (21.0, 13.8)**	**7.28**	0.61	0.26	<**0.001 (21, 19.4)**	**13.53**
Dissolved organic carbon (mg/L)	0.119	2.43	0.643	0.21	**0.001 (7.25, 11)**	**11.4**
Total nitrogen (mg/L)	0.053	3.75	0.309	1.04	**0.009 (0.96, 1.23)**	**6.73**
Total phosphorus (mg/L)	0.425	0.64	0.309	1.04	<**0.001 (0.08, 0.25)**	**18.45**
Maximum Bd concentration from filter (zoospore equivalents/L)	NA	NA	NA	NA	**0.043 (0, 2.3)**	**4.1**

magnitude higher than water concentrations, providing limited support for the idea of sediments as a source of pesticide occurrence in overlying waters (Kolok et al., 2014).

At most sites, pesticides were detected more often in tissue or sediment than in water even though MDLs for water analyses were 2 to 3 orders of magnitude lower than for sediment or tissue (Fig. 2). This finding could be because aquatic occurrence of pesticides is expected to be relatively transient, and the timing of our sample collections was not targeted at peaks in pesticide applications or runoff events. As expected, some pesticides with lower log K_{ow} values such as atrazine or clomazone were detected frequently in water, but were not detected in sediment and tissue, whereas some more hydrophobic pesticides such as bifenthrin or the pesticide degradate DDE were detected only in sediment or tissue and not in water (Fig. 2). However, in general, log K_{ow} or log K_{oc} values were not strong predictors of whether a pesticide would be detected in water, sediment, or tissue.

3.2. Bd occurrence

Bd was detected in 14 of 86 (16%) water filter samples and in at least one water filter sample from 9 of 20 (45%) sites. Bd densities ranged from <10 to >340 zoospore equivalents per liter of filtered water. These concentrations are many orders of magnitude lower than what has been used to produce chytridiomycosis in laboratory experiments (Gervasi et al., 2013). Of 192 frog swabs, 34% tested positive for Bd, and one or more frog swabs tested positive for Bd at 14 of 19 (74%) sites where swabs were collected (Table S3). This overall percentage of Bd-positive frogs is similar to what has been reported in the Rocky Mountains (24% of frogs and 64% of sites, Muths et al., 2008), in the southeastern US (39% of frogs and 44% of sites, Rothermel et al., 2008), and in the Pacific Northwest (28% of frogs and 43% of sites, Pearl et al., 2007). Bd densities on frog swab samples ranged from <5 to >17.000 zoospore equivalents (Table S3). These concentrations are generally less than the threshold thought to cause chytridiomycosis related population declines (~10,000 zoospore equivalents) (Vredenburg et al., 2010), but different species are likely to be affected differently by Bd exposures (Searle et al., 2011).

Seven species of frogs were swabbed and the frequency of Bd detection varied by species. Thirty-eight of 62 (61%) *Pseudacris regilla*, 1 of 41 (2%) *Rana clamitans*, 12 of 34 (35%) *Rana catesbeiana*, 12 of 30 (40%) *Pseudacris triseriata*, 2 of 18 (11%) *Rana sphenocephala*, 0 of 5 *Rana pipiens*, and 1 of 2 (50%) *Lithobates septentrionalis* tested positive for Bd (Table S3).

Bd was detected more frequently in water filter samples and on frog swab samples from California than in water filters or on frog swabs from the other States. Bd was detected in water filter samples from 75% of the California sites, but at only 37% of the sites in other States. Bd was detected on 63% of frog swabs from the California sites, but only on 22% of frog swabs from the other six States (Table S3). Some of the Bd water filter sample results may be biased towards non-detection by as much as 14% because our protocol for Bd-water sampling specified three filter samples per site, and recent work within our study area suggests that four to six samples per site may be needed to ensure 95% confidence Bd will be detected when it is present (Chestnut et al., 2014).

Table 4
Spearman's rho and significance (p) values of correlations between concentrations of Bd on frog swabs and concentrations of pesticides in water, sediment, and tissue; nutrient concentrations, other water quality parameters, and land use in 2-km circular buffers around study sites. Statistically significant results ($p \leq 0.05$) are indicated by bold text.

Explanatory variable	Bd swabs (84 Bd-negative and 54 Bd-positive Swabs for tissue correlations; 126 Bd-negative and 66 Bd-positive swabs for all other correlations)	
	p	rho
Pesticides in tissue (μg/kg)		
All Pesticides	0.271	0.09
Insecticides	0.572	0.05
Herbicides	0.226	0.1
Fungicides	0.834	0.02
Pesticides in water (μg/L)		
All pesticides	0.687	0.03
Insecticides	0.796	−0.02
Herbicides	0.501	−0.05
Fungicides	0.086	−0.12
Pesticides in sediment (μg/kg)		
All pesticides	0.473	0.05
Insecticides	**0.002**	**−0.22**
Herbicides	0.554	0.04
Fungicides	**0.018**	**0.172**
Land use (percent of 2-km buffers)		
Cropland	0.518	−0.05
Grassland or pasture	**0.029**	**0.16**
Forest	0.422	0.06
Wetlands or water	0.18	−0.1
Urban Land	0.263	0.08
Barren or shrubland	<**0.001**	**0.29**
Other variables (various units)		
PH	<**0.001**	**−0.37**
Specific conductance (μS/cm)	0.656	0.03
Water temperature (deg C)	<**0.001**	**−0.29**
Dissolved organic carbon (mg/L)	<**0.001**	**0.24**
Total nitrogen (mg/L)	**0.014**	**0.18**
Total phosphorus (mg/L)	<**0.001**	**0.31**
Maximum Bd concentration from water filter (zoospore equiv./L)	0.146	0.11

3.3. Interactions Between Pesticide and Bd Occurrence

Kruskal-Wallis tests were used to compare water, sediment, and tissue pesticide occurrence at the 11 sites where Bd was not detected in water filter samples, with the 9 sites where Bd was detected, to identify site-level differences in pesticide occurrence relative to Bd occurrence, with the expectation that there would be no differences. There were no statistically significant differences in total (all) pesticide concentration, total fungicide concentration, or total insecticide concentration in water or sediment between sites with and without Bd (Table 3). There were differences between sites with and without Bd and total pesticide concentration in tissue samples ($p = 0.028$) and in total herbicide concentrations in sediment samples ($p = 0.039$), with generally higher values associated with Bd negative sites. Similarly, the water, sediment, and tissue pesticide occurrence at the 5 sites where Bd was not detected on a swabbed frog was compared with occurrences from the 14 sites where one or more frog swabs tested positive for Bd. There were no statistically significant differences in water, sediment, or tissue pesticide occurrence between sites with only Bd negative swabs and sites with some Bd positive swabs (Table 3).

Kruskal-Wallis and Spearman's rho tests were used to compare water, sediment, and tissue pesticide occurrence associated with the 126 frog swabs (only 84 have associated tissue pesticide chemistry) on which Bd was not detected, with the 66 frog swabs (54 with tissue pesticide chemistry) where Bd was detected to identify potential individual-animal level interactions between, pesticide occurrence and Bd occurrence, with the expectation that there would be interactions. There were no significant differences in the tissue concentrations of total pesticides, fungicides, insecticides, or herbicides between frogs associated with Bd positive versus Bd negative swabs (Table 3), nor were there significant correlations between pesticide occurrence in frog tissue and Bd concentrations on frog swabs (Table 4). There was some indication of a difference in total fungicide concentration in water and Bd occurrence on frog swabs with generally higher concentrations associated with frog swabs that tested positive for Bd ($p = 0.027$, medians both zero but mean for positives was higher) (Table 3). However, the importance of this interaction is questionable because the correlation between total fungicide concentration in water and Bd concentrations on frog swabs was not significant (Table 4).

Bd may reside and persist in bed sediments in the absence of an amphibian host (Garmyn et al., 2012; Chestnut et al., 2014), so it is possible that Bd could interact with pesticides on sediments either independent of a host or while attached to a host. There were differences in the sediment concentrations for total insecticides and total fungicides and Bd occurrence on frog swabs. In general, we found lower insecticide and higher fungicide concentrations associated with frog swabs that tested positive for Bd (Table 3). The importance of these two differences is supported by the negative correlation between total insecticide concentrations in sediments and Bd concentrations on frog swabs, and the positive correlation between total fungicide concentrations in sediments and Bd concentrations on frog swabs (Table 4).

A negative correlation between pesticides concentrations in general, and fungicides concentrations more specifically in water or sediment, with Bd concentrations on frog swabs was expected due to their presumed negative effect on Bd (Hanlon and Parris, 2014). The positive correlation between fungicide concentrations in sediment and Bd concentrations on frog swabs, and finding higher fungicide concentrations in water associated with Bd positive frog swabs are counterintuitive. Our results are in general agreement with the findings of several laboratory-based studies, which have found exacerbating effects, mitigating effects, and no interactive effects between pesticide exposure and Bd effects or Bd infected amphibians (Gaietto et al., 2014; Hanlon and Parris, 2014; Hayes et al., 2010; McMahon et al., 2013; Paetow et al., 2012; Rumschlag and Boone, 2015).

3.4. Interactions between water quality and Bd occurrence

Several other interactions were observed between basic measures of water chemistry (Table S4) and the occurrence of Bd at the site level and individual frog level. Water temperatures were generally higher at sites where Bd was not detected in a water filter sample ($p = 0.007$, Table 3). At the individual animal level, there were differences between Bd positive and Bd negative frog swabs and water pH, water temperature, DOC, TN, and TP. Water temperature and pH were generally lower in association with Bd positive frog swabs, whereas the concentrations of DOC, TN, and TP were generally higher in association with Bd positive frog swabs. The importance of these five interactions is supported by significant negative correlations between pH and water temperature and Bd concentration on frog swabs, and positive correlations between DOC, TN, and TP concentrations and Bd concentrations on frog swabs (Table 4).

The strong correlations between Bd occurrence and site water chemistry contrast with the results of Strauss and Smith (2013) who found no significant relations, perhaps due to the limited geographic extent of that study. The relation between water temperature and Bd in

Table 5
Spearman's rho and significance (p) values of correlation between concentrations of pesticides in frog tissue, water, and sediment; with land use within 2-km circular buffers around study locations. Statistically significant results ($p \leq 0.05$) are indicated by bold text.

Total concentrations of:	Cropland		Grassland and pasture		Forest		Wetlands or water		Urban		Barren or shrubland	
	p	rho	p	rho	p	rho	p	rho	p	rho	p	rho
Frog tissue ($n = 138$)												
All pesticides	**<0.0001**	**0.46**	0.095	−0.14	**0.052**	**−0.17**	**<0.0001**	**0.60**	**<0.0001**	**0.37**	0.131	−0.13
Insecticides	**<0.0001**	**0.51**	0.475	−0.06	**0.003**	**−0.25**	**<0.0001**	**0.56**	**<0.0001**	**0.38**	**0.042**	**−0.17**
Herbicides	**0.008**	**0.22**	0.708	−0.03	0.674	−0.04	**<0.0001**	**0.41**	**0.045**	**0.17**	**0.004**	**−0.24**
Fungicides	0.299	0.09	**0.014**	**−0.21**	0.233	0.10	**0.038**	**0.18**	0.861	0.02	0.349	0.08
Water ($n = 37$)												
All pesticides	0.264	0.20	0.106	−0.29	0.148	−0.26	0.138	0.26	0.99	<0.01	**0.01**	**−0.44**
Insecticides	**0.051**	**0.34**	0.787	0.05	0.508	0.12	0.189	−0.23	0.29	0.19	0.863	−0.03
Herbicides	**0.052**	**0.34**	0.797	−0.05	**0.032**	**−0.37**	**0.051**	**0.34**	0.561	0.11	**<0.0001**	**−0.63**
Fungicides	0.738	−0.06	**0.003**	**−0.50**	0.761	0.05	0.338	0.17	0.78	−0.05	0.514	0.12
Sediment ($n = 30$)												
All pesticides	0.682	−0.08	0.643	−0.10	0.679	0.09	0.124	−0.31	0.2	−0.26	**0.011**	**0.49**
Insecticides	0.198	0.26	0.969	−0.01	0.245	0.24	0.946	0.01	0.759	−0.06	0.063	−0.37
Herbicides	0.161	0.28	0.598	−0.11	0.419	0.17	0.957	−0.01	0.354	−0.19	0.424	−0.16
Fungicides	0.095	−0.33	0.706	−0.08	0.656	−0.09	0.255	−0.23	0.352	−0.19	**0.001**	**0.60**

this study supports observations by Forrest and Schlaepfer (2011) and Raffel et al. (2013) of a negative relation between Bd prevalence or growth on frogs and water temperature.

3.5. Interactions Between Land Use and Pesticide and Bd Occurrence

There were few significant interactions between pesticide occurrence in water samples and land cover in a 2-km buffer around the sites. Total insecticide and herbicide concentrations in water were positively correlated to percent cropland; however there was no relation between total pesticide concentration in water and cropland (Table 5). Conversely, total fungicide concentrations in water were negatively correlated with percent grassland or pasture and no significant correlation to cropland was observed. In sediment, total pesticide and total fungicide concentrations were both positively correlated with percent barren or shrubland (Table 5). Barren land can include fallow cropland and shrubland can include managed rangeland. Land cover within the drainage areas associated with each sampling location would likely be better predictors of water (Battaglin and Goolsby, 1997, 1998) and sediment occurrence of pesticides than was land cover in the 2-km buffers since a large portion of these areas are likely to be outside the drainage areas and a large portion of the drainage areas are likely to be outside the 2-km buffers.

There were numerous significant interactions between pesticide concentrations in tissue and land cover around the sites. Total pesticide, total insecticide and total herbicide concentrations in frog tissue were positively correlated with percent cropland, wetland, and urban land (Table 5). Total pesticide and insecticide concentrations were negatively correlated with percent forest, and total insecticide and total herbicide concentrations were negatively correlated with percent barren or shrubland. Total fungicide concentrations were positively correlated with percent wetland and negatively correlated with percent grassland or pasture.

Pesticide use on adjacent land, both urban and agricultural, is a primary driver of pesticide concentrations in frog tissues. However, there are multiple potential mechanisms of exposure, and the primary routes remain unclear and may differ at the site level based on the type of habitat assessed. Adult frogs use the terrestrial environment and could have direct exposure in upland environments. Sample collection of adult frogs during the study may have coincided with the consumption of terrestrial insects that could have been exposed to pesticides in either the aquatic or terrestrial landscape. Both larval and adult insects can be sources of pesticides to their predators because they can retain or concentrate the chemicals they were exposed to (Kraus et al., 2014; Walters et al., 2008); in addition, amphibians are known to uptake pesticides directly through their skin (Van Meter et al., 2014). Therefore, both the aquatic and terrestrial environments can be a source of pesticides to the frogs that may not be detected with single wetland sampling events.

4. Conclusions

Frogs are of particular ecological importance because they are often top predators in aquatic ecosystems and can represent substantial biomass. They are considered sentinel organisms because they can be exposed to contaminants in both the aquatic and terrestrial environments. Our research confirms that pesticides occurred widely in both the water and sediment of amphibian habitats, and in the tissue of the amphibians occupying those habitats; however, in many cases the pesticides found in frog tissue did not match the pesticides found in their aquatic habitats. Fungicides are used in smaller quantities than herbicides or pesticides but were detected widely in water, and accumulated in sediments and the tissue of frogs. Pesticide occurrence in frog tissue and the water of their habitats was influenced by the proximity to agricultural and urban land with compounds detected more frequently or in higher concentrations in samples from the sites that were surrounded by greater amounts of cropland and urban land. However, land cover within a 2-km buffer around each site was a better predictor of pesticide concentrations in frog tissues than of pesticide concentrations in the water and sediment of aquatic frog habitats, suggesting that terrestrial pesticide exposure may be an important stressor for amphibians, and that management actions designed to reduce pesticide use on land surrounding amphibian habitat could positively affect amphibian health.

The fungal pathogen, Bd, occurred widely and was detected in water and on individual frogs at more than half of the study sites. There were no differences in surrounding land cover between the sites where Bd was detected in water or on frogs and the sites where Bd was not detected. The total pesticide concentrations in frog tissue samples and total herbicide concentrations in sediment samples were higher at sites where Bd was not detected in the water. There were no other differences in pesticide occurrence between Bd positive and Bd negative sites. Total fungicide concentrations in water and sediment samples were higher in association with individual frogs that tested positive for Bd, whereas insecticide concentrations in sediment samples were lower in association with individual frogs that tested positive for Bd. There were no other differences in pesticide occurrence between individual frogs that were positive or negative for Bd. The concentrations of Bd on frog swabs were negatively correlated with pH and water temperature; and positively correlated with DOC, TN, and TP concentrations. These results indicate that in many of the habitats we sampled amphibians are being exposed to Bd. Our results further suggest that Bd occurrence in these habitats is more affected by differences in basic water-quality properties or pesticide occurrence, than by differences in the surrounding land use.

Ours is the first field-based study to identify interactions of Bd occurrence with the concentrations of pesticides in water, sediment, and frog tissue samples as well as with other water-quality properties such as DOC, TN, and TP. We provide information about the complexities of the interactions among common pesticides and the presence of the amphibian chytrid fungus Bd, in a variety of landscapes representing several amphibian habitat types. Our results provide some insight into the potential for interaction among these pervasive stressors. The robustness of Bd occurrence across the landscape, with minimal affect by pesticides, suggests that Bd is tolerant to a wide range of environmental conditions and that Bd life history allows it to adapt to multiple stressors. The means by which Bd moves between locations and persists in the environment remains an important gap in our knowledge of Bd dynamics.

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