Ecological Applications, 13(4), 2003, pp. 1083–1093

COMBINED EFFECTS OF UV-B RADIATION AND NITRATE FERTILIZER ON LARVAL AMPHIBIANS

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Abstract. As part of a global loss of biodiversity, amphibian populations are declining worldwide. Numerous factors may be involved in these declines, including environmental changes and the spread of contaminants. Ultraviolet-B (UV-B) radiation (280–315 nm) and chemical pollution are two factors that have become increasingly important as contributing to amphibian mortality and, perhaps, to amphibian population declines. Therefore, we studied the combined effects of ambient UV-B radiation and nitrate fertilizer pollution on larval amphibians in outdoor experiments at low and high elevation sites in Oregon. Recent studies have shown that UV-B radiation and nitrate fertilizer pollution have differential effects on amphibians. Some species are more susceptible than others when exposed to either UV-B or to nitrate. Moreover, depending upon other environmental conditions, UV-B levels are often greater in intensity at higher elevation sites compared to lower elevation sites. Therefore, our experiments were designed to include amphibians from both low and high elevations. Very little is known about interpopulational variation regarding the effects of environmental stressors. We tested the combined effects of UV-B and nitrate on larval survival, mass, and length in Pacific treefrogs (*Hyla regilla*) and long-toed salamanders (*Ambystoma macrodactylum*). In the low elevation experiment, we found that UV-B and nitrate together reduced the mass of larval *H. regilla*. In the high elevation experiment, we found that UV-B and nitrate together reduced the survival of larval *H. regilla*. In both the low elevation and the high elevation experiment, nitrate increased the mass of larval *A. macrodactylum*. However, in the high elevation experiment, this result occurred only when UV-B was blocked. This result indicates that the effects of nitrate addition could depend upon the presence of other factors such as UV-B. Results emphasize the importance of considering the effects of multiple stressors.

Key words: Ambystoma macrodactylum; *amphibian decline; fertilizer;* Hyla regilla; *multiple stressors; nitrate; Oregon, USA; Pacific Northwest; UV-B.*

INTRODUCTION

As part of a global decline in the Earth's biodiversity, amphibian populations are declining worldwide (Alford and Richards 1999, Houlahan et al. 2000, Davidson et al. 2001, Blaustein and Kiesecker 2002). Several factors have been linked to amphibian population declines, including global climate change, disease, chemical contaminants, increasing ultraviolet radiation (particularly UV-B; wavelength range 280–315 nm), and introduced nonnative species (Alford and Richards 1999, Daszak et al. 1999, Pounds et al. 1999, Sparling et al. 2000, Blaustein et al. 2001, Kiesecker et al. 2001, Blaustein and Kiesecker 2002).

In nature, animals are exposed to a complex array of environmental insults. Therefore, interactions between two or more stressors may contribute to amphibian population declines (e.g., Hatch and Burton 1998, Alford and Richards 1999, Hatch and Blaustein 2000, Blaustein et al. 2001, Kiesecker et al. 2001, Blaustein and Kiesecker 2002). However, few studies have considered interactions of contaminants with other stressors, such as UV-B radiation. Moreover, interpopulational variation in susceptibility to environmental change and contaminants is not well known, although some research suggests that amphibians from different populations might vary in their response to environmental stressors such as nitrate and UV-B radiation (Johansson et al. 2001, Belden and Blaustein 2002). Accumulating evidence suggests that UV-B radiation and nitrate fertilizer pollution, among others, are both important stressors that harm amphibians (Rouse et al. 1999, Blaustein et al. 2001). Therefore, this study investigated the effects of ambient UV-B radiation and nitrogen-based fertilizer pollution alone and in combination with one another on amphibians from several different populations.

Several biological characteristics of amphibians make them particularly vulnerable to the effects of environmental degradation. For example, many amphibian species must remain near water throughout migration, frequently making them susceptible to habitat fragmentation (Blaustein et al. 1994*a*). The typical biphasic life history of amphibians exposes them to anthropogenic influences in both the terrestrial and aquatic environments (Bishop 1992, Blaustein et al. 1994*b*).

Manuscript received 27 August 2001; revised 8 October 2002; accepted 1 December 2002. final version received 23 December 2002. Corresponding Editor: H. B. Shaffer.

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Moreover, amphibians have thin skin that may be highly permeable to environmental contaminants (Bishop 1992, Blaustein et al. 1994*a*).

The intensity of UV-B radiation reaching aquatic ecosystems at the Earth's surface is increasing due to anthropogenic influences including ozone depletion, acidification, and climate change (Herman et al. 1996, Yan et al. 1996, Pienitz and Vincent 2000, Middleton et al. 2001). UV-B radiation penetrates aquatic habitats to biologically significant depths (Häder et al. 1998). Depending upon other environmental conditions, it is generally assumed that UV-B levels are greater in intensity at higher elevations (Blumthaler et al. 1997, Xenopoulos and Schindler 2001). This relationship between UV-B intensity and altitude has been measured at several locations including the United States, Switzerland, Austria, and Germany (Xenopoulos and Schindler 2001).

Several studies have shown that ambient UV-B is lethal to amphibian embryos and larvae of some species (e.g., Blaustein et al. 1994*b*, Anzalone et al. 1998, Lizana and Pedraza 1998, Broomhall et al. 2000, Häkkinen et al. 2001). In addition, UV-B may have sublethal effects on several species, which may be manifested in slower growth and development (e.g., Belden et al. 2000, Smith et al. 2000, Pahkala et al. 2001). Sublethal effects in larvae such as reduced growth may ultimately affect the general condition of adult amphibians (Smith 1987, Semlitsch et al. 1988, Bervin 1990). Several other studies have examined the effects of UV-B on developing amphibians and found little or no significant effects (e.g., Van de Mortel and Buttemer 1996, Corn 1998, Starnes et al. 2000). While there are potential differences in methodology and species tolerance between studies, the different results emphasize the need to consider the potential interactive effects of UV-B with other environmental contaminants and interpopulational variation in sensitivity. Larvae that are not damaged by UV-B alone may be damaged by UV-B in combination with other environmental agents (e.g., Long et al. 1995, Zaga et al. 1998, Hatch and Blaustein 2000).

The use of nitrogen fertilizers has greatly increased in the past several decades (Vitousek 1994, Tilman 1999). Fertilizers are applied in forests and agricultural areas. For example, in Oregon, $>79 \times 10^6$ kg of nitrogenous fertilizers were applied commercially in 2000 (U.S. Department of Agriculture data, available online).[2] This application can have several impacts on amphibians (Oldham et al. 1997, Marco et al. 1999, Hatch et al. 2001, Johansson et al. 2001). Nitrate may kill developing amphibians or induce sublethal effects (Hecnar 1995, Oldham et al. 1997, Marco et al. 1999, Johansson et al. 2001). Nitrate degradation products including ammonia and nitrite are also toxic to developing amphibians (Jofre and Karasov 1999, Marco and Blaustein 1999). Nitrite affects the behavior and physiology of tadpoles (Marco and Blaustein 1999). Furthermore, nutrient enrichment from nitrogen-based fertilizers may alter community dynamics by increasing the abundance of herbivores such as snails. Snails are secondary parasitic hosts for trematode parasites, and the cysts of these parasites have been linked to deformities in developing amphibians (Johnson et al. 2002).

As discussed, several studies have documented the adverse effects of UV-B and nitrogen fertilizers alone on amphibians. However, no studies in amphibians have considered their combined effects. In this study, we investigated the combined effects of UV-B radiation and nitrate on the survival and mass of developing amphibians. Moreover, we investigated interpopulational variation in response to these two important environmental stressors. Few studies have investigated interpopulational variation, although this is an important aspect to consider in determining the role of various environmental agents in amphibian population declines.

Methods and Materials

General overview

We tested the combined effects of ambient UV-B and nitrate on two species of amphibians native to the Willamette Valley (lower elevation; study site ~10.2 m) and Cascade Mountain (higher elevation; study site ~1022 m) regions of Oregon, USA: long-toed salamanders (*Ambystoma macrodactylum*) and Pacific treefrogs (*Hyla regilla*; see Plate 1). We exposed larvae of each species to UV-B and nitrate in a full factorial experimental design for 3 wk, and then assessed their survival and growth (length and mass). We selected the 3-wk exposure time, rather than allowing animals to be exposed to the treatments until metamorphosis, for several reasons. First, preliminary work indicated that effects on growth in amphibian larvae would be evident after this length of exposure (A. C. Hatch, *unpublished data*). Second, we chose to run all experiments for the same length of time for a comparison among species and experimental locations. Because of differences in the timing of breeding, the experiments at the two elevations (Willamette Valley and Cascade Mountain) could not be completed simultaneously. Previous work indicated that the time to metamorphosis varied for the different species at the different sites (A. C. Hatch, *unpublished data*). By selecting a predetermined time to terminate experiments, we ensured that animals of both species at both sites were exposed to the experimental variables for the same length of time. Finally, we chose to add nitrate only once to mimic a run-off event. Running the experiment for a longer time period would likely have required water changes, confounding the nitrate exposure regime.

We controlled for UV-B using clear plastic filters that either transmit UV-B (acetate; Hillcor Plastics,

[2] URL: ⟨http://www.nass.usda.gov/or/bu16101.pdf⟩

PLATE 1. (Left) Eggs of the Pacific treefrog (*Hyla regilla*) collected at Parish Pond (Cascade Mountains) in the early stages of development. (Right) Adult Pacific tree frog. Photographs by A. Hatch.

Baldwin Park, California, USA) or block UV-B (Mylar; Hillcor Plastics, Baldwin Park, California, USA). Acetate filters (''with UV'') typically transmit 80% of ambient UV-B radiation and 95% of UV-A radiation, while Mylar filters (''without UV'') typically transmit 5% of ambient UV-B radiation and 30% of UV-A radiation (Blaustein et al. 1994*b*).

We added nitrate as sodium nitrate with an initial dose at the appropriate initial concentration, to mimic a single runoff event that might occur in the field after the application of fertilizer. Runoff containing nitrate from agricultural or urban application may often contaminate groundwater in nontarget areas (Owens et al. 1994, Bruce and McMahon 1996, Griffith et al. 1997). In some cases, the levels of nitrate found in the groundwater may exceed safe standards for drinking water (Owens et al. 1994, Bruce and McMahon 1996). Some work in the Pacific Northwest indicates that nitrate contamination in groundwater from intensive agriculture is typically below 4 mg/L (Griffith et al. 1997). However, other work indicates that nitrate can contaminate groundwater or ponds at levels up to ~20 mg/L (e.g., Owens et al. 1994).

We added nitrate as sodium nitrate ($NaNO_3$). The nitrate concentrations given in the text refer to nitrate only (NO_3^-; calculated by molecular mass). Other researchers investigating nitrate toxicity in amphibians have concluded that sodium nitrate is less toxic than ammonium nitrate (NH_4NO_3), which has been used in several studies with amphibians (Johansson et al. 2001). The toxicity of ammonium nitrate could be caused by ammonium cation (NH_4^+) or un-ionized ammonium (NH_3) (Johansson et al. 2001). Our experiments using sodium nitrate eliminated the potential for this confounding factor. Moreover, it is unlikely that sodium contributed to toxic effects in our study. Several researchers (e.g., studies cited in Devillers and Exbrayat 1992) have routinely used water with 625 mg NaCl and 96 mg $NaHCO_3$ per liter of water as control test water for toxicity tests in amphibians. In our experiments, the highest concentration tested had 20 mg nitrate/L and 7.3 mg sodium/L. Therefore it is highly unlikely that toxicity in our experiments could be attributed to the effects of sodium. Water in the treatments (at the appropriate nitrate concentrations initially) was not renewed during the experiment.

Animal care

After collection (described below for each experiment), we reared embryos in the laboratory until hatching (*H. regilla*, stage 26 [Gosner 1960]; *A. macrodactylum*, stage 46 [Harrison 1969]). *Hyla regilla* larvae were reared in 38-L tanks filled with dechlorinated water with ~50 animals per tank. Half of the water was changed twice per week, and larvae were fed a mixture of ground alfalfa pellets and TetraMin flakes (Tetra, Blacksburg, Virginia, USA) ad libitum. *Ambystoma macrodactylum* larvae were reared in 4-L plastic boxes (29 × 16 cm in area, 12 cm deep) at a density of 10 larvae per container. Larvae were fed newly hatched brine shrimp ad libitum and half of the water was changed twice per week. All animals were maintained at room temperature (21–24°C) under a constant photoperiod of 16 h light to 8 h dark using fluorescent light tubes. Under these conditions larvae of both species hatched in approximately three weeks.

We collected animals from the field and reared them in the laboratory so that experiments could begin with animals at the same stage in development at each of the study sites. All experiments began when animals were newly hatched. However, one caveat to our approach concerns the potential development of protective mechanisms (e.g., melanin production, photolyase induction). Some of these mechanisms might not have been induced in animals that were reared in the laboratory for a period of time. As a result, it is possible

TABLE 1. Ultraviolet radiation measurements for experiments on two species of amphibians at two field sites in Oregon.

Site and species	UV-A (mW/cm^2)	UV-B ($\mu W/cm^2$)
Willamette Valley (low elevation)		
H. regilla	1.16–3.68	3.88–7.34
A. macrodactylum	2.41–4.11	3.18–9.28
Cascade Mountain range (high elevation)		
H. regilla	2.4–5.03	18.7–21.2
A. macrodactylum	2.88–4.43	7.77–15.3

Notes: Values represent the range (minimum and maximum) measured at the water's surface weekly during experiments. Dates of the experiments are given in *Methods and Materials*.

that our experimental animals might have become more sensitive to stressors such as UV-B than animals directly collected from the field.

Willamette Valley experiment

In the Willamette Valley, both species tested typically breed in late winter (January–February) in roadside ditches or temporary ponds (Nussbaum et al. 1983) and often co-occur. *Ambystoma macrodactylum* typically breed earlier (~1–2 weeks) than *H. regilla* (Nussbaum et al. 1983). Therefore our experiments with the two species were not completed simultaneously. The *H. regilla* experiment in the Willamette Valley ran from 7 March to 28 March 2000 and the *A. macrodactylum* experiment ran from 8 April to 29 April 2000. We collected egg masses (5–7 of each species) from ponds located ~5.5 km west of Tangent in Linn County, Oregon.

We used outdoor mesocosms (55-L galvanized steel cattle watering tanks lined with plastic) as experimental units. We filled tanks with 50 L of well water (alkalinity 88 mg $CaCO_3$/L; hardness 102 mg $CaCO_3$/L; nitrate 2 mg/L; pH 7.2; conductivity 177 μs/cm; DO 11.1 mg/L at 12°C). We exposed larvae of each species separately to combinations of UV-B and nitrate for 3 wk and measured larval growth (length and mass). For the *H. regilla* experiment, we used two levels of UV-B (with, without) and three levels of nitrate (0, 5, 20 mg/L) for a total of six treatments. For each treatment we had six replicate cattle tanks with 10 larvae per replicate. For the *A. macrodactylum* experiment, we used two levels of UV-B (with, without) and two levels of nitrate (0, 10 mg/L) for a total of four treatments. For each treatment we had four replicate cattle tanks with six larvae per replicate. We arranged mesocosms in a randomized block design with respect to treatment.

To provide food for *H. regilla*, we added alfalfa pellets (~0.5 g) to each tank every week. To provide food for *A. macrodactylum*, we collected zooplankton from the original pond where animals were collected. We added ~100 mL of water containing zooplankton (at a density of ~15 zooplankton per mL) twice per week to each tank. At the completion of the 3-wk exposure, we measured algal growth in the *H. regilla* experiment and zooplankton abundance in the *A. macrodactylum* experiment. We measured algal growth by placing three ceramic tiles (11 cm on each side) in each mesocosm and quantifying the percent cover of the tiles by algae. We measured zooplankton abundance by sieving zooplankton from tank water, preserving zooplankton in 70% ethanol, then identifying and counting the various orders of zooplankton in each tank.

Cascade Mountain experiment

We tested the combined effects of ambient UV-B and nitrate on *H. regilla* and *A. macrodactylum* at a field site in the Cascade Mountains (Parish Pond; 62 km east of Lebanon in Linn County, Oregon; elevation 1022 m). In the Cascade Range, both species breed when snowmelt fills breeding ponds in the spring (April–June) (Nussbaum et al. 1983). Both species often breed in temporary ponds that dry completely before the end of the summer. In general, UV-B levels are higher at the higher altitude Cascade Mountain range sites, in comparison to sites in the lower elevation Willamette Valley (Table 1). The *H. regilla* experiment at the Cascade Mountain site ran from 14 June to 5 July 2000. The *A. macrodactylum* experiment at the Cascade Mountain site ran from 1 June to 21 June 2001. We collected *H. regilla* egg masses from Parish Pond, allowed eggs to hatch in the laboratory and used these larvae for the Cascade Mountain experiment. We collected newly hatched *A. macrodactylum* larvae from Susan's Pond (21 km south of Sisters in Deschutes County, Oregon; elevation ~1903 m) and used these larvae for the Cascade Mountain experiment with this species.

We used 4-L plastic buckets filled with 3.8 L of pond water as experimental units. We exposed larvae to combinations of UV-B (with, without) and nitrate (0, 10 mg/L) for a total of four treatments. We used four replicate buckets per treatment, with ten *H. regilla* larvae per replicate or seven *A. macrodactylum* larvae per replicate. UV-B treatments were achieved by using clear plastic filters clipped over the buckets, as described for the Willamette Valley experiment. We added nitrate as an initial pulse of 10 mg/L. We arranged buckets in a randomized block design in the pond. We exposed animals for 3 wk and then assessed effects on survival and growth (length and mass) because preliminary experiments suggested that effects on survival might be important in the Cascade Mountain experiment.

Water quality

We measured UV-B, nitrate, and other characteristics of water quality in at least one container from each treatment at regular intervals. We measured UV-B levels in the water of the containers under the filters at least once per week using a model 2100 PMA (personal

TABLE 2. Water quality measurements for experiments in Oregon on the effects of UV-B and nitrate in two populations of *Hyla regilla* and *Ambystoma macrodactylum*.

Site and species	Conductivity (μs/cm)	DO (mg/L)	pH	Alkalinity (mg $CaCO_3$/L)	Hardness (mg $CaCO_3$/L)
Willamette Valley					
Hyla regilla	60–250	6.3–13.8	7.2–9.1	16–114	22–116
Ambystoma macrodactylum	88–150	6.6–10	7.7–9.0	110–116	128–134
Cascade Mountain					
Hyla regilla	58–207	4.6–6.0	6.5–7.8	26–62	14–50
Ambystoma macrodactylum	25–91	5–8.2	7.1–8.6	32–62	20–62

Notes: Values represent the range (minimum and maximum) measured weekly at the water's surface during experiments. DO = dissolved oxygen.

measurement assistant) meter with model 2102 UV-B detector (Solar Light, Philadelphia, Pennsylvania, USA). UV-A was quantified using the same meter and a PMA2111 detector. Additionally, we measured UV levels at the water's surface weekly during each experiment. We measured nitrate levels in the water twice per week using an Orion pH/ISE nitrate probe (model 290A; Orion Research, Beverly, Massachusetts, USA). Once per week we measured pH, hardness, alkalinity, and conductivity in the water. We measured these variables because we wanted to determine whether our experimental manipulations (nitrate addition or UV-B exposure) altered other aspects of water quality, and to provide more information about our experimental conditions. The pH was measured using an Orion 290A pH/ISE (ion selective electrode) meter with a pH electrode. Conductivity was measured using a handheld conductivity meter (Hanna Instruments, Woonsocket, Rhode Island). Dissolved oxygen was calculated using the Winkler titration method (American Public Health Association 1995). Water hardness and alkalinity were measured by titration with EDTA and 0.02 mol/L HCl, respectively (American Public Health Association 1995). Temperature in the experimental treatments (at least one container of each UV-B treatment: acetate or Mylar filter) was recorded hourly (in the Willamette Valley experiment) and every 4 h (Cascade Mountain experiment) with Onset dataloggers (Onset Computer, Bourne, Massachusetts, USA).

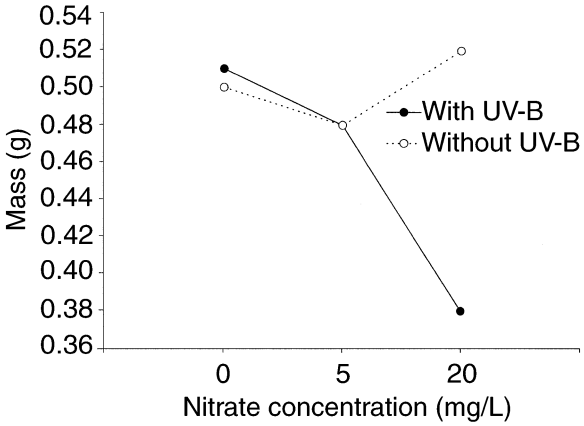

FIG. 1. Willamette Valley experiment: mass of *Hyla regilla* when exposed to UV-B and nitrate. Symbols represent mean mass.

Data analysis

For all experiments, we assessed the effects of UV-B, nitrate, and the interaction between UV-B and nitrate on larval survival, mass, and length. We checked that data met the assumptions for parametric analysis (normality, homogeneity of variance) visually. ANOVA *F* tests were used to analyze results. Container means were used as the units of statistical analysis. Data for the proportion surviving were arcsine square-root transformed prior to analysis. For all experiments, we first tested for effects due to block before proceeding with the analysis of the effects of experimental treatments. Because we did not find any significant block effects, we proceeded to analyze for effects due to the treatments (UV-B, nitrate, and UV-B × nitrate) and we report these results in the following section. All statistical tests were completed using SAS version 6.12 for Windows (SAS Institute 1999).

To determine whether algal percent cover differed among treatments in the Willamette Valley experiment with *H. regilla*, we checked for differences in the mean percent cover between treatments using nonparametric ANOVA on the ranked data because the data was not normally distributed. To determine whether zooplankton abundance or composition differed among treatments in the Willamette Valley experiment with *A. macrodactylum*, we checked for differences in the relative composition between treatments using ANOVA.

RESULTS

All experiments

Table 1 summarizes UV measurements for all of the experiments. In all experiments, Mylar filters transmitted ~10–22% of UV-B and 45% of UV-A radiation that penetrated the water column. Acetate filters transmitted ~75–95% of UV-B and 85–100% of UV-A radiation that penetrated the water column. Water quality

TABLE 3. ANOVA results for experiments on the effects of UV-B and nitrate in two populations of *Hyla regilla* and *Ambystoma macrodactylum*.

Source	Length				Mass				Survival			
	df	MS	*F*	*P*	df	MS	*F*	*P*	df	MS	*F*	*P*
Willamette Valley												
Hyla regilla												
Nitrate	2	0.130	1.16	0.327	2	0.011	1.47	0.247	2	0.101	2.08	0.142
UV-B	1	0.151	1.34	0.256	1	0.023	3.21	0.084	1	0.165	3.40	0.075
Nitrate × UV-B	2	0.033	0.30	0.746	2	0.025	3.44	0.045	2	0.084	1.73	0.194
Ambystoma macrodactylum												
Nitrate	1	0.014	0.10	0.761	1	0.010	10.04	0.008	1	0.006	0.21	0.653
UV-B	1	0.001	0.00	0.959	1	0.004	4.24	0.062	1	0.037	1.28	0.280
Nitrate × UV-B	1	0.014	0.10	0.761	1	0.004	3.62	0.082	1	0.006	0.21	0.653
Cascade Mountains												
Hyla regilla												
Nitrate	1	0.001	0.02	0.898	1	0.001	0.79	0.401	1	0.254	30.58	0.001
UV-B	1	0.035	0.47	0.512	1	<0.001	0.00	0.974	1	0.219	26.40	0.001
Nitrate × UV-B	1	0.034	0.45	0.512	1	0.003	2.62	0.144	1	0.544	65.53	<0.001
Ambystoma macrodactylum												
Nitrate	1	0.032	2.88	0.118	1	<0.001	4.46	0.058	1	0.047	1.26	0.285
UV-B	1	<0.001	0.00	0.951	1	<0.001	0.50	0.494	1	0.002	0.05	0.820
Nitrate × UV-B	1	0.001	0.98	0.343	1	0.001	7.13	0.022	1	0.174	4.64	0.054

measurements for all of the experiments are summarized in Table 2.

Willamette Valley experiment: H. regilla

Larval mass was affected by the interaction between UV-B and nitrate (Fig. 1, Table 3). Without UV-B, nitrate increased larval mass. However, nitrate did not increase larval mass in the presence of UV-B (Fig. 1). Overall these results suggest an effect of the combined treatments on the mass of larval *H. regilla*; mass was lower in the combined presence of both UV-B and high nitrate levels (20 mg/L).

Temperature ranged from 5.3° to 29°C with a 12°C range in any one day. There was no difference in temperature between acetate and Mylar-covered mesocosms in either experiment in the Willamette Valley. Algal growth differed in the various treatments (Kruskal-Wallis ANOVA on ranks, $H_5 = 14.7$, $P = 0.012$). There was less algal growth in the two UV-B treatments with nitrate added and in the no UV-B treatment with high nitrate compared to the no UV-B control treatment. This suggests that either algal growth was reduced in these treatments or that tadpoles consumed more algae. There was very little algal growth in the water column; rather, algae grew on the bottom of the tanks.

Nitrate in low treatments ranged from 5.2 to 6.4 mg/L in week 1, 3.3 to 4.0 mg/L in week 2, and 1.1 to 2.1 mg/L at week 3. Nitrate in high treatments ranged from 18.8 to 20.1 mg/L at the beginning of the experiment, 8.3 to 10.4 mg/L after the first week, 7.4 to 9.1 mg/L after the second week, and 2.4 to 3.2 mg/L at the end

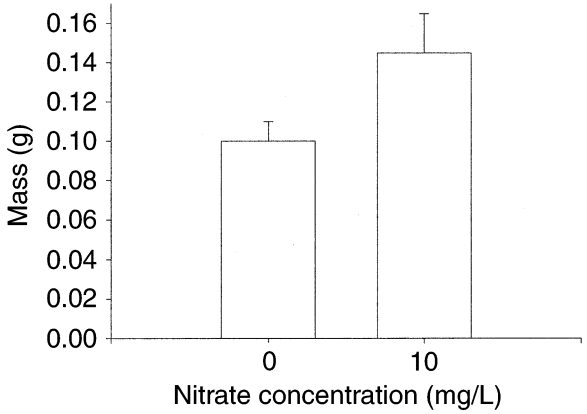

FIG. 2. Willamette Valley experiment: mass of *Ambystoma macrodactylum* when exposed to nitrate. Bars represent mean mass; error bars represent one standard error.

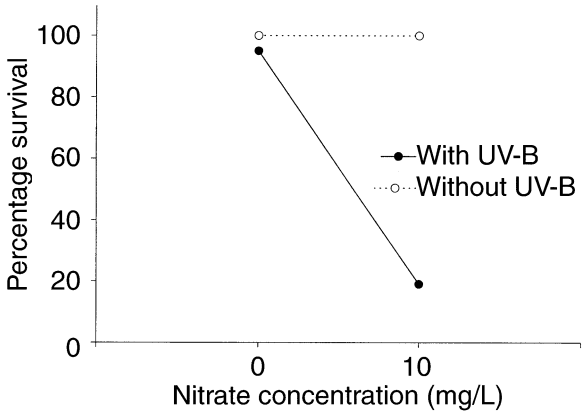

FIG. 3. Cascade Mountains experiment: survival of *Hyla regilla* when exposed to UV-B and nitrate. Symbols represent mean survival.

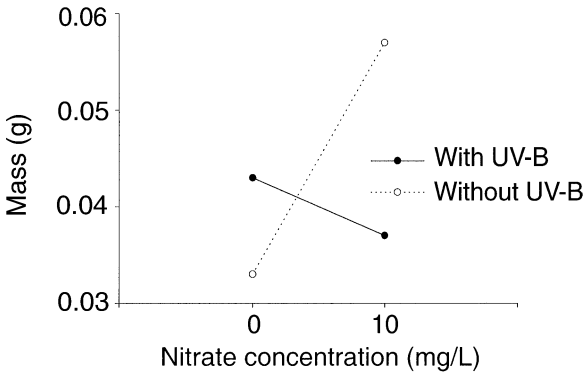

FIG. 4. Cascade Mountains experiment: *Ambystoma macrodactylum* mass when exposed to UV-B and nitrate. Symbols represent mean mass.

of the experiment. Nitrate in control treatments ranged from 0.85 to 1.89 mg/L throughout the experiment.

Willamette Valley experiment: A. macrodactylum

Nitrate increased larval mass (Table 3, Fig. 2). Temperature ranged from 8° to 32°C with a 15°C range in any one day. There was no difference in zooplankton composition between treatments in the *A. macrodactylum* experiment. Nitrate in nitrate treatments ranged from 9.1 to 10.1 mg/L at the beginning of the experiment, 6.0 to 6.8 mg/L after the first week; 4.2 to 6.4 mg/L after the second week, and 2.4 to 3.2 mg/L at the end of the experiment. Nitrate in control treatments ranged from 0.70 to 2 mg/L throughout the experiment.

Cascade Mountains experiment: Hyla regilla

Survival was reduced by both nitrate and UV-B in combination, resulting in the loss of three containers in one treatment (Fig. 3). Temperature was cooler over the first 5 d of the experiment compared to the remaining exposure time. Temperature ranged from 6° to 12°C the first 5 d, with a 4°C range in any one day. For the remaining 16 d of the experiment, temperature ranged from 10° to 26.5°C, with a 12°C range in any one day. There was no difference in temperature between acetate and Mylar-covered enclosures.

Water collected from the pond at the initiation of the experiment had <1 mg/L nitrate; <0.1 mg/L ammonia; pH 6.5; conductivity 29 μs/cm; alkalinity 15 mg $CaCO_3$/L; and hardness 10 mg $CaCO_3$/L. In the nitrate treatments, nitrate levels decreased from 10 mg/L at the beginning of the experiment, to 6 mg/L after the first week, to 5 mg/L after the second week, and to 2 mg/L after the third week. There was no detectable nitrate in the control treatments.

Cascade Mountains experiment: A. macrodactylum

Mass was increased by the addition of nitrate when UV-B was blocked (Fig. 4, Table 3). Temperature ranged from 7° to 33°C, with an 18°C range in any one day. There was no difference in temperature between acetate and Mylar-covered enclosures. Water collected from the pond at the initiation of the experiment had <1 mg/L nitrate; <0.1 mg/L ammonia; pH 7.8; conductivity 20 μs/cm; alkalinity 15 mg $CaCO_3$/L; and hardness 10 mg $CaCO_3$/L. In the nitrate treatments, nitrate levels decreased from 10.5 mg/L at the beginning of the experiment, to 7.1 mg/L after the first week, to 4.2 mg/L after the second week, and to 2.5 mg/L after the third week. There was no detectable nitrate in the control treatments.

DISCUSSION

Understanding the combined effects of multiple stressors is one of the most important problems facing ecologists and environmental risk assessors (Breitburg et al. 1998, Blaustein and Kiesecker 2002). Traditionally, risk assessment has focused on understanding the effects of environmental stressors acting alone, and this information is necessary to evaluate their effects. In some cases, one perturbation may follow another, resulting in compounded effects over time (Paine et al. 1998). In other instances, animals are simultaneously exposed to multiple environmental threats. Nontoxic levels of a stressor may induce detrimental effects when animals are simultaneously exposed to other environmental contaminants (e.g., Long et al. 1995, Hatch and Blaustein 2000). Some recent work on amphibians has indicated the potential for the effects of environmental stressors to vary depending on surrounding ecological conditions (e.g., Boone and Semlitsch 2001, Relyea and Mills 2001). For example, low levels of carbaryl can become highly toxic to tadpoles in the presence of predator chemical cues (Relyea and Mills 2001). Another study concluded that carbaryl contamination could increase tadpole growth because of its toxicity to competing zooplankton (Boone and Semlitsch 2001). Therefore, multifactorial studies that consider several variables, such as the one we present here, are particularly important in addressing the role of various agents in contributing to amphibian population declines.

Our study considered the single and combined effects of two important environmental agents: UV-B and nitrate. Multifactorial studies such as this one are useful in assessing the effects of environmental stressors. While UV-B radiation did not directly impact amphibians in our experiments, UV-B may be important to consider in combination with other environmental agents such as nitrate. In particular, *H. regilla* exhibited a negative response when simultaneously exposed to UV-B and nitrate in both the Willamette Valley and the Cascade Mountain experiments. Nitrate caused increased mass in *A. macrodactylum*, but in the higher elevation Cascade Mountain experiment, this response did not occur in the presence of UV-B.

While UV-B radiation alone did not impact *H. regilla* in our experiments, we have shown that UV-B is important to consider in combination with other environmental agents such as nitrate. Previous work indicates

TABLE 4. Summary of experimental results for *Hyla regilla* and *Ambystoma macrodactylum* larvae exposed to nitrate and UV-B in combination.

Region and species	Effect of nitrate alone?	Effect of UV-B alone?	Combined effects?
Willamette Valley (low elevation)			
Hyla regilla	no	no	yes: decreased mass with simultaneous exposure to high nitrate and UV-B
Ambystoma macrodactylum	yes: mass increased with increased levels of nitrate	no	no
Cascade Mountains (high elevation)			
Hyla regilla	no	no	yes: decreased survival with simultaneous exposure to high nitrate and UV-B
Ambystoma macrodactylum	no	no	yes: without UV-B, increased mass with increased levels of nitrate

that *H. regilla* eggs are resistant to the direct effects of UV-B, and have relatively high levels of the photorepair enzyme photolyase compared to other amphibians from the Pacific Northwest (Blaustein et al. 1994*a*). In the current study, we found combined effects caused by UV-B and nitrate in *H. regilla* from both experiments (Figs. 1 and 2, Tables 3 and 4). In the Willamette Valley experiment, mass of *H. regilla* was affected by exposure to high levels of nitrate in the presence of UV-B. *Hyla regilla* mass was reduced in the treatment with high nitrate and UV-B. At higher elevations with higher levels of UV-B, *H. regilla* in the nitrate treatment suffered mortality over a 3-wk exposure, whereas at lower elevations *H. regilla* survival was not affected. The most likely explanation for this result was the higher levels of UV-B radiation at higher elevations (Table 1). Alternatively, the different responses in the two habitats could also suggest a difference in sensitivity of the populations to UV-B or to nitrate. *Hyla regilla* populations in the mountains may be more sensitive to the effects of environmental agents such as fertilizers. Another study concluded that populations of common frogs (*Rana temporaria*) differed in sensitivity to nitrate depending on previous exposure (Johansson et al. 2001). In considering our results, it is important to note that our study design was limited in that only one site at each elevation was considered. Therefore, differences in response could be attributed to some other factor besides UV-B levels. Future work could include multiple study sites at the different elevations to more effectively quantify elevational effects.

For *A. macrodactylum*, results from the experiments in the two study sites were similar: nitrate increased larval mass (Figs. 2 and 4, Table 4). In the Cascade Mountain experiment, this effect occurred only when UV-B was blocked. This result indicates that the effects of nitrate addition could depend upon the presence of other factors such as UV-B radiation. This effect could be due to increased nutrients (nitrate) increasing the amount of zooplankton available as salamander food. If algae or zooplankton are sensitive to UV-B, they may not exhibit increased growth in the presence of UV-B, and this could explain the different results of *A. macrodactylum* mass in the treatment with UV-B. The overall implications of the combined effects of UV-B and nitrate for *A. macrodactylum* at the population level are uncertain. However, our results suggest that this might be an interesting area for research at multiple trophic levels. Certainly a reduction in salamander food base could result in reduced larval growth, with potential consequences at metamorphosis.

In the Willamette Valley experiment with *A. macrodactylum*, zooplankton abundance and composition did not differ by treatments. However, we did find decreased abundance of algae with nitrate addition in the *H. regilla* experiment, particularly in the presence of UV-B. Therefore, it is possible that UV-B alone or in combination with nitrate may have affected the salamander's food source. Other work has shown the potential for UV-B to impair amphibian food sources such as algae (Rogers et al. 2001), and several studies have found direct effects of UV-B on amphibian food sources. UV-B can damage bacterioplankton (Häder et al. 1998), phytoplankton (Hessen et al. 1997, Häder et al. 1998), rotifers (Vinebrooke and Leavitt 1999), and freshwater algae (Arts and Rai 1997, Rogers et al. 2001). In other examples, UV-B can affect a particularly sensitive component of the aquatic food chain, with the potential for alterations at the community level. For example, herbivorous macroinvertebrates were more sensitive to UV-B than algae, resulting in increased algal growth in the presence of UV-B (Bothwell et al. 1994). Zooplankton fed from a UV-B treated experimental microcosm had impaired growth and survival compared to those fed from a microcosm that was not UV-B treated, although there were no direct effects of UV-B on phytoplankton, zooplankton, periphyton, or macroinvertebrates (DeLange et al. 1999). Other experiments have shown that UV-B exposure reduced diatom cell size of periphyton without affecting the qual-

ity of the periphyton as food for snails (McNamara and Hill 2000).

For *H. regilla*, the combination of UV-B and nitrate had a sublethal effect at the lower UV-B intensity in the lower elevation Willamette Valley experiment, and this effect was greater for *H. regilla* in the higher UV-B intensity in the Cascade Mountains experiment. We do not know of a direct mechanism by which one of these factors would alter the toxicity of the other. Therefore, we suggest that our results are caused by reduced ability to compensate for the effects of one stressor in the presence of another. For example, in the Selyean model of stress, organisms experience increased energetic costs upon exposure to a stressor (Selye 1956). According to the Selyean general adaptation syndrome, organisms can persist in the presence of a stressor such as chemical pollution or UV-B radiation until the stressor reaches a critical level. In our experiment, larval amphibians may be compensating for the physiological costs of exposure to a stressor such as UV-B, and this might not become apparent until exposure to a second stressor such as nitrate pollution occurs.

Our study considered the interpopulational variation and susceptibility to stressors in low and high altitude populations. Population differences in response to stressors such as UV-B are important to consider (e.g., Belden et al. 2000, Belden and Blaustein 2002). In previous work with salamanders, *A. macrodactylum* from lower elevation sites exhibited high mortality upon UV-B exposure, while animals from high elevation sites exhibited no mortality but slower growth caused by UV-B exposure (Belden et al. 2000, Belden and Blaustein 2002). Our experiments at two different study sites provide further evidence of the potential differences in response to UV-B radiation between populations that have historically been exposed to different levels of UV-B.

We observed effects on larval amphibians that may potentially affect later developmental stages. For example, reduced mass in *H. regilla* could result in smaller animals at metamorphosis, and therefore smaller adults. In some cases amphibians that are small at metamorphosis have delayed sexual maturity, possibly reducing their fitness compared to larger animals (e.g., Smith 1987, Semlitsch et al. 1988). Therefore, environmental agents that reduce growth in larvae may potentially affect the species at the population level if exposed animals have reduced fitness (e.g., Bridges 2000). In other cases, the reduced survivorship of some larvae may benefit survivors by reducing competition between them (e.g., Morin 1986). Therefore, if some animals in a population are eliminated by a toxicant, the survivors may benefit from reduced competition (e.g., Liess 2002). The effects of this scenario on the population remain unclear. Overall, the relationship between negative effects in larval amphibians to adult fitness, and the implications for the population, deserves further study.

Both UV-B and nitrate contamination are likely to exist in breeding ponds at the same time as amphibians. Furthermore, amphibians are increasingly likely to encounter both of these environmental agents because of increasing human impacts on sensitive habitat. Although there are variations at any given location, overall the intensity of UV-B is increasing due to global change, including ozone depletion, acidification, and climate change (Herman et al. 1996, Yan et al. 1996, Häder et al. 1998, Pienitz and Vincent 2000). Additionally, nitrate runoff is increasing with increasing input from application of fertilizers (Vitousek 1994, Tilman 1999). Amphibians breeding in shallow temporary ponds, particularly those impacted by nitrogenous fertilizer runoff, may frequently be exposed to both of these factors simultaneously in the field during their sensitive developmental stages. We suggest that the combined effects of environmental agents such as UV-B and nitrate must be considered when determining acceptable levels of contamination in sensitive amphibian breeding areas.

Acknowledgments

We thank L. Belden, I. Downie, E. Arroway, P. Joss, and J. Romansic for all their help. We are indebted to B. A. Menge, H. B. Shaffer, M. Boone, and an anonymous reviewer for insightful comments that greatly improved the manuscript. We thank C. Schreck and employees at the Oregon State University Salmon Disease Laboratory for access to land for the mesocosm portions of this study. We thank the EPA STAR Graduate fellowship program, the American Museum of Natural History, the Katharine Bisbee II Fund of the Oregon Community Foundation, and the National Science Foundation (IBN-9904012) for funding. We also thank Robert G. Anthony and the Biological Resources Division, U.S. Geological Survey through Cooperative Agreement No. 14-45-0009-1577, Work Order No. 17 for financial assistance.

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