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Assessment of pollution in road runoff using a *Bufo viridis* biological assay

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Highway runoff has detrimental effects on the development of B. viridis larvae.

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ABSTRACT

Road runoff is a major source of environmental pollution, significantly threatening nearby aquatic habitats. Chemical analyses indicate high pollutant concentrations in the road's "first flush", but bioassays are more advantageous for addressing the cumulative effects of the numerous pollutants within the runoff. We used *Bufo viridis* embryos and larvae to assess the toxicity of road runoff from two major highways in Israel. We show, for the first time, that exposure to midseason runoff not only has an adverse effect on growth and development rates of *B. viridis* larvae but can also lead to increased rates of morphological deformations. Seasonal first flushes, despite having higher metal concentrations, did not adversely affect the toad larvae, apparently due to a counter effect of organic matter that potentially served as a supplementary energy resource. Road runoff can be a major cause for a qualitative decrease in the quality of aquatic habitats threatening amphibians in Israel.

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1. Introduction

1.1. Pollution in road runoff and its effect on aquatic organisms

Road construction and traffic adversely affect the environment by altering the physical and chemical characteristics of nearby land, air, and water bodies (Gunderson et al., 2005). Water pollution by road-borne chemicals has received little attention compared with other deleterious effects. Numerous factors may affect the quality of a road runoff, but since vehicles are the main source of pollution, traffic volume is usually proportional to the quantity of pollutants in runoff (Driscoll et al., 1990 cited in Barrett et al., 1995, 1998; Drapper et al., 2000). Higher concentrations of pollutants are often observed in the "first flush" of a rain storm; this is especially true for highly soluble pollutants such as nutrients, organic compounds and ionic metals (Barrett et al., 1995). Consequently, in locations with a defined rainy season, increased concentrations of pollutants have been found in the seasonal first flush, e.g. in Israel, in the first autumn rain (Kayhanian and Borroum, 2000; Lee et al., 2004).

The chemical constituents of road runoff have been identified worldwide (U.S.: Driscoll et al., 1990, cited in Barrett et al., 1995, 1998; Australia: Davies et al., 2000; Drapper et al., 2000; Europe: Legret and Pagotto, 1999; Mangani et al., 2005; Revitt et al., 2004; Van Bohemen and Janssen Van De Laak, 2003). However, the potential environmental threat of the numerous pollutants in the runoff can be assessed only by measuring their cumulative effects on biological systems.

Of the many constituents of road runoff (see Buckler and Granato, 1999), heavy metals are of major concern because of their persistence in the environment and accumulation in living tissues, resulting in increasing concentrations at higher levels of the food chain (Beasley and Kneale, 2002; Forman and Alexander, 1998). Road runoff primarily affects aquatic habitats and organisms located alongside roads (Beasley and Kneale, 2002; Forman and Alexander, 1998; Maltby et al., 1995; Spellerberg and Morrison, 1998), however, metals' lethal effect on fish has been observed as far as 8 km downstream from a runoff inlet.

1.2. Amphibians as bio-indicators for pollution in road runoff

We studied the biological effects of road runoff using embryos and larvae of the green toad *Bufo viridis*, a local aquatic vertebrate. This species is commonly exposed to runoff, and has recently experienced population decline along the heavily developed coastal plain of Israel (Elron et al., 2005). The global decline of amphibian populations, partially as a result of human actions (Blaustein et al., 2003; Stuart et al., 2004), might imply a high sensitivity of this class to environmental stress in general and to water quality deterioration in particular. Amphibians have been

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used in many studies to monitor the quality of water bodies exposed to pollutants of various sources (Freda et al., 1990; Harris et al., 1998; Haywood et al., 2004; Herkovits et al., 1996; Hopkins, 2006; Lefcort et al., 1998; Loumbourdis and Wray, 1998; Rasanen et al., 2003; Rouse et al., 1999 cited in Blaustein et al., 2003), but studies on biological effects of road runoff are scarce. In the eastern United States, high concentrations of toxic lead were reported in tadpoles exposed to highway runoff (Birdsall et al., 1986). Polycyclic aromatic hydrocarbons (PAHs), a common constituent of road runoff, in combination with the sunlight UV-A radiation were extremely toxic for tadpoles (Blaustein and Kiesecker, 2002). The current study attempts to quantify, for the first time, the biological effect of road runoff on aquatic vertebrates in Israel.

2. Materials and methods

2.1. Sites description

We sampled road runoff water from two high-traffic road sections during the 2005–2006 rainy season: Highway 4, between Geha and Morasha interchanges on the central coast of Israel (HW4); and Highway 70, between the Fureidis junction and the Bat Shlomo interchange on the northern coast (HW70). Road structure was similar at the two sites, made up of asphalt surface with concrete shoulders and storm-drains. HW4 was characterized by higher daily traffic flow (81 200 cars/day compared with 43 500 cars/day on HW70, according to the Israel National Road Company data). In addition, HW4 had a larger drainage area and mixed industrial, residential, and agricultural land use adjacent to the road, all potentially contributing greater runoff pollution compared with the HW70 site (rural mixed with agricultural land use). Both sites have a Mediterranean climate characterized by a short, cool-wet winter and a long, hot-dry summer (Table 1, Israel New Atlas, 1995).

2.2. Road runoff sampling

We sampled each road twice, at the beginning and again in the mid-rainy season. We collected the first seasonal road runoff in October 2005 at the beginning of the first seasonal rain (precipitation volume of 2 mm or more, after at least five dry months). We collected the midseason runoff, during December 2005, after multiple rains had washed the road surface (about 20 days of at least 1 mm of rain according to the Israeli meteorological service data) and a few hours after the rain event had started.

The runoff was collected in polypropylene containers (18 L) placed at the bottom of storm-drains on both sides of the road. We collected a total of ~234 L of first runoff and ~117 L of midseason runoff at each site. The containers had a float element made of PVC pipe and a polystyrene ball that sealed them once they were filled and prevented the inflow of additional water, thus ensuring the collection of the very first road runoff only.

We took a sample for chemical analyses of heavy metals and major ions (described in Section 2.3). The sample was kept in a refrigerator at 4 °C until analyzed. We then transferred the remaining collected water into clean polypropylene bottles (1.5 L) and stored them under −18 °C within 24 h. Before each test we thawed the necessary volume and kept it in a refrigerator at 4 °C for no longer than two weeks (preliminary examination found similar metal contents prior to and subsequently to freezing the runoff in the described bottles).

2.3. Chemical analyses

Chemical analyses were performed by the electrochemistry laboratory at Tel-Aviv University. Total metal concentration as well as soluble metals and metals in the sediment of road runoff (copper, lead, cadmium and nickel) were determined by voltammetric methods (ASV and AdSV) with an accuracy of about 100 ppt (0.1 μg/L) (Bonfil and Kirowa-Eisner, 2002). Soluble metal concentrations in water fraction of runoff were examined after filtering with a 0.45 μm mesh filter. All samples were acidified prior to quantification of metals using HNO_3/H_2SO_4. The concentrations of cations (Ca^{+2} and Mg^{+2}) were determined using potentiometric titration, with EDTA and EGTA as titrate agents. Concentrations of anions (F^-, Cl^-, Br^-, NO_3^-, and SO_4^{-2}) were determined using ion chromatography (Dionex Ion Chromatograph DX 500 with IONPACK Analytical column AS4A-SC). (For full description of analytical methods see Rechtman, 2006.)

Table 1
Precipitation data of the beginning of the rainy season in the Mediterranean coast of Israel between the years 1981 and 2000 (Israel Meteorological Service, IMS, 2009).

Month	Mean monthly precipitation (mm)/Mean number of rainy days (>1 mm)			
	September	October	November	December
Haifa	1.2/0.2	28/2.8	77.4/6.3	135.5/10.1
Tel-Aviv	0.4/0.1	26.3/2.4	79.3/6	126.4/9

2.4. Embryo production

Adult *B. viridis* were collected from a pristine pool during the breeding season of winter 2005. Mating of adults was allowed in breeding containers made of polyethylene filled with aged tap water (500 L, 3–5 cm depth) that were kept outdoors (see Supplementary data, Pic. 1). We collected the egg strands of two pairs of adults during the morning after mating occurred and immediately removed their gelatinous coats using Cysteine solution (2% L-Cysteine Hydrochloride) and verified their embryonic developmental stage using a dissecting microscope (Zeiss Stemi 2000-C) (see Supplementary data, Pic. 2). All cleaving embryos (stages 8 and 9, Gosner, 1960 [8–9GS]) were pooled from each pair in a sterilized container, mixed gently, and randomly distributed into five replicates of 10 embryos each (50 embryos in total) to each of the experimental setups. Thus all tested embryos were of a common parentage in each of the experiments (below).

2.5. Experimental design

We assessed the effects of road runoff on development and survival of *B. viridis* embryos and tadpoles throughout their metamorphosis. The experimental system included two media sets ($HW4_x$ and $HW70_x$, representing water collected from Highway 4 and Highway 70, respectively): seasonal first runoff ($HW4_{1st}$, $HW70_{1st}$); midseason runoff ($HW4_m$, $HW70_m$); synthetic solutions of metals (Cu, Pb and Ni) in similar concentrations to the first runoff ($HW4_s$, $HW70_s$); and a Modified Marc's Ringer (MMR) solution (see Section 2.6) as a control ($HW4_c$, $HW70_c$, Table 2). Larvae were exposed to all media simultaneously in all experiments. Media were used in a static-renewal fashion in experiments A and B (below). In Experiment C, due to the large volumes needed, tap water was used instead of MMR (in the synthetic and control media), and media were not renewed, but purified water (18.3 MΩ cm) was added to compensate for the water lost in evaporation.

We examined three levels of ontogenetic development, using three sequential experiments, respectively:

A Days 1–12: embryonic and young tadpoles until the transition to feeding and free-swimming tadpoles (8–25GS).
B Days 13–37: from the free-swimming pre-metamorph tadpoles through the earlier stages of the metamorphosis, until full elongation of the hind limb bud (26–30GS).
C Days 38–65: from early metamorphosis through later stages of metamorphosis until transformation of young metamorphs (31–46GS).

The duration of each test was determined according to the number of days needed to reach an average larval stage in a given treatment. Tests were performed in a sequential manner, thus, larvae were exposed to the same conditions throughout their entire development (65 days) under the three experimental stages.

In all experiments, four parameters for the effects of runoff were assessed: survival (proportion), body length (cm ±0.5 mm), stage of development (days to reach a given GS) and morphological deformities (proportion).

Simultaneously, we performed a second set of Experiment A using embryos produced by different parental couples to confirm that results are not biased by genetic source of the parents. These tadpoles were discarded at the end of the experiment.

2.6. Bioassay conditions: experiment solutions

We used 0.3 MMR (Modified Marc's Ringer, Peng, 1991), an embryo rearing solution, as a standard medium for the synthetic solutions and the control. The hardness of solutions was adjusted and metals were added in equivalent concentrations to those found in the soluble fraction of the first road runoff. Metal solutions were made up from copper chloride ($CuCl_2$), lead and nickel nitrates [$Pb(NO_3)_2$ and $Ni(NO_3)_2$, respectively] (Table 2). No metals were added to the control solution. All solutions were made of analytical grade chemical salts and ultra purified water (18.3 MΩ cm) (Barnstead EASYpure™RF).

2.7. Bioassay conditions: experiment criteria

Dead tadpoles, identified by their unresponsiveness to tactile stimuli, were recorded and removed daily. The media were renewed every two days at the time when the living tadpoles were scored. We selected a random sample of seven tadpoles from each replicate and recorded the above parameters (Section 2.5). Morphological deformities included aberrant development of the eyes and mouth parts, defective or broken tail and edemas. A tadpole exhibiting one of the above

Table 2
Soluble metal concentrations and Hardness parameters of first runoff ($HW4_{1st}$, $HW70_{1st}$), midseason runoff ($HW4_m$, $HW70_m$), synthetic solutions ($HW4_s$, $HW70_s$), and control media ($HW4_c$, $HW70_c$).

Medium	Metal concentration[a] (ppb, mean ± standard deviation)			Hardness parameters (mM)			
	Cu	Pb	Ni	Ca^{+2}	Mg^{+2}	HCO_3	Hardness[b] (mg/L)
$HW4_{1st}$	136.8 ± 67.4	26.5 ± 23.9	18.75 ± 2.3	0.65	0.1	1.9	75
$HW70_{1st}$	39.6 ± 2.2	4.1 ± 2.8	21.6 ± 25.3	1.5	0	1.9	150
$HW4_m$	9.4 ± 3.6	1.7 ± 1.2	29.45 ± 34	0.33	0	0.8	33
$HW70_m$	11 ± 0	3 ± 2.3	24.9 ± 31.6	0.4	0	0.65	40
$HW4_s$	180	34	17	0.65	0.1	1.9	75
$HW70_s$	25	3.3	27	1.5	0	1.9	150
$HW4_c$	0	0	0	0.65	0.1	1.9	75
$HW70_c$	0	0	0	1.5	0	1.9	150

[a] Values represent an average of several analyses of different samples except for HW4s and HW70s (Cadmium [Cd] concentrations were ∼0 ppb in all tested media).
[b] Hardness (mg/L) = [Ca^{+2} (mM) + Mg^{+2} (mM)] × $CaHCO_3$ molecular weight (=100.09) × 1000.

defects was scored as deformed. Sterile pipettes were used to transfer tadpoles between containers that were cleaned in advance using acid wash (10–15% HCl solution). At the time of media replacement, we also controlled the following abiotic parameters: water and air temperature (20 ± 1 °C), dissolved oxygen (>4 mg/L) and pH of the medium (7–8.5 units). Tadpoles of 25GS and higher were fed daily with a frozen commercial food for fish, based on algae (Spirulina Formula by Ocean Spray Ltd). The amount of food needed for development was evaluated and provided according to the number of tadpoles in each replicate and their mean developmental stage.

2.8. Bioassay conditions: experiments description

Experiment A of embryos and young tadpoles (days 1–12) had 10 replicates for each treatment, starting with 50 embryos in each replicate. Embryos were kept in 90 mm plastic Petri dishes filled with 40 ml of rearing medium. The dishes were placed randomly in an incubator with L_{12}:D_{12} photoperiod conditions.

Experiment B included pre-metamorphs (days 13–37) and had 10 replicates. Twelve tadpoles were chosen randomly from each replicate of Experiment A, discarding the deformed tadpoles. Each new replicate was kept in a 0.9 L polypropylene container filled with 0.25 L of rearing medium and maintained at the above temperature and photoperiod in an air conditioned controlled room.

Experiment C included young metamorphs (days 38–65) and had four replicates, 5–12 individuals in each. Individuals were randomly chosen from replicates of Experiment B and kept under natural outdoor conditions (natural temperature and photoperiod conditions) within 40 L polyethylene containers filled with 10 L rearing medium. Media effects were scored once a week using a random sample of five individuals from each replicate.

2.9. Evaluation of organic level in the highway runoff

Since large amounts of solid particles accumulated as sediment in the first flush treatments, we measured the organic level of the runoff and experiment media as this could provide a possible food source for the tadpoles. The organic level for all experiment media was measured using a Total Organic Carbon (TOC) analysis.

2.10. Data analysis

Road runoff effects on embryos and tadpoles were compared among treatments using one way ANOVA or Kruskal–Wallis tests (when data were not normally distributed according to Shapiro–Wilk and Kolmogorov–Smirnov tests). Significance of differences between treatments was identified using Post-Hoc Bonferroni multiple comparisons. In all tests, a statistical significance level of $p < 0.05$ was chosen.

To compare differences of young tadpoles body length, we calculated in Experiment A the mean difference between each treatment and the control. In addition, we used a second power transformation (X^2) of the data obtained from the HW4 bioassay on Day 12 to achieve normal distribution.

3. Results

3.1. Survival

The exposure to road runoff and synthetic metal solutions did not affect the survival of the toad embryos and tadpoles, despite the relatively high metal concentrations in the first flush, reaching up to 400 μg/L (including the metals in the sediment; see also in Section 4.3). Survival of young tadpoles was not affected in Experiment A (average ± s.e. of 97–99 ± 0.7–1% in HW4 treatments [including the control] and 96–98 ± 0.5–3% in HW70 treatments; an exception was survival of 31 ± 3.5% recorded on $HW70_{1st}$ on Day 11 of the exposure). Survival moderately decreased during Experiment B, to a similar extent across all treatments (22.5–42.5 ± 12–25% in HW4 treatments; 11–44 ± 10.4–18.3% in HW70 treatments). Young metamorphs in Experiment C survived all treatments (94–100 ± 0–1.6%) except for those in $HW70_s$ that showed decreased survival rate from Day 41, ending with 37 ± 10.3% alive at the end of the experiment (Fig. 1).

3.2. Body length

HW4 and HW70 bioassays showed similar effects of runoff on tadpole length. The body length of young tadpoles (Experiment A) exposed to the first runoff treatments ($HW4_{1st}$, $HW70_{1st}$) and control ($HW4_c$, $HW70_c$) were similar while tadpoles exposed to the midseason runoff ($HW4_m$, $HW70_m$) and to the synthetic solutions ($HW4_s$, $HW70_s$) showed a significant reduction in body length beginning Day 7 of the exposure (HW4: $F_{2,27} = 4.47$, $p < 0.05$; HW70: $\chi^2_2 = 18.126$, $p < 0.001$; Fig. 2). On Day 12, tadpoles exposed to $HW4_m$ and $HW4_s$ were 2 ± 0.2 mm and 2.5 ± 0.1 mm shorter

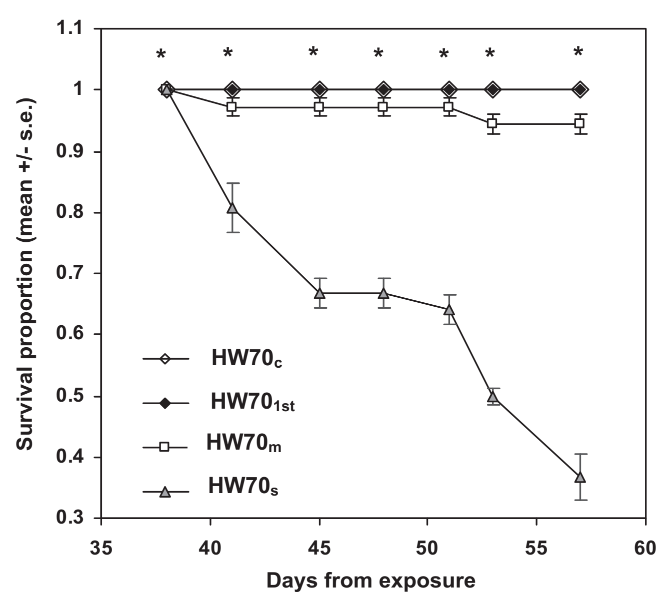

Fig. 1. Survival proportions (mean ± s.e.) of young metamorphs treated with first runoff ($HW70_{1st}$), midseason runoff ($HW70_m$), synthetic solution of metals with equivalent composition to the first road runoff ($HW70_s$), and control ($HW70_c$, $n = 4$) in highway 70 bioassay. *$p < 0.05$.

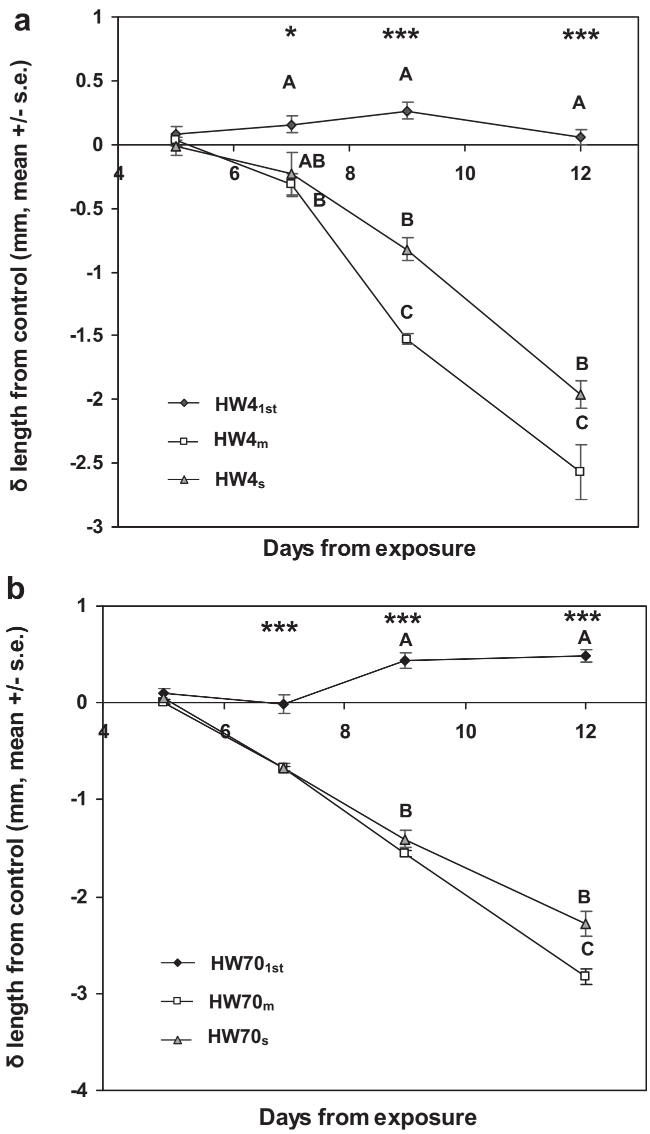

Fig. 2. Deviations from control of young tadpoles' body length (mean ± s.e.) treated with first runoff ($HW4_{1st}$, $HW70_{1st}$), midseason runoff ($HW4_m$, $HW70_m$), synthetic solution of metals with equivalent composition to the first road runoff ($HW4_s$, $HW70_s$) ($n = 10$) in highway 4 (a) and highway 70 (b) bioassays. $^{**}p < 0.01$, $^{***}p < 0.001$. A,B,C denote statistically significant difference between averages according to Post-Hoc Bonferroni multiple comparisons test.

than the control tadpoles' mean length, respectively ($F_{2,27} = 48.09$, $p < 0.001$, Fig. 2a), corresponding to 17% and 21.5% from the control tadpoles' total length, respectively. Tadpoles exposed to $HW70_m$ and $HW70_s$ were 2.8 ± 0.08 mm (24%) and 2.3 ± 0.1 mm (20%) shorter than the control tadpoles, respectively ($F_{2,27} = 346.273$, $p < 0.001$, Fig. 2b).

Pre-metamorphs (Experiment B) were up to 3 ± 0.3 mm longer in $HW4_{1st}$ and up to 3.4 ± 0.3 mm longer in $HW70_{1st}$ compared to all other treatments (Post-Hoc Bonferroni multiple comparisons, $p < 0.001$). Control tadpoles were significantly longer than those of $HW4_m$ and $HW70_m$, and $HW4_s$ and $HW70_s$ at the beginning of the experiment (up to 2 ± 0.3 mm and 2.4 ± 0.25 mm longer, respectively, Post-Hoc Bonferroni multiple comparisons, $p < 0.001$) but length differences between these treatments were reduced in the course of the experiment and similar length was recorded by Day 23 in both bioassays for control, midseason, and synthetic solution treatments.

Young metamorphs (Experiment C) of all treatments had similar body length, except for those exposed to the $HW70_s$ that were significantly shorter. Average growing rate of these tadpoles was only 1 mm in 7 days, compared with 4.5 mm in the control, and 14.5 ± 1.2 mm shorter than other tadpoles by the end of the experiment.

3.3. Metamorphosis rate

Tadpoles of all treatments, except for $HW70_s$, completed their development during Experiment A by becoming free-swimming, self-feeding tadpoles (25GS). Tadpoles of first runoff and control treatments exhibited increased rate of metamorphosis beginning on Day 9 (HW4: $\chi^2_3 = 32.5$, $p < 0.001$, HW70: $\chi^2_3 = 32.48$, $p < 0.001$) and consequently completed their development at least one day before the tadpoles of other treatments.

By the end of Experiment A, the developmental stage of $HW70_s$ tadpoles was significantly lower than that of tadpoles of all other treatments (25.75 ± 0.1 compared to 26.4 ± 0.07 mean GS in $HW70_s$ and in $HW70_c$, respectively, $\chi^2_3 = 22.292$, $p < 0.001$).

Developmental rates of pre-metamorphs in Experiment B and young metamorphs in Experiment C were similar in all treatments (and also within treatments, standard error <0.15 mean GS in all treatments) except for $HW70_s$ tadpoles. The development of these tadpoles ceased at 25GS. On Day 25 in Experiment B, the mean GS of $HW70_s$ tadpoles was 25.61 ± 0.07 while that of $HW70_c$ was 29.4 ± 0.07 ($\chi^2_3 = 25.938$, $p < 0.001$). Higher though more variable development rate was recorded among surviving $HW70_s$ metamorphs in Experiment C. At the end of this experiment the mean GS of $HW70_s$ tadpoles was 39.5 ± 1.98 while that of $HW70_c$ was 45.6 ± 0.3 ($\chi^2_3 = 8.194$, $p = 0.042$).

3.4. Morphological deformities

$HW4_s$ and $HW70_s$ tadpoles had noticeable deformities (mainly tail deformities and edemas) beginning on Day 7 from the exposure in Experiments A and throughout Experiment B ($HW4_s$: $\chi^2_3 = 8.565$, $p < 0.05$, Fig. 3a rowsep="1"; $HW70_s$: $\chi^2_3 = 28.826$, $p < 0.001$, Fig. 3b). Control tadpoles exhibited normal deformity rate of 0–4% throughout the experiment (see Supplementary data, Pic. 3).

On Day 9 significant increase in the incidence of deformities was recorded among $HW4_m$ and $HW70_m$ tadpoles due to deformed and/or late eye development (100% in $HW4_m$, $\chi^2_3 = 35.747$, $p < 0.001$; 90 ± 1% in $HW70_m$, $\chi^2_3 = 31.272$, $p < 0.001$, Fig. 3a,b). These deformities, however, were transient (short-term) (Fig. 3a,b). Similar eye deformities were recorded in $HW4_s$ and $HW70_s$ tadpoles. $HW4_s$ tadpoles recovered from their deformities starting Day 19 and by Day 21 exhibited no significant difference in deformity rates (Day 21: 6.8 ± 3% in $HW4_s$ compared to 2 ± 1.2% in $HW4_c$, $\chi^2_3 = 6.195$, $p = 0.103$, Fig. 3a).

Tadpoles of the midseason runoff and synthetic solutions exhibited deformities of the tail (in severe cases tails were amputated) and mouth parts, resulting in awkward, inefficient movement and decreased utilization of food provided.

The extreme toxic effect of $HW70_s$ was found also in Experiment C. Incidence of deformities of young metamorphs in this treatment was 100% throughout the most of the experiment, while the incidence of deformities decreased to control levels (~0%) in all other treatments (Fig. 4). Deformity types in Experiment C were different from those exhibited in the former experiments. While development of the eyes and absorbance of the larvae's tail were successfully complete by this developmental stage, deformed front and/or hind limbs of the young metamorphs were discernable in the form of limbs turned with their ventral side upwards (see Supplementary data, Pic. 4).

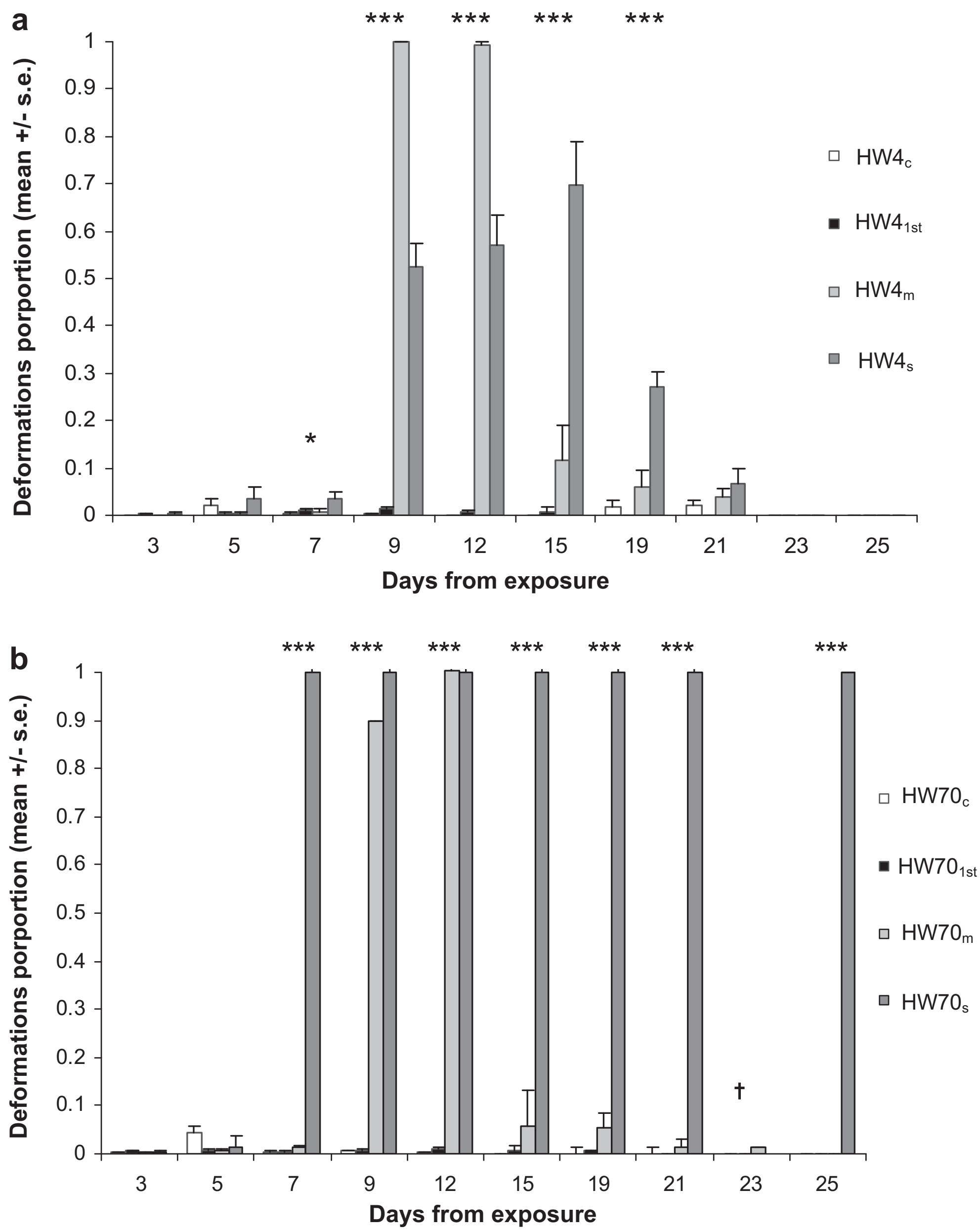

Fig. 3. Morphological deformities proportion (mean ± s.e.) of tadpoles treated with first runoff ($HW4_{1st}$, $HW70_{1st}$), midseason runoff ($HW4_m$, $HW70_m$), synthetic solution of metals with equivalent composition to the first road runoff ($HW4_s$, $HW70_s$), and control ($HW4_c$, $HW70_c$, $n = 10$) in highway 4 (a) and highway 70 (b) bioassays. *$p < 0.05$, ***$p < 0.001$. †HW70s data are missing.

A second set of Experiment A, performed concurrently on embryos of different genetic source, showed similar results to the above. Larvae treated with first road runoff and control media were longer, developed faster, and had normal deformity rate, compared to larvae treated with midseason runoff or with synthetic solutions, which showed elevated rates of body, tail, and eye deformities, as indicated above (for detailed results see Dorchin, 2007).

3.5. *Evaluation of organic level in the highway runoff*

Total Organic Carbon (TOC) analysis revealed relatively high levels amounting to 336 mg/L and 127.6 mg/L in $HW4_{1st}$ and $HW70_{1st}$, respectively, compared with undetected levels in all other media.

4. Discussion

4.1. *Effects of road runoff on B. viridis*

Highway runoff collected from two different roads had negative effects on the development of *B. viridis* embryos and larvae, even though survival remained unaffected. Many studies pointed to direct relationships between the antecedent dry period (ADP) of a storm event and the concentrations of soluble pollutants in subsequent road runoff (Barbosa and Hvitved-Jacobsen, 1999; Barrett et al., 1995, 1998; Drapper et al., 2000; Kayhanian and Borroum, 2000; Lee et al., 2004; Li et al., 2005; Mangani et al., 2005). Unexpectedly, we found that midseason runoff, collected in the middle of the flush event, after the road had been initially washed, resulted in decreased developmental rates and higher

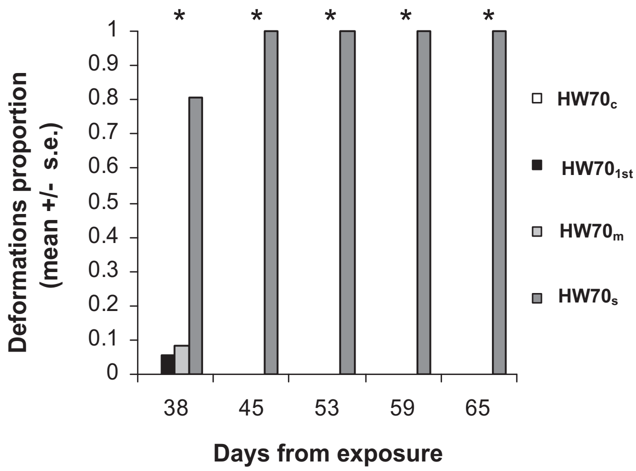

Fig. 4. Morphological deformities proportion (mean ± s.e.) of young metamorphs treated with first runoff ($HW70_{1st}$), midseason runoff ($HW70_m$), synthetic solution of metals with equivalent composition to the first road runoff ($HW70_s$) and control ($HW70_c$, $n = 4$) in highway 70 bioassay. $^*p < 0.05$.

morphological deformities of tadpoles (Figs. 2 and 3). This is the first evidence showing that midseason runoff is potentially harmful to aquatic vertebrates. Although bioassays usually show higher toxicity potential of seasonal first flushes (Kayhanian and Borroum, 2000), or of heavily polluted road runoff (Beasley and Kneale, 2002; Buckler and Granato, 1999; Maltby et al., 1995), midseason runoff may have greater environmental impact considering the numerous storm events that occur during the rainy season, as opposed to the single event of the seasonal first flush.

4.2. Effects of synthetic solutions on *B. viridis*

In our experiments, the increase in deformity incidence among tadpoles reared on the synthetic solutions, having first flush equivalent metals concentrations, indicates a potential toxicity to aquatic organisms of these runoff. Similarly, exposure to copper (Cu) and lead (Pb) in comparable concentrations to the defined road runoff concentration range (<160 μg/L, Table 2) appears to have no influence on survival of amphibians embryos and tadpoles, but may have deleterious effects on time-related variables, such as development rate (Chen et al., 2006; Haywood et al., 2004; Lefcort et al., 1998; Parris and Baud, 2004). However, studies have also showed that combined exposure to different metals can have synergistic effects on mortality of different amphibian larvae (Horne and Dunson, 1995; Lefcort et al., 1998).

4.3. Effects of road "first flushes" on *B. viridis*

The highest metal concentrations were recorded in the first flush, reaching up to 400 μg/L (including metals in the soluble fraction and in the sediment), a concentration that is lethal to amphibian embryos (Haywood et al., 2004). These levels were up to 30 times higher than levels of metals found in the runoff's soluble fraction and in their analogical synthetic solutions (Table 2). Nevertheless, no deleterious effects of the first runoff were observed, while tadpoles in the synthetic solutions did show deformities. We suggest that despite the possible consumption of metals in the sediment, they were not available for the tadpoles as they commonly adsorb to solid particles (Aryal et al., 2005; Barrett et al., 1995; Li et al., 2005). Also, aluminum, cadmium and copper in dissolved organic compounds were found non-toxic to fish and amphibian larvae (Freda et al., 1990). In our study, first flushes had large amounts of solid particles and TOC levels of 336 mg/L and 127.6 mg/L in highways 4 and 70, respectively, compared with undetected levels in all other media. Other variables, such as water hardness and acidity, which may affect availability of metals (Blaustein et al., 2003; Freda et al., 1990; Horne and Dunson, 1995), were controlled throughout the experiments. We also suggest that since tadpoles of the first runoff were feeding regularly on the rich organic sediment they had an additional energy source for growth and development and for regulating the excessive load of metals. Accumulation patterns of metals in tadpoles suggest limited absorption via food uptake (Lefcort et al., 1998). Accumulation of toxic metals was found mainly in the tadpoles' gut (Burger and Snodgrass, 2001; Rice et al., 2001; Sparling and Lowe, 1996), but tadpole feeding data indicate (Rice et al., 2001) that being continual feeders they maintain a continuous rate of food uptake while eliminating pollutants from their gut through defecation.

The different effects that midseason and first flush treatments had on tadpoles may also arise from effects of non-metal toxic chemicals, such as polycyclic aromatic (PAHs) hydrocarbon compounds, a common road runoff pollutant (Beasley and Kneale, 2002; Buckler and Granato, 1999; Van Dolah et al., 2005). PAHs quick volatilization (Barrett et al., 1995) and association with suspended solids (Aryal et al., 2005; Mangani et al., 2005) may provide greater bio-availability of soluble concentrations (Buryskova et al., 2006; Marquis et al., 2006) in the midseason flushes rather than in the first flushes.

An alternative explanation for the midseason runoff effect is the contribution of toxic nickel (Ni). We cannot account for the unusual high Ni concentration found in the midseason runoff (Table 2).

4.4. Effects of $HW70_s$ solution on *B. viridis*

The dramatic effects of the HW70 synthetic solution ($HW70_s$) on tadpoles' development and morphology may be attributed to a lack of magnesium (Mg, Table 2), an essential element in early development of amphibians (Brown and Gurdon, 1964; Garber, 2002). Since we observed lethal effect of $HW70_s$ during later developmental stages (Experiment C, Fig. 1), and since metamorphosis rates were similar in HW70 control treatment (which was identical to $HW70_s$, excluding the metals) and in the HW4 control treatment, we assume that the combined effect of magnesium deficit, metal stress and reduced feeding caused the dramatic $HW70_s$ effects.

4.5. Sublethal effects of road runoff

In agreement with previous studies we show increased susceptibility of larval rather than adult amphibians to pollutants in water (Chen et al., 2006; Freda et al., 1990; Gross et al., 2007; Haywood et al., 2004; Horne and Dunson, 1995). We did not observe reduced survival under road runoff. However, sublethal effects, including reduced growth and development, morphological deformities and behavioral changes can become lethal under natural conditions where competition and predation are major factors for animals' survival (Blaustein and Johnson, 2003; Bridges, 1999; Hayes et al., 2006; Mills and Semlitsch, 2004). Reeves et al. (2008) observed higher abnormality prevalence of Alaskan wood frogs (*Rana sylvatica*) in sites close to roads and concluded that road-associated contaminants may reduce tadpoles' size or fitness thus increasing their risk of predation. Growth and developmental rates are very important life-history traits, especially for species like *B. viridis* which exploit ephemeral pools for breeding and has limited time and resources for development (Wilbur, 1997; Wilbur and Collins, 1973; Semlitsch, 2000; Semlitsch and Wilbur, 1988). In addition, exposure to toxic chemicals may have later external expression, for example, interference in hormonal systems that may hinder reproduction in mature amphibians (Blaustein et al., 2003; Hayes et al., 2006).

4.6. Effects of road runoff on aquatic environments

The pollutant concentrations in the road first runoff were 0.57–7.86 times higher than concentrations in urban first runoff from the city of Ashdod, Israel (Asaf et al., 2004). Despite the apparent threat to aquatic biota, and therefore the relatively high environmental pollution potential from road runoff in Israel, no control measures are currently in use. Elimination and deterioration of aquatic habitats in Israel due to human activity have already caused the apparent global extinction of one amphibian species and the upgrade to endangered status of three temporary pool amphibian species (Blaustein and Schwartz, 2001). A decline in *B. viridis* population size has recently been documented along the coastal plain of Israel (Elron et al., 2005). This species has consequently been declared an endangered species (Gafny, 2004). Elron et al. (2005) suggested that decreased habitat availability in heavily developed urban areas has lead to exploitation of water bodies of limited suitability for the toad reproduction. However, no acute effect of pollutants on habitats' water quality has been identified (Elron, 2007). We suggest that road runoff pollution can be a major cause for a qualitative decrease of temporary pools used by amphibians, especially in heavily developed urban areas. We recommend an immediate implementation of control actions for road runoff in roads located near water bodies. This is especially important in Israel and in other arid and semiarid countries where water resources are scarce.

5. Conclusions

Road runoff collected from major highways in Israel during two consecutive rainy seasons showed detrimental temporal effects on the development of *B. viridis* embryos and tadpoles in laboratory toxicity tests. While typically higher concentrations of heavy metals were found in the "first flush", midseason runoff, collected from the same roads, had greater adverse effect on the toads, and are therefore of higher environmental threat. Nevertheless, experiments with synthetic solutions of metals indicated a potential toxicity of road first flushes to aquatic organisms. Recent studies identified roads as a major source of pollution for aquatic habitats (Beasley and Kneale, 2002; Reeves et al., 2008). Additional laboratory and field testings are therefore necessary to determine the possible threat of road runoff pollution to amphibian populations and to aquatic environments in general.

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Appendix. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.envpol.2010.08.004.

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