Environmental characterization of surface runoff from three highway sites in Southern Ontario, Canada: 2. Toxicology

ABSTRACT
Highway runoff is a significant source of contaminants entering many freshwater systems. A battery of bioassays was used to assess the degree of runoff toxicity. The toxicity results are described in the spatial and temporal context and are linked to the runoff chemistry. Runoff samples from three sites, representing different classes of highways (high, intermediate and low traffic intensity), were used to assess the degree of runoff toxicity. Runoff from the major multilane divided highway, with the highest traffic intensity (92,000 vehicles/24 h), had the highest levels of contaminants and displayed the greatest toxicity. Variations in toxic responses were observed both seasonally and throughout runoff events. The runoff samples containing high concentrations of road salts from winter maintenance were acutely toxic to Daphnia magna. In general, a sharp decline in runoff toxicity over time showed that the ‘first flush’ was the most toxic. Road solids present in runoff showed moderate to severe toxicity using a nematode bioassay. Consistently, a significant mixed function oxidase (MFO) induction was observed in rainbow trout exposed to runoff with high concentrations of polycyclic aromatic hydrocarbons (PAHs). The data show that vehicular operation, road maintenance and metal highway structures were significant contributors to contaminant-associated toxicity in road runoff.

Key words | acute toxicity, bioassays, chronic toxicity, ‘first flush’, highway runoff, toxicity tests

INTRODUCTION
Highway runoff has been identified as a significant contributor of non-point source pollution containing a broad variety of contaminants, which enter receiving waters and add to loads from other sources. Many of these substances, which originate from the operation of automobiles, corrosion of highway structures and maintenance of highways, are known to occur at levels that can be toxic to freshwater organisms. Increased emphasis on controlling the non-point sources of pollution has intensified the need to discern the toxicological effects of complex mixtures, such as highway runoff.

A broad variety of compounds in highway runoff make the assessment of toxicity and identification of potential toxicants complicated. In a study of road runoff in England, Maltby et al. (1995) used toxicity identification evaluation (TIE) procedures to isolate and identify potential toxicants. A similar approach was used in a recent study of highway runoff by Kayhanian et al. (2008) in an urban area of Los Angeles, CA, USA. The TIE approach is frequently used to identify the toxicity of risk-causing chemicals in complex mixtures (Ankley & Schubauer-Berigan 1994; Carr et al. 2001; Custer et al. 2006). The TIE procedures utilize toxicity-based manipulation schemes (USEPA 1991a, b, 1996) to characterize, identify and confirm substances responsible for sample toxicity (Ankley & Schubauer-Berigan 1995). These procedures, however, have

some limitations because of their effectiveness and resulting uncertainty (some toxicity remains typically after each treatment, Science Applications International Corporation 2002). Due to the excessive chemical manipulations, TIEs are unlikely realistically to assess true field conditions (Burton & Nordstrom 2004a, b). Moreover, the TIE procedures are expensive and time consuming and not suitable for a large number of samples. These issues contributed to their slow adoption in ecological risk assessment. The present study relied on a more traditional combination of chemical analyses and toxicity testing, but employing a battery of toxicity tests (Marsalek et al. 1999b) to help determine chemicals of concern.

A seasonal study of highway runoff was carried out in Southern Ontario, one of the most densely populated areas in Canada, to establish the linkage between the chemistry and toxicity of highway runoff in a cold climate. While the first of the two companion papers (Mayer et al. 2011) addressed the first objective of the study, the highway runoff chemistry, this second companion paper addresses the second and third objectives of the study: (ii) to establish the linkages between the chemistry and toxicity of runoff by relating the toxicity of runoff to its chemical characteristics and (iii) to address the temporal and inter-site variation in highway runoff toxicity.

The chemical composition of runoff from three highway sites characterized by different intensity and type of traffic was compared to the toxic effects observed on various organisms exposed to this runoff in the laboratory. Because different classes of contaminants exert different effects on biota, various organisms and endpoints were used to assess runoff toxicity. Metals, polycyclic aromatic hydrocarbons (PAHs) and road salts are the most abundant contaminants in highway runoff and, hence, the toxicity of runoff was related to the levels of these contaminants.

**MATERIALS AND METHODS**

**Study sites and sample collection**

Three sites, each located on a highway with different traffic characteristics, were selected for the study. The sites differed in traffic intensity (Mayer et al. 2011) and the type/composition of vehicular traffic. Two of the study sites were located on major provincial highways, the Queen Elizabeth Way (QEW) and Highway #2 (Hwy 2), which at the time of the sample collection (1996–1998) were the major transportation routes in Southern Ontario. The QEW is a multilane divided highway with four lanes in each direction and, at the study site, had the highest intensity of traffic (92,000 vehicles/day) and the greatest number of trucks (Mayer et al. 2011). Hwy 2 with two lanes in each direction was at the time of our sampling a major arterial road between Hamilton and Brantford, Ontario, with a traffic intensity of 31,100 vehicles/day. The third site, with a traffic intensity of 15,460 vehicles/day, was located on an urban/residential road with two lanes in each direction (Plains Road) in Burlington, Ontario, a city with a population of 150,000. While the exact breakdown between the trucks and passenger vehicles was not available for the studied highways, the major highway sites were characterized by a substantially heavier volume of truck traffic than the site from the urban/residential road. The sampling site on the QEW was located at the north foot of the James N. Allen Burlington Bay Skyway Bridge (referred to herein as Skyway Bridge) and is denoted here as the SW site. The sampling site on Hwy 2 was located on the bridge over the Fairchild Creek (just east of Brantford) and is referred to further as the FCC site. Lastly, the sampling site in Burlington was situated on the bridge near the Royal Botanical Gardens parking lot and is referred to further as the RBG site. Further details about the sampling sites are provided in Mayer et al. (2011).

As reported in the companion article, the study was conducted over a period of 24 months (1996–1998) encompassing all seasons. At each site, samples were collected by diverting flow from the bridge deck drains into large (200-L) plastic barrels. The runoff samples were taken from the barrels immediately after the individual events and sub-samples were split for chemical and toxicological analysis. Runoff samples used for toxicity testing were stored in 20-L stainless steel containers or 4-L glass bottles at 4 °C until the biotesting began (within 48 h of sample collection). Road solids used for toxicity tests were collected from the bottom of the runoff barrels after emptying the barrels. These sub-samples were left to settle for approximately 12 h at 4 °C, and then the aqueous phase was decanted.
In total, 47 samples were collected and analysed. Not all samples collected were analysed for all runoff constituents nor used for all toxicity tests (Table 1). For intra-event sampling, the time at the end of each collection interval during the time series was used in the charts to present the results of the toxicity tests. During the study, wetter-than-normal conditions were experienced; this reduced the antecedent build-up period to 3 days or fewer. Even small events (e.g. 2 mm) produced runoff from these well-drained bridge sites.

Bioassays

The toxicity of runoff was assessed using a battery of tests, which evaluated different endpoints in both vertebrates and invertebrates. Each test was selected to relate the biological effects of runoff to specific classes of toxicants. *Daphnia magna* (water flea) static non-renewal toxicity tests were used to screen samples for acute toxicity. Replicates of three were run for each exposure concentration. Each test was selected to relate the biological effects of runoff to specific classes of toxicants.

*Daphnia magna* (water flea) static non-renewal toxicity tests were used to screen samples for acute toxicity. Replicates of three were run for each exposure concentration. In these tests, *D. magna* less than 24 h old were introduced to test solutions. Ten neonates were used per test cup in 15 mL of test solution. The tests were carried out at 22°C with 16/8 h light/dark photoperiods. After 48 h, mortalities were recorded for undiluted effluent. Where the runoff toxicity was very high, a dilution series was performed with de-chlorinated municipal tap water (purged by air) and an EC50 (the runoff dilution at which 50% mortality to *D. magna* occurred) was reported (Dutka 1988; Marsalek et al. 1999b).

Sub-mitochondrial particle (SMP) assays, forward or conventional electron transport (SMP-CET) and reverse electron transport (SMP-RET) were also used to detect bioavailable toxicants in some samples (Dutka 1988; Rochfort et al. 1997). This procedure has been successfully used to determine different classes of chemicals (Argese et al. 1998) and has proved useful in relating the physico-chemical properties of toxicants to their biological activities (Blondin et al. 1987; Knobeloch et al. 1990). The procedure utilizes beef heart bioparticles and is based on the ability of these particles to carry out the enzymatic processes of electron transport and oxidative phosphorylation. The conventional electron transport involves forward movement of electrons, which converts the reduced nicotinamide adenine dinucleotide (NADH) into the oxidized nicotinamide adenine dinucleotide (NAD) complex. The CET is the direction of normal flow of electrons through these enzymes during cellular respiration. The conversion of NADH to NAD (ratio of reduced/oxidized NAD) was

<table>
<thead>
<tr>
<th>Sampling site</th>
<th>Date</th>
<th>Type of toxicity test</th>
<th>Objectives to be determined</th>
</tr>
</thead>
<tbody>
<tr>
<td>SW</td>
<td>Six dates shown in Table 2</td>
<td><em>C. dubia, D. magna</em> mortality and reproduction, trout mortality, EROD activity, Solid Phase Microtox™</td>
<td>Temporal differences</td>
</tr>
<tr>
<td></td>
<td>17 September</td>
<td><em>C. dubia</em> mortality and reproduction, trout mortality, EROD activity, Solid Phase Microtox™</td>
<td>Intra-event differences</td>
</tr>
<tr>
<td></td>
<td>22 September</td>
<td>Whole runoff Microtox™, SMP test</td>
<td>Intra-event differences</td>
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<tr>
<td></td>
<td>20 June</td>
<td>EROD activity</td>
<td>Temporal differences</td>
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<tr>
<td></td>
<td>17 July</td>
<td>EROD activity</td>
<td>Temporal differences</td>
</tr>
<tr>
<td></td>
<td>2 December</td>
<td>EROD activity</td>
<td>Temporal differences</td>
</tr>
<tr>
<td>SW</td>
<td>8 August</td>
<td><em>C. dubia</em> reproduction</td>
<td>Spatial differences</td>
</tr>
<tr>
<td>FCC</td>
<td>8 August</td>
<td><em>C. dubia</em> reproduction</td>
<td></td>
</tr>
<tr>
<td>RBG</td>
<td>8 August</td>
<td><em>C. dubia</em> reproduction</td>
<td></td>
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<tr>
<td>SW</td>
<td>27 September</td>
<td><em>C. dubia</em> reproduction</td>
<td></td>
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<tr>
<td>FCC</td>
<td>27 September</td>
<td><em>C. dubia</em> reproduction</td>
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<td>RBG</td>
<td>27 September</td>
<td><em>C. dubia</em> reproduction</td>
<td></td>
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<tr>
<td>SW</td>
<td>21 July</td>
<td>EROD activity</td>
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<td>FCC</td>
<td>21 July</td>
<td>EROD activity</td>
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</tr>
<tr>
<td>RBG</td>
<td>21 July</td>
<td>EROD activity</td>
<td></td>
</tr>
</tbody>
</table>
measured spectrophotometrically at 340 nm and is reported here as % activity. Increased activity of the bioparticles (in either reverse or normal electron flow) is an indication of less toxic runoff.

The commercially available standard toxicity bioassay, Microtox™ test, based on the reduction of bioluminescence of the marine bacterium Vibrio fischeri, was also used to assess the toxicity of runoff. In this test the re-hydrated bacteria are incubated at 15 °C with various dilutions of the sample (prepared using a Microtox™ diluent solution) for 15 and 30 min. The samples were read in a Microtox 500 M reader with computer print out and the concentration (% of whole effluent sample) at which a 50% normalized light loss occurred for a certain exposure time was determined graphically and reported as the EC50 (effective concentration for 50% light loss) of the toxicant ( Dutka 1997). The Microtox™ and the SMP tests were performed on samples concentrated 10 times by flash evaporation, as is commonly done in aquatic toxicity testing in order to compensate for dilution required during many test procedures.

Rainbow trout (Oncorhynchus mykiss) acute toxicity tests were conducted, in which fish were exposed to effluent at various dilutions (25, 50% and undiluted), for 96 h. Three replicates of each exposure concentration were performed, with five fish (1–10 g) per tank. A total of 12 L of test solution were placed in 15 L aquaria for each replicate. Fish survival in the exposed and control tanks was used to determine an LC50 utilizing the trimmed Spearman/Karber method. Rainbow trout exposures were conducted using a static renewal and non-renewal system at 15 °C and 16/8 h light/dark photoperiod with continuous aeration according to the standard protocol ( Parrott et al. 1999). Negative control tanks and all dilutions used tap water de-chlorinated with sodium thiosulfate.

Estimates of sub-lethal toxicity were performed using Ceriodaphnia dubia (water flea) chronic tests and rainbow trout mixed function oxidase (MFO) tests. Ceriodaphnia dubia 7-day reproductive impairment tests were carried out on samples with no or low acute toxicity. A C. dubia neonate less than 24 h old was placed in each test cup. A total of ten 20-mL replicates for each dilution tested were used, and compared against a set of 10 replicate controls. The control and dilution water was tap water that had been de-chlorinated by continuous aeration for at least 4 days. A series of dilutions (100, 50, 25, 12.5 and 6.25%) of the whole test solution was made for several tests from each site. Daily renewals of the test solutions were performed over the 7-day period of the test, at which time the neonates had matured and produced three broods of young ( Rochfort et al. 1998). Just as for the D. magna acute toxicity test, the light regime was 16/8 h light/dark photoperiod, but the exposure temperature was 25 °C. The results are reported as the concentration of whole effluent required to produce a 25% effect on reproduction (IC25) and the concentration of whole effluent at which there was no-observed-effect (NOEC). The IC25s were determined through either the Bootstrap method or, where this was not possible, linear interpolation. The NOEC values were interpolated graphically from the series of dilutions (100, 50, 25, 12.5 and 6.25%) at which there was no significant difference between the control brood size and the brood size for that particular concentration.

Because PAHs are abundant in highway runoff and are potent MFO inducers ( Parrott et al. 1999), we used MFO induction in fish to assess the biological effects of these contaminants. A commonly used standardized MFO enzyme assay, ethoxyresorufin-O-deethylase (EROD), was utilized to measure the activity in rainbow trout livers. Hepatic EROD activity was measured in small rainbow trout (average weight of 2 g) exposed to runoff for 96 h ( Parrott et al. 1999). Exposures were performed at either 100% concentrations or at varying concentrations (e.g. 10, 25, 50 and 100%).

A different experiment was used to determine the optimum time for removal of liver tissue following a pulse exposure. These pulse exposures are more realistic than exposing an organism for 96 h in the concentrated effluent, as the fish are more likely to be subject to a high initial dose, followed by clean water resulting from upstream dilution. With each exposure a negative control (de-chlorinated tap water) and a positive control, 10 μg/L solution of β-naphthoflavone (BNF) were used. The conditions for the laboratory fish exposures were the same as those for the rainbow trout acute toxicity tests outlined earlier. Logarithmic transformations of EROD activities provided homogeneous variability distributions. Multiple general linear models of analysis of variance were used to determine if significant induction had occurred. Systat (Evanston, Illinois, USA) was used for the statistical analyses of data. Where the
induction was significant, Tukey’s test showed which treatments were elevated compared to water controls and showed their probabilities of significance (p values).

Fish caging experiments were carried out to determine in-stream receiving water impacts. Cages with 15 fish (average weight of 5 g) were anchored in pairs upstream and downstream from the road (source of highway runoff) for 8 days. Twenty-four hours after the storm event, cages were removed, the fish were sacrificed and livers and bile taken for analysis.

Toxicity of the runoff solids was determined using the nematode (*Panagrellus redivivus*) test and Microtox™ solid phase test. For the nematode test, solid phase samples were extracted with M9Y (a growth medium) and then incubated for 96 h at 21 °C. Ten replicates with 10 organisms in each replicate were used to determine toxicity. Organism growth stage and survival were then documented and compared against negative control (*McInnis* 1997). A detailed description of the assay protocol can be found in *McInnis* (1997).

The Microtox™ Solid Phase test was performed on the same equipment and used the same principle as the liquid Microtox™ acute test. A small sample of sediment was exposed directly to the bacterium, allowing the test organisms to come into direct contact with the particle bound toxicants in aqueous suspension. After a brief incubation period, the response of the organism was measured, and an EC50 value was determined in a similar way as in the routine Microtox™ test described earlier. These tests are more suitable for identifying the relative differences between samples, as non-toxic (control) sediment for highway runoff cannot be obtained (*Microbics* 1992).

The reproducibility of the bioassays was tested by *Marsalek et al.* (1999b) and was found to be acceptable for water management decisions with the coefficients of variations ranging between 7 and 51%.

**RESULTS**

**Whole runoff toxicity**

Highway runoff is a complex mixture of many chemical constituents. These chemicals may exhibit a wide range of toxic effects, ranging from acute or cellular mortality to chronic (low-level/long-term) toxicity. Furthermore, the complex chemical mixtures may exert synergistic and additive effects, making toxicant identification difficult and toxicity test responses variable (*Rochfort et al.* 1997). A battery of bioassays, both whole organism and *in vitro* bioassays, using different endpoints, were employed to determine the toxicity of samples, as different bioassays are sensitive to different toxicants (*Rokosh et al.* 1997).

*Daphnia magna* and *C. dubia* whole organism tests were used to assess the acute and chronic toxicity of runoff. Both of these organisms were shown to be sensitive to elevated concentrations of salts and metals in runoff (*Novotny & Witte* 1997; *Rokosh et al.* 1997). The results of the toxicity tests are presented in *Table 2*, where EC50 indicates the median effective concentrations resulting in 50% mortality of *D. magna*, IC50 and IC25 indicate inhibiting concentrations (dilutions of the whole effluent) at which 50% and 25% lower reproduction of *C. dubia* occurred and NOEC indicates concentrations at which no effects were observed. The data in *Table 2* show that the *C. dubia* chronic test was more sensitive than the *D. magna* acute test when evaluating toxicity of runoff resulting from road salts. Generally, higher toxicities, and hence lower IC25, EC50 and NOEC values (*Table 2*), were observed with increasing conductivities of samples, corresponding to high concentrations of chloride. Moderate to strong acute and chronic toxicity responses were also generated by runoff samples (*Table 2*) where the analytical data indicated elevated levels of Zn (*Table 2* in *Mayer et al.* 2011).

The changes in acute and chronic toxicity over the course of the runoff event are shown in *C. dubia* survival and reproduction tests (*Figure 1*) using the runoff from the SW site. Each of the samples was tested at 100% concentration. Acute mortality in *C. dubia* was reduced and the number of neonates produced increased over the duration of the storm, indicating a decline in toxicity of runoff during the storm event. The decline in runoff toxicity may likely be again attributed to changes in Zn concentrations (*Figure 1*), as the majority of toxic metals, except for Zn, did not decrease appreciably. The decline in toxicity is consistent with the decreasing concentration of Zn in runoff from 1.01 mg/L Zn at the beginning of the event to 0.17 mg/L Zn at the end of the event. The toxicity tests...
clearly show that survival and reproduction of these organisms are severely impaired by elevated concentrations of road salt constituents and dissolved Zn, with the main sources of Zn being tyre wear and galvanized metal highway structures (USEPA 1985).

Similar toxic response was also observed in exposures of rainbow trout (O. mykiss) to highway runoff. In these exposures, the high initial acute toxicity (96 h rainbow trout acute lethality) of runoff also decreased with the time into the event (Figure 1). It is, however, important to note the differences between the responses in C. dubia and rainbow trout. While there is no longer any mortality to C. dubia after 30 min into the rain event, the toxicity still remains high to rainbow trout, indicating different sensitivities of organisms to toxicants present in the runoff.

To confirm the changes in runoff toxicity within a single runoff event, two additional short-term in vitro bioassays, the Microtox™ and SMP test, were also conducted on samples from the SW site collected on September 17. A good agreement (Figure 2) was observed between the bioassays. The SMP-RET samples tested at 10× concentrations were so acutely toxic that no responses could be measured, and therefore the 1× concentration results were used.

### Table 2 | Temporal changes in runoff toxicity, as indicated by seasonal variation in Daphnia magna and Ceriodaphnia dubia toxicity tests – Skyway Bridge (SW)

<table>
<thead>
<tr>
<th>Date</th>
<th>Conductivity (μS/cm)</th>
<th>Cl mg/L</th>
<th>C. dubia IC25 (%)</th>
<th>IC50 (%)</th>
<th>NOEC (%)</th>
<th>EC50 (%)</th>
<th>Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dec 1</td>
<td>n/a</td>
<td></td>
<td>35</td>
<td>58</td>
<td>12.5</td>
<td>n/a</td>
<td>Rain</td>
</tr>
<tr>
<td>Feb 18</td>
<td>39,000</td>
<td>19,135</td>
<td>L&lt;sup&gt;+&lt;/sup&gt;</td>
<td>L&lt;sup&gt;+&lt;/sup&gt;</td>
<td>&lt;3.12</td>
<td>12.5</td>
<td>Rapid snowmelt</td>
</tr>
<tr>
<td>Apr 7</td>
<td>23,000</td>
<td>10,960</td>
<td>4.5</td>
<td>7</td>
<td>1.75</td>
<td>20</td>
<td>Rain on snow</td>
</tr>
<tr>
<td>Apr 14</td>
<td>1,800</td>
<td>718</td>
<td>65</td>
<td>84</td>
<td>25</td>
<td>&gt;100</td>
<td>Rain</td>
</tr>
<tr>
<td>Apr 17</td>
<td>5,000</td>
<td>2,410</td>
<td>47</td>
<td>58</td>
<td>25</td>
<td>&gt;100</td>
<td>Rain</td>
</tr>
<tr>
<td>Aug 8</td>
<td>390</td>
<td>115</td>
<td>41</td>
<td>62</td>
<td>&lt;25</td>
<td>n/a</td>
<td>Rain</td>
</tr>
</tbody>
</table>

The values in tables show dilutions of whole effluent obtained from interpolation when the effect occurred.

IC25 <40% – indicates severe acute effect.

IC25 <70% – indicates moderate acute effect.

IC50 >100 – indicates non-toxic sample.

L<sup>+</sup> – 48h median lethal concentration, IC50 4.5%.

IC25 – inhibiting concentration of whole stormwater effluent at which 25% lower rate of reproduction of C. dubia occurred.

IC50 – median effective concentration of whole stormwater effluent at which 50% lower reproduction of C. dubia occurred.

EC50 – median effective concentration of whole stormwater effluent at which 50% of mortality in D. magna occurred.

NOEC – No Observed Effect Concentration.

n/a – no sample available.

### Figure 1 | Changes in runoff toxicity during the course of the runoff event as indicated by Time Series, from Skyway Bridge (SW), of Ceriodaphnia dubia survival and reproduction and rainbow trout acute toxicity tests (17 September). The time at the end of each collection interval of the event is used in this graph to present the results of the toxicity tests and is indicated on the x-axis as the sample collection time. Italicized numbers in brackets are the concentrations (mg/L) of dissolved Zn in runoff.

### Figure 2 | Changes in the toxicity of runoff from Skyway Bridge (SW) over the course of the event (22 September) as indicated by the whole runoff Microtox™ and SMP toxicity tests. The time at the end of each collection interval of the event is used in this graph to present the results of the toxicity tests and is indicated on the x-axis as the sample collection time.
SMP-RET was notable in that it was the most sensitive to toxicants in runoff. The measurements obtained from both tests (Figure 2) indicate a decrease in toxicity from the onset of the event, when 58% dilution of the sample resulted in EC50 (as measured by Microtox™), but in 30 min the same effect required 100% concentration. Similarly, a decline in toxicity was observed in the SMP test; however, because the SMP test was more sensitive, the concentrations of runoff producing EC50 towards the end of the event were lower than those obtained from the Microtox™ test. Other bioassays were generally non-indicative after short periods of time.

Toxicological studies (Parrott et al. 1999; Servos et al. 1999) have shown that MFO induction in fish may be used as a sensitive endpoint to measure the exposure of fish to various organic contaminants. Hence, EROD induction in the liver of trout exposed to highway runoff is thought to indicate the effect of PAHs on the hepatic system of rainbow trout. Several laboratory exposures were used to determine if highway runoff caused EROD induction. Initial testing involved runoff from the SW site at various serial dilutions (Figure 3(a), (b) and (c)) from three different rain events. These tests showed strong MFO induction for all dilutions, although there was no significant ($p > 0.972$) difference between the induction at 50 and 100% (Figure 3(a)). Additional experiments with similar dilutions (Figure 3(b)), showed no significant differences between 50, 50 and 100% concentrations, but the 10% dilution produced significantly less ($p < 0.001$) induction than the higher concentrations. All dilutions, however, showed significantly higher induction than the positive control (BNF). The lack of difference between the 30 and 50% dilutions and undiluted runoff may indicate that a maximum level of induction could be obtained at or near the 30% concentration of the runoff solution or that the optimum temporal period necessary for greater induction at this concentration was not met. The last exposure (Figure 3(c)) showed that although the induction was present in undiluted runoff (100%), at 50% dilution the runoff did not induce EROD in the fish livers. The differences in runoff toxicity between the three events may be explained by different concentrations of PAHs in runoff solids, resulting from differences in event characteristics. Runoff with the highest toxicity had the highest concentrations (74.2 mg/kg) of 16 priority PAHs in solids, followed by runoff solids containing 36.6 and 26.6 mg/kg of 16 priority PAHs.

The samples collected for the time series experiment (17 September), discussed previously, were also used to determine if there were changes in induction over the course of the storm event. It was observed that although acute toxicity was reduced over time (Figure 1), there was no notable difference in MFO induction, even though the concentrations of particulate PAHs dropped from 93 to 12 mg/kg. All samples showed very high levels of induction (Figure 4),
suggesting a strong exposure to PAHs and probably an insufficient time interval between the runoff collections to substantially reduce the hepatic response caused by the PAHs.

Strong MFO induction occurred at all three sampling sites (Figure 5). All samples were collected on the same day (hence under similar conditions) and diluted in the lab. Good dose–response relationships were noted in the runoff from the FCC and RBG sites, although the response may not be linear. The runoff at the SW site was acutely toxic at 100%, and this acute toxicity likely caused some inhibition in the production of EROD at the higher exposure concentrations (Figure 5).

An in-laboratory depuration exposure was used to identify the peak time for production of MFO enzymes under pulsed loading conditions. This information was essential for field confirmation studies. As fish in the laboratory had been exposed to SW runoff (a known MFO inducer) for only 2 hours (a typical summer storm duration), it was presumed that a strong induction would probably not occur. The experimental results show that even after a long depuration period, MFO induction was still highly significant (Figure 6). It was also noted that the peak induction occurred between 24 and 48 h after the exposure. This information was then used to determine the optimum time after a rain event for removal of the fish cages exposed in the receiving stream.

The in-stream tests showed that there was very little inducible MFO activity in fish and there were no discernible differences between the upstream and downstream sites. Fish survival was 100% at both sites. The in-stream tests, therefore, demonstrated that dilution and attenuation of highway runoff by the stream flow at this test site was significant.

Toxicity of runoff particulates

Many contaminants (e.g. metals, PAHs) commonly present in highway runoff are known to have a strong affinity for particulates. Hence, highway runoff particulates act as an important transport pathway for contaminants originating from transportation corridors. Therefore, solid phase toxicity testing should be an important component of highway runoff toxicity assessment. This becomes particularly

![Figure 4](https://iwaponline.com/wqrj/article-pdf/46/2/121/379761/121.pdf)

**Figure 4** | Time Series exposures, showing changes in EROD activity in runoff collected from Skyway Bridge (SW) on 17 September. BNF is the positive control solution (10 μg/L) of β-naphthoflavone.

![Figure 5](https://iwaponline.com/wqrj/article-pdf/46/2/121/379761/121.pdf)

**Figure 5** | EROD activities of fish exposed for four days to runoff from different road sites (21 July). BNF is the positive control solution (10 μg/L) of β-naphthoflavone. PAHs were measured only in undiluted runoff (italicized numbers in brackets are total PAH concentrations in runoff solids).

![Figure 6](https://iwaponline.com/wqrj/article-pdf/46/2/121/379761/121.pdf)

**Figure 6** | EROD activity in rainbow trout after a two-hour exposure and different depuration periods. BNF is the positive control solution (10 μg/L) of β-naphthoflavone.
important if trophic transfer of contaminants is concerned. Benthic invertebrates from receiving water bodies can take up bioavailable toxicants from sediments by ingestion of contaminated particles and concentrate them in their tissues. Organisms further up the food chain can then biomagnify the toxicants when they consume these benthic organisms.

Two bioassays, namely the Microtox™ solid phase test and the nematode test, were conducted to assess the toxicity of runoff solids. Solids from runoff samples collected for the time series experiment from the SW site (for the same experiment as the C. dubia and rainbow trout MFO time series) were used to evaluate the quality of solids washed off during different portions of the storm event. The results of the Microtox™ sediment tests performed on these samples were indicative of increasing toxicity over time (Figure 7), a trend that was opposite to that displayed by the toxicity of the aqueous portion of runoff, which rapidly diminishes with time.

Road solids collected from the three locations were also used to carry out the nematode test to assess the effect of traffic intensity on the toxicity of runoff solids. The nematode bioassay provides up to five endpoints including survival, growth, maturation and reproduction. Under extreme conditions the organisms will die or stop growing. Under sub-lethal conditions, the organisms grow poorly and will not reproduce as well as the controls. The test results show the lowest survival rate of nematodes in SW solids, and the highest survival rate at the FCC site (Figure 8), a trend consistent with that of the toxicity of the whole runoff.

The toxicity of sediment in these cases was likely to be slightly underestimated, as solids were collected at each site from the bottom of the runoff barrels after emptying, and some loss of fine suspended sediment may have occurred during this process. The fine particles (clay and silt) would have contained higher concentrations of toxicants, as they have greater adsorption capacity than coarser sediments (sand and gravel), due to their greater surface area.

**DISCUSSION**

**Seasonal and inter-event variation**

As shown in our companion paper, a broad variety of factors, both climatic and anthropogenic, account for seasonal and intra-event runoff variation in chemical composition of the runoff. This in turn translates into a variation in toxicological responses. Furthermore, toxicological responses are dependent on the sensitivity of the organisms to the particular class of toxicant, type of exposure (ingestion, absorption), the form of the toxicant (dissolved, particulate, free ion, elemental), the dose and the length or frequency of exposure (Hollenberg 2010).

Seasonal variation in highway runoff toxicity generally occurs as a result of changes in chemical composition of runoff, resulting from changes in road maintenance. To maintain safe driving conditions during the winter months, chemical de-icers, typically salts such as sodium chloride and (or) calcium chloride, are applied on the Ontario roads. These salts add to pollutant loads in runoff, which
augment the underlying toxicity of the highway runoff during the winter months and contribute to the adverse impacts of runoff on the receiving environment (Mayer et al. 1999; Ramakrishna & Viraraghavan 2005). The results of toxicity tests and the analytical data (Table 2 and Mayer et al. 1998) confirm that the most notable variation in toxic responses was attributable to the presence of de-icing salts. The runoff samples with the highest Cl concentrations were also most toxic to test organisms (Table 2 and Mayer et al. 1998). No organisms survived in undiluted samples, which were collected from the snowmelt with high input of road salts (February 18 and April 7).

During winter, pollutants accumulate in snow and ice berms, which form on road shoulders (Westerlund et al. 2005). Accumulated solids, metals, PAHs and road salts may then be collectively washed off in major snowmelt events, as those observed during our study (Table 2 and Mayer et al. 1998). This can result in a large toxic load (usually of de-icing salts, metals and oil) in the runoff. Another factor affecting the release of road salts from urban snow banks is the preferential elution of dissolved constituents during freeze-thaw cycles (Westerlund & Viklander 2011). High levels of chlorides from the application of de-icing salts not only cause osmotic imbalance in organisms, they also increase the solubility of toxic metals, particularly Cd, Zn and Cu (Mayer et al. 2008, 2011), thus increasing their total dissolved concentrations in runoff. Although the free metal ion (Me$^+$) is the most toxic form of metals (Borgmann et al. 2004, 2005; Norwood et al. 2007; Hollenberg 2010), elevated total dissolved metal concentrations also result in higher free metal concentrations and higher runoff toxicity overall. Hence, chlorides also contribute indirectly to heavy metal toxicity. Direct uptake is most likely the main route of exposure for dissolved metals by test organisms. Although the perceived impact of this runoff during winter and early spring is low, damage could occur to hibernating or overwintering aquatic organisms and benthic invertebrates (Marsalek et al. 1999a).

In the late spring, summer and fall, the runoff remains toxic, but it no longer exerts the same acute toxicity as runoff from winter/early spring months. It is important to note that during the summer period, there are relatively few events when D. magna registers a strong acute response.

The importance of the event characteristics on the magnitude of runoff toxicity was clearly shown from the variability of MFO induction from the SW site (Figure 3(a)–(c)). Samples collected at this site for different events exhibited differences in the magnitude of the EROD induction. These differences may be explained by the rainfall characteristics, which affect the contaminant loads in runoff. Generally, higher rainfall events wash off more contaminants from the roads and generate more toxic runoff (Marsalek et al. 1999a, b). Meland et al. (2010) found that heavy rainfall substantially increased the concentrations of PAHs and other contaminants in runoff, except road salt, which was diluted. The runoff with the highest MFO induction was collected from the precipitation event with the highest rainfall, 24.2 mm and 0 day antecedent dry period. As shown by the chemistry of runoff, this runoff had the highest concentration (74.2 mg/kg) of 16 priority PAHs in the runoff solids. Runoff with the second highest MFO induction and the second highest concentration of PAHs (36.6 mg/kg, Mayer et al. 2011) was collected from the rainfall event characterized by 9.8 mm rainfall and 3 days of antecedent dry period. Lastly, runoff with the lowest MFO induction and the lowest concentration of PAHs (26.6 mg/kg) in runoff solids was collected from the lowest rainfall, 5.4 mm and 0 day antecedent period. The total concentration of 16 priority PAHs in aqueous phase appeared to be inversely related to runoff toxicity, although the number of samples was too small to establish any statistically significant relationship. A higher proportion of the more soluble low-molecular-weight PAHs in the least toxic runoff was the likely explanation of the observed effect. These compounds are less toxic and mutagenic than the low-solubility, high-molecular-weight PAH compounds. Conversely, the toxicity of runoff increased with the increasing concentrations of high-molecular-weight PAHs in runoff solids, particularly with increasing concentrations of potent mutagens and carcinogens such as benzo[a]pyrene (from 0.73 to 5.28 mg/kg) and indeno[1,2,3-cd]pyrene (from 0.34 to 2.8 mg/kg). The high-molecular-weight PAHs are products of incomplete combustion at high temperatures and their source in road and highway runoff is automobile exhaust (Buckler & Granato 1999), especially from diesel engines. Additional sources of PAH-enriched particles, as seen in our study, can be asphalt pavements (Mayer et al. 2011).
Although the toxicity of runoff from the same site varied, depending on the magnitude of the rainfall event, the results clearly show that highway runoff is a potent inducer of EROD and that this effect often disappears with dilution of the runoff.

**Intra-event variation**

The primary pollutant removal mechanism during periods of wet weather is stormwater runoff generated by rainfall events. The efficiency of pollutant removal by rainfall is dependent on the characteristics of the rainfall event (duration and intensity) and on the length of the antecedent dry weather (ADW) period; the pollutant build-up increases with an increasing ADW period, but reaches an upper limit after about 5–7 days, when any new pollutant additions are mostly offset by pollutant removal by traffic-generated wind or pollutant decay (Pitt et al. 2004). Accumulation rates are site dependent and can vary based on traffic density, speed, construction and exposure to weathering factors such as temperature, wind and rainfall intensity and duration. Rainfall drops mobilize pollutants, which accumulate on the road surface during the ADW period. When a significant rainfall occurs, the highest pollutant concentrations occur during the early stages of the runoff event (Marsalek et al. 1999b), making this part of the runoff the most toxic. This is known as the ‘first flush’ and has been observed in several studies (Marsalek et al. 1999b; Shinya et al. 2000; Kayhanian et al. 2008). The most mobile substances (e.g. soluble salts, released by preferential elution) wash off the roadway first, while some other materials (such as old oil deposits and larger particles) require larger volumes of water or greater intensity to mobilize. It has been shown (Shinya et al. 2000) that more than half of the total pollutant load can be mobilized during the initial phase of runoff.

The *C. dubia* test (Figure 1) reveals that both acute and chronic toxicity of runoff samples collected at the SW site decrease as a function of time during the event. The decline in toxicity is consistent with the decreasing concentration of Zn in runoff. The *C. dubia* test is known to be sensitive to elevated Zn and Cu concentrations (Rokosh et al. 1997; Kayhanian et al. 2008). The targets for metal toxicity are generally cellular molecules, macromolecules, membranes or organelles, and the toxic effect of the metal usually involves the initial interaction of the free metal ion with the toxicological target (Hollenberg 2010). Comparable results were obtained from the Microtox™ and SMP tests (Figure 2), which also show the gradual decline in runoff toxicity due to decreasing concentrations of soluble toxicants. Similar conclusions were also derived from rainbow trout toxicity tests, which show that acute toxicity to rainbow trout decreased over time (Figure 1), although the undiluted highway effluent still exhibited some toxicity after 60 min into the rain event. Even though the concentrations of PAHs in runoff solids decreased within the event from 93 mg/kg to 12 mg/kg, they remained sufficiently high to cause substantial sub-lethal toxicity to rainbow trout (Figure 4). While the analytical data show that most of the PAHs (91%, Mayer et al. 2011) were associated with solids and are thought to be less bioavailable than the dissolved PAHs, the toxicity tests indicate that given a sufficiently long exposure, a sufficient portion of PAHs were present in solution to exert a significant EROD induction. This assumes that the pathway for PAH uptake is through soluble forms and not through ingestion of particles, as only soluble PAHs cause EROD induction in fish livers.

While the overall toxicity of runoff declined as the event progressed (Figure 1), the tests have shown that the opposite was true for the runoff solids toxicity (Figure 7). This observation has been largely attributed to the nature of the storm event, which was of high intensity for the first 30 min, followed by a period of reduced intensity. This would allow coarse particulates, containing fewer toxicants (Horowitz 1991), to be washed off quickly, followed by finer particulates, which likely contain higher concentrations of metals and PAHs. Heavy metals and other contaminants tend to associate with the finer particles (<62 μm) (Herngren et al. 2006), as they have a larger surface area, and hence more sites, available for adsorption. The proportion (by mass) of coarse particles (>45 μm) in SW runoff sediments was reported as two orders of magnitude larger than that of fine particles (<45 μm) and thus those coarser particles contribute higher overall loads of contaminants (Marsalek et al. 1997). These coarser particles are more likely to be trapped by stormwater best management practices (BMPs) (Stone & Marsalek 1996). Only a small portion of the total metal load is weakly bound to...
sediiments and thus bioavailable to receiving water organ-
isms (Irvine et al. 2009).

The stormwater events sampled in this study were quite small by comparison to typical events, with wetter-than-
average conditions during the sampling period. As such, the runoff results may not be reflective of different types of storms (e.g. thunderstorm-type events after a build-up of 14 days or more). Given the traffic conditions and the antecedent dry period, it would appear that chemical loading could have been lower than expected in a typical event/year.

It should also be emphasized that the study discussed here deals with highway runoff at the edge of the pavement, which does not fully reflect receiving water conditions. These bridge sites were ideal for our study as they are designed to drain quickly (hence even small rainfall events produce runoff), and there was no impact from storage and little opportunity for adsorption, filtration or transformation of contaminants; this may be one reason why the ‘first flush’ toxic effect was strongly evident at these sites. Further downstream, as runoff is transported by conveyance elements or passes through BMPs designed to improve runoff quality, runoff toxicity would decline. Indeed, fine runoff particles containing toxic contaminants can be trapped by such BMPs as grassed swales and stormwater management ponds along the contaminant transport route, resulting in the attenuation of runoff toxicity. The US Federal Highway Administration Report on Management Practices for Mitigation of Storm Water Runoff Pollution (Maestri & Lord 1987) states that highway runoff is not likely to have adverse effects on receiving waters if it is conveyed by overland flow in an unlined or grassed channel over a distance of 60 m or more prior to discharge into the receiving stream.

The intra-event toxicological data clearly show that the toxicity of runoff diminishes over time, exhibiting the ‘first flush’ effect. Collecting a sample late in a runoff event or using a time-averaged composite sample may lead to underestimation, or dilution of contaminants and the associated toxicity. As such, the sample may not be identified as toxic, when in fact the ‘first flush’ runoff may be acutely toxic. The receiving waters could provide enough dilution or attenuation to help mitigate the toxicity of such highway runoff. These observations have important implications for stormwater quality management, as it is obvious that sub-
stantial improvement in runoff quality could be attained by controlling or capturing the first flush and ensuring an ade-
quate BMP size.

**Inter-site variation**

Traffic intensity and vehicle type distribution were thought to have a strong influence on the toxicity of highway runoff (Viklander 1999). To address this issue, the chemical and toxicological data from the three different sites were compared. The data show that there were indeed measurable differences between the investigated sites. The C. dubia IC25 for samples collected during the same rain event on 8 August ranged from 32% at the RBG site to 60% at the FCC site, despite the SW site having the greatest conduc-
tivity. On September 27, the same trend was noticed, with IC25 ranging from 1% at RBG to 50% at FCC. The variation in toxicity, in this case, does not correlate with the variation in traffic intensity. The examination of data showing chemical composition of runoff indicates that the RBG site had the highest levels of dissolved Zn, although the traffic intensity was the lowest. Input of Zn from the galvanized bridge drain is believed to have augmented the toxicity of runoff at the RBG site, and this may have con-
founded the chemical and toxicity test results. Higher toxicity of runoff, associated with elevated Zn concent-
trations at the RBG site, is consistent with findings of Kayhanian et al. (2008), who found copper and zinc to be the primary causes of toxicity in about 90% of runoff samples. These results clearly show that in addition to traf-
cic intensity and vehicular composition, highway structures have a significant (possibly localized) effect on the runoff quality and associated toxicity.

The spatial differences in runoff toxicity as measured by the MFO responses were also readily apparent. The runoff from the SW site, which was the most potent MFO inducer, had the highest concentrations of PAHs (Table 4 in Mayer et al. 2011), followed by the RBG and FCC sites. It is interest-
ing to note that, although the FCC site had a higher intensity of traffic than the RBG site at the time of the study, both the toxicity tests and chemical analyses indicated that the oppo-
site was true for the runoff quality. Factors such as the slope, condition of pavement and length of road between the
drains may play a role in this inconsistency. The segment of the road drained at the RBG site (54.7 m, Mayer et al. 2011) was substantially longer than that at the FCC site (6.30 m, Mayer et al. 2011) and, considering that most traffic by-products deposit in a narrow band along the curb, the longer site would have a higher input of pollutants. This RBG site had also been resurfaced with fresh asphalt prior to sampling.

The spatial differences in the toxicity of road solids were also evident from the results of the nematode test (Figure 8). Runoff solids from the site with the highest traffic intensity (SW) were severely toxic to the test organisms, while the remaining two sites (FCC and RBG) were moderately toxic, indicating the linkage between the traffic intensity and levels of toxicity associated with the particulate phase. The sediment from the RBG site was slightly more toxic than the sediment from the FCC, even though the opposite would be expected from the traffic intensity. This situation again could be due to the input of Zn from the bridge drain at this site, which may have elevated the toxicity of the sample and higher PAH concentrations in runoff solids. The above toxicity data reveal important spatial differences in runoff toxicity attributable largely to traffic intensity and to the conditions of the road infrastructure (i.e. presence and corrosion of metal structures and pavement condition).

The information presented here clearly demonstrates substantial toxicity of highway runoff caused by various classes of contaminants. However, the in-stream experiments, which show no inducible MFO activity in rainbow trout, reveal that short-term effects on organisms in receiving waters were not observed in this case, provided that sufficient dilution of runoff by receiving waters occurred. This observation is consistent with a conclusion of the US Federal Highway Administration Report on Management Practices for Mitigation of Storm Water Runoff Pollution (Maestri & Lord 1987), which states that highway runoff is not likely to have adverse effects on receiving waters if there is sufficient dilution of highway runoff by receiving streamflow (dilution ratio at least 100 to 1). Repeated exposures to stormwater toxicants (unlike the single event captured in this case) may result in increased MFO responses of the fish to persistent toxicants in the runoff (Burton et al. 2000).

CONCLUSION

A battery of bioassays with different endpoints, along with the supporting chemical analyses of runoff samples, clearly show that several classes of contaminants contribute to highway runoff toxicity. The effect of these contaminants on biological communities depends on the size and type of receiving waters, and possible runoff treatment along the path from the highway pavement to the receiving waters. The toxicants determined in our study included metals, PAHs and road salts. The results show that surface runoff from the major multilane divided highway, with the highest traffic intensity, had the highest levels of contaminants and that this runoff exerted the highest acute and chronic toxicity on aquatic organisms in laboratory bioassays. Differences were also observed on a temporal basis, both during individual events and seasonally, with the overall reduction in toxicity in later stages of events and typically high toxicity of winter and early spring runoff containing some snowmelt.

As documented by our results, the sources of contaminants and contaminant-associated toxicity are vehicular operation, road maintenance and pavement surface conditions (age and material) and highway metal structures. Different sources contribute different classes of contaminants to runoff. For instance, elevated toxicity of winter and spring runoff is primarily associated with applications of chemical de-icers, which considerably increase the concentrations of road salt constituents in runoff. Likewise, the data show that corrosion of highway structures (e.g. bridge drains, guardrails, sign posts) may contribute significantly to metal loads and consequently augment the toxicity of runoff. Strong MFO induction in rainbow trout has shown that this test is sensitive enough to determine the potential toxicity of PAHs in runoff. It is important to note that the results of this study are indicative, rather than conclusive, because more spatial and temporal data would be needed to confirm the observed trends.

Finally, the information presented in this study represents the ‘worst case scenario’, in which untreated highway runoff would directly enter a small receiving water body, with little dilution. The fate and transport characteristics of contaminants, the method of their conveyance to the receiving water bodies and controls of runoff quality (e.g. by BMPs)
will affect contaminant concentrations in drainage effluents entering the receiving water body. This investigation provides valuable information necessary for planning and implementation of remediation strategies for the mitigation of impacts of highway runoff pollution.

ACKNOWLEDGEMENTS

We thank J. Richardson and R. Neureuther for field and laboratory assistance. The cyanide analyses by V. Ferraro of MOE and PAH analyses by B. Brownlee and G. McInnis are gratefully acknowledged. Thanks are due to the late Dr S. Brown whose valuable comments and constructive criticism strengthened and substantially improved the manuscript. The paper benefited from thorough review and salient comments by Dr U. Borgmann. Funding for this project was provided by Environment Canada.

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First received 16 December 2010; accepted in revised form 20 August 2011