An Assessment of the Phototoxicity of Contaminated Groundwater

Using Whole Effluent Toxicity Bioassays on *Daphnia magna* and *Pimephales promelas* with UVR Supplemented Lighting

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Cover Photos: (Clockwise) Groundwater sampling; Underwater spectroradiometer used to measure local solar radiation; Laboratory design.

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by
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Table of Contents

List of Figures and Tables

Page

Figures

Page

Tables

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Abbreviations and Acronyms

°C	degrees Celsius
µg	microgram
µE	microEinstein
µW	microwatt
ASTM	ASTM International
CL	confidence limits
cm	centimeter
CWP	Sylvania cool white plus fluorescent lamp
DO	dissolved oxygen
Ecology	Washington State Department of Ecology
ft-c	footcandle
J	joule
L1	lamp configuration L1: (CWP, CWP, UVA-340, CWP, CWP, UVA-340, CWP,
L2	lamp configuration L2: (UVA-340, CWP, CWP, UVA-340, CWP, CWP, UVA-340)
LC25	concentration lethal to 25% of test population
LC50	median lethal concentration
Lighting Guide	ASTM Standard Guide for use of lighting in laboratory testing
LL	laboratory lighting
LL+UVR	laboratory lighting plus ultraviolet radiation
LSP	Little Squalicum park
LOEC	lowest observed effects concentration
MDL	Method detection limit
mg	milligram
ml	milliliter
MLE	maximum likelihood estimate

NEI	noise equivalent irradiance
NIST	US National Institutes of Standards
nm	nanometer
NOEC	no observed effects concentration
NPDES	National Pollutant Discharge and Elimination System
Oeser	The Oeser Company, Inc.
PAHs	polycyclic aromatic hydrocarbons
PCP	pentachlorophenol
PDT	Pacific Daylight Time
pMSD	percent minimum significant difference
ppPAHs	potentially phototoxic polycyclic aromatic hydrocarbons
ppt	parts per thousand
R1	round 1 laboratory light measurements
R2	round 2 laboratory light measurements
RI/FS	remedial investigation/feasibility study
T1-LL	test replicate T1, standard laboratory light treatment
T1-LL+UVR	test replicate T1, standard laboratory light plus UVR
T2-LL	test replicate T2, standard laboratory light treatment
T2-LL+UVR	test replicate T1, standard laboratory light plus UVR
USEPA	United States Environmental Protection Agency
UVA	ultraviolet A radiation (320-400 nm)
UVA-340	Q-lab UVA fluorescent lamp
UVB	ultraviolet B radiation (280-320 nm)
UVC	ultraviolet C radiation (200-280 nm)
UVR	ultraviolet radiation (200-400 nm)
WET	whole effluent toxicity
WWU	Western Washington University

Glossary

Absorption	An interaction of energy and matter in which energy is converted to different forms of energy.
Daily dose	Time integrated irradiance at a surface for one day. Determined by multiplying irradiance times the light portion of a 24 hour photoperiod (in seconds). Term is used to define quantity of radiant exposure for a specified waveband. Units are J m^{-2} or J cm^{-2}.
Einstein	1 Einstein = 6.022 X 10^{23} photons = 1 mole of photons = 6.022 x 10^{23} quanta.
Energy	Integral over time of power. Unit is the Joule (J).
Flux density	Electromagnetic radiation per second per unit area. Units for radiant flux density are W m^{-2} or μW cm^{-2}. Units are for photon flux density are μE s^{-1} m^{-2} or μmol s^{-1} m^{-2}.
Foot-candle	Lumens per ft^2
Illuminance	Visible flux density. Measured in lux (lumens per square meter) or foot-candles (lumens per square foot).
Instantaneous irradiance	Irradiance. Term sometimes used to emphasize the difference between irradiance, which is based on energy per unit area per second, and total daily irradiance and total daily dose, which are measurements of cumulative exposure to radiant energy over time.
Irradiance	Radiant flux incident on a unit area. Also known as radiant flux density. Units are W m^{-2} or μW cm^{-2}.
Joule (J)	SI unit of energy. 1 J = 1 W s.
Lumen	Measurement of the brightness of light emitted by a point source of 1 candela. The lumen is the photometric equivalent of the Watt.
Lux	Lumens per m^2.
Noise equivalent irradiance	The level of irradiance required to produce a signal equal to instrument noise. Determined for each wavelength.
Optical radiation	Portion of electromagnetic spectrum that lies between radio waves and X-rays, including UVR and visible radiation.

Photometry	Science of measurement of light based on how the human eye perceives the brightness of a light source.
Photon	One single indivisible packet (quanta) of light or radiant energy.
Photon flux	Amount of photons of radiation emitted, transmitted, or received per unit time. Units are s^{-1} (sometimes given as $\mu E\ s^{-1}$ or $\mu mol\ s^{-1}$).
Photon flux density	Photon flux per unit area. Units are $\mu E\ s^{-1}\ m^{-2}$ or $\mu mol\ s^{-1}\ m^{-2}$.
Phototoxicity	An increase in the toxicity of a substance resulting from exposure to natural or artificial electromagnetic radiation.
Power	Rate of flow of energy with respect to time. Unit is the Watt (W).
Quanta	Single indivisible packet of light or radiant energy. Photon.
Radiant energy	Time integral of the radiant flux over a given duration. Units are W s.
Radiant flux	Measure of energy flow per unit time. Units are W or $J\ s^{-1}$
Spectral distribution	Description of relative amounts of electromagnetic radiation as a function of wavelength.
Spectral irradiance	Description of relative amounts of radiant energy as a function of wavelength. Units are $\mu W\ cm^{-2}\ nm^{-1}$ or $W\ m^{-2}\ nm^{-1}$
Tier I	Range-finding groundwater toxicity test(s)
Tier II	Definitive groundwater toxicity test(s)
Total irradiance	Waveband irradiance.
Watt (W)	SI unit of radiant energy. $1W = 1\ J\ s^{-1}$.
Waveband	Continuous segment of the electromagnetic spectrum (320-320 nm represents one waveband).
Waveband irradiance	Total irradiance for a specific waveband, determined by integrating irradiance over the entire waveband with respect to wavelength.

Executive Summary

The primary goal of this project was to determine if supplementing standard laboratory lighting with ultraviolet radiation (UVR) during Whole Effluent Toxicity (WET) tests could modify the toxicity of contaminated groundwater samples to aquatic organisms. Research has shown the toxicity of some polycyclic aromatic hydrocarbons (PAH) to aquatic organisms increases significantly in the presence of sunlight or artificial UVR, a phenomenon known as photoactivated toxicity or phototoxicity. WET tests are frequently employed to evaluate the hazards of surface water and provide a standardized means to characterize the toxicity of those waters; WET testing protocols can also be used for groundwater samples. Despite the known interaction of UVR with PAHs, however, WET test protocols do not require the presence of UVR during testing. The results of the phototoxicity studies suggest that the lack of specific guidance for lighting conditions may result in inaccurate estimates of *in situ* toxicity when photoactive PAHs are present and UVR is not specifically included in the laboratory lighting.

Two types of light treatments were designed for use during WET testing in this study. The first type represented typical laboratory lighting conditions (LL). The second type provided typical laboratory lighting conditions supplemented with UVR (LL+UVR). The LL+UVR treatment design included both UVA (320 – 400 nm) and UVB (280-320 nm) radiation and incorporated optical radiation characteristics that are more representative of local solar conditions than those found in typical laboratory lighting. Outdoor solar measurements were taken in two freshwater environments and one marine environment, all within 10 km of Little Squalicum Park, Bellingham, WA. These were used to evaluate the habitat-specific spectral irradiance characteristic of local solar radiation and to guide the development of the LL+UVR light treatment design.

Groundwater samples contaminated with PAHs were collected from Little Squalicum Park between June and November, 2007. The acute toxicity of those samples to *Daphnia magna* and *Pimephales promelas* were characterized under both lighting treatments in duplicate. The groundwater samples were analyzed to confirm the presence and concentrations of PAHs.

Results show that the toxicity of PAH-contaminated groundwater increased significantly to *D. magna* under laboratory lighting supplemented with UVR. In the absence of significant UVR, LC25s were 22.1 and 25.7%, compared to 0.59 and 0.68% when UVR was present. A 17-fold difference in the NOEC and LOEC values, also supports a greater toxicity under laboratory lighting augmented with UVR. NOEC values were 6.25% under LL treatments and 0.38% under LL+UVR treatments, and the LOEC values were 12.5% and 0.75% under LL and LL+UVR treatments, respectively. Although trends in the *P. promelas* results suggest greater groundwater toxicity occurred to *P. promelas* when UVR was present, these trends were not statistically significant. There are several possibilities for the lack of significant differences in the *P. promelas* tests, including a low concentration of potentially phototoxic PAHs (ppPAH) in the definitive WET tests. The concentration of ppPAHs in the definitive *P. promelas* test was 60.1 µg/L compared to 869 µg/L in the preliminary *P. promelas* test. These tests should be repeated to confirm the findings that UVR does not significantly increase the toxicity of groundwater to *P. promelas* under typical laboratory lighting supplemented with UVR.

The study results are important for many reasons. First, the results demonstrate that PAH-contaminated field samples can be phototoxic to *D. magna*. Previous studies have focused primarily on the phototoxicity of single substances or mixtures created in the laboratory. This study contributes a better understanding of phototoxic hazards associated with field samples. In particular, the findings demonstrate that a phototoxic hazard can exist in samples with multiple co-contaminants, such as pentachlorophenol. The findings also suggest that the presence of known phototoxic compounds in samples can be indicative of phototoxic hazard, which may be used to inform bioassay design. Finally, the results show that phototoxicity can occur under WET testing conditions when UVR is present, which suggests that WET tests can be used to effectively evaluate phototoxic hazard with a consistent approach. Managers who use WET testing or other bioassay designs may, therefore, find it important to consider the influence lighting choices may have on toxicity estimates on a case-by-case basis.

Introduction

Arguably, the most fundamental principle in the field of environmental toxicology is that biological and chemical factors interact in the environment to ultimately produce effects to organisms, populations, and communities. Physical factors can also influence the nature and magnitude of these effects and typically are considered as part of a standardized method when evaluating the hazards posed by environmental contaminants. Although most standardized test methods place an emphasis on requirements related to physical factors like temperature, there is often incomplete guidance on the type of lighting that should be used during testing. Artificial light sources are commonly used to conduct toxicity tests, but laboratory lighting is not standardized, nor is it comparable to light conditions in natural environments (ASTM, 2002; Fortner, 2009). Research suggests that disregarding the influence of lighting conditions on organism and contaminant interactions during testing may result in inaccurate estimates of sample toxicity when phototoxic compounds are present.

Project Background

Polycyclic aromatic hydrocarbons (PAHs) are persistent and ubiquitous environmental contaminants that have been detected in animal and plant tissue, sediments, soils, air, surface water, industrial effluents, well water, drinking water, and groundwater (USEPA, 1980; Eisler, 1987). Research has shown the toxicity of some PAHs to aquatic organisms increases significantly in the presence of sunlight or artificial ultraviolet radiation (UVR), a phenomenon known as phototoxicity or photoactivated toxicity (Diamond, 2003). Two reaction mechanisms, photosensitization (Barron, 2000; Lampi et al., 2005) and photomodification (Huang et al., 1993; Lampi et al., 2005), have been proposed to explain this enhancement of toxicity. Photosensitization is currently the most investigated, identified, and widely accepted mechanism of phototoxic events. For both photosensitization and photomodification mechanisms, a photoactive chemical and radiation of sufficient spectral quality and magnitude to induce phototoxic effects must be present.

In their extensive reviews of the phototoxicity literature, Arfsten et al. (1996) and Diamond (2003) support that the phototoxic potential of PAHs has been demonstrated in the laboratory using a diversity of methods and materials. Most of this research has focused on defining the phototoxic potential of single PAHs to individual species (Diamond, 2003). Less attention has been given to: 1) evaluating the phototoxic potential of environmental samples; and 2) evaluating the phototoxic potential of these samples using standard regulatory toxicity test methods.

Environmental samples containing PAHs are often characterized as mixtures of many individual compounds occurring at low concentrations in association with other co-contaminants that are not phototoxic (Grote et al., 2005a). Researchers have demonstrated the phototoxic potential of some types of environmental media using *in situ* UVR exposure and laboratory bioassay methods (Diamond, 2003 and references therein), although the phototoxic potential of PAH-contaminated groundwater has not been investigated using standard regulatory test methods. It is also possible that the co-contaminants present in environmental samples may obscure phototoxic effects in these samples (Grote et al., 2005a). This confounds a clear understanding of whether groundwater contaminated with mixtures of PAHs could be more toxic when UVR is used during testing.

The Washington State Department of Ecology (hereafter referred to as Ecology) regulates the release of effluents, many of which contain complex mixtures of contaminants, to surface waters of Washington State under Section 402, National Pollutant Discharge Elimination System (NPDES), of the Clean Water Act (Hudiburgh, 1995). Under NPDES, Ecology enforces specific numeric criteria for toxic pollutants and uses Whole Effluent Toxicity (WET) testing to evaluate the toxicity of point-source discharges to surface waters. In some cases, Ecology may also employ WET test results to establish water quality criteria or cleanup levels for non-point pollution sources such as contaminated groundwater (David Sternberg, Ecology, Olympia, WA, personal communication). Since PAHs are common constituents of both point and non-point contaminant sources, WET testing could represent a standardized approach for evaluating the phototoxic potential of waste streams containing PAHs.

Despite the known interaction of UVR with specific PAHs, WET methods do not currently require the use of UVR during testing. Specifically, the guidance is incomplete regarding the quality and quantity of illumination that should be used when conducting these tests. For example, under WET test protocols, the United States Environmental Protection Agency (USEPA) recommends the use of ambient laboratory lighting during WET tests. This recommendation is given in terms of photon flux density or illuminance (10-20 $\mu E/m^2/s$ or 50-100 ft-c, respectively) (USEPA, 2002a). No recommendations are made, however, for: 1) light source spectral distribution; 2) spectral irradiance levels; 3) presence of UVA and UVB light; and 4) dose of light over time. The protocols also indicate that plastic or glass should be used to cover test chambers during testing, a procedural element that could alter the spectral character and level of optical radiation reaching test solutions.

Polycyclic Aromatic Hydrocarbons (PAHs) – Properties, Occurrence, and Toxicity

Polycyclic aromatic hydrocarbons (PAHs) have received considerable attention because of well documented toxic effects to many classes of organisms (Eisler, 1987). The pervasiveness of these compounds in the environment is considerable. An estimated 6,000 metric tons of total production-related PAH waste is generated annually in the United States (USEPA, 2008a) and PAHs are major contaminants at over half of the USEPA's National Priorities List of Superfund sites (Diamond, 2003).

PAHs are recognized as pollutants of concern by the USEPA, Environment Canada (Environment Canada, 1994; CEPA, 1999), and the European Union (Manoli and Samara, 1999). Under the Clean Water Act, USEPA currently lists 16 unsubstituted PAHs as priority pollutants (Figure 1) (USEPA, 2008b). Environmental concern has focused primarily on PAHs that range in molecular weight from 128.16 (naphthalene, 2-ring structure) to 300.36 (coronene, 7-ring structure) because many of these PAHs have been shown to be mutagenic, carcinogenic, or teratogenic to a variety of organisms, including fish and other aquatic species (Eisler, 1987). Generally, the aqueous solubility of PAHs is greater at lower molecular weights, a factor that can increase the bioavailability and mobility of these contaminants in aquatic environments (Eisler, 1987; Arfsten et al. 1996).

In the absence of UVR, PAHs do not generally cause acute toxicity to aquatic organisms at concentrations less than their aqueous solubilities (Arfsten et al., 1996; Lampi et al., 2005; Diamond et al., 2006). Acute effects tend to occur instead at high concentrations as a result of narcosis. Since dissolved PAH concentrations in some of the most heavily polluted waters are several orders of magnitude less than those historically recognized to cause acute effects in the laboratory (Burgess et al., 2003), it would seem that PAHs pose little acute hazard to aquatic organisms *in situ*. This assumption, however, does not consider the potentiating influence of UVR on PAH toxicity.

Phototoxicity

Phototoxicity is a phenomenon that has been recognized since the early 1900s, with the majority of studies conducted since the early 1980s. A variety of chemicals have been reported to be phototoxic, including constituents of petroleum, some plastics and pharmaceuticals, the pesticides carbaryl and α-terthienyl, the flame retardant chemical sodium ferrocyanide, the heterocyclic azaarenes, chloroform, carbon tetrachloride, dinitrotoluenes, and some organic dyes like erythrosin-B (Arfsten et al. 1996; Zaga et al., 1998; Bleeker et al., 2002; Calfee and Little, 2003; Little and Fabacher, 2003; Diamond, 2003 and references therein).

In general, the phototoxicity of PAHs has been studied most extensively, an emphasis which stems from concern that these relatively ubiquitous environmental contaminants can be acutely toxic at levels once considered safe for aquatic organisms (Lampi, 2005). According to Weinstein and Diamond (2006), the most potent phototoxic PAHs tend to be compounds that have three to five rings (e.g. fluoranthene, pyrene, anthracene, and benzo-a-pyrene), although factors such as the spectral distribution of photoactivating radiation and radiation dose can affect the phototoxic potential of a PAH (Diamond, 2003). The toxicity of some of these compounds has been shown to increase as much as three orders of magnitude in the presence of UVR (Arfsten et al., 1996 and references therein; Spehar et al., 1999 and references therein).

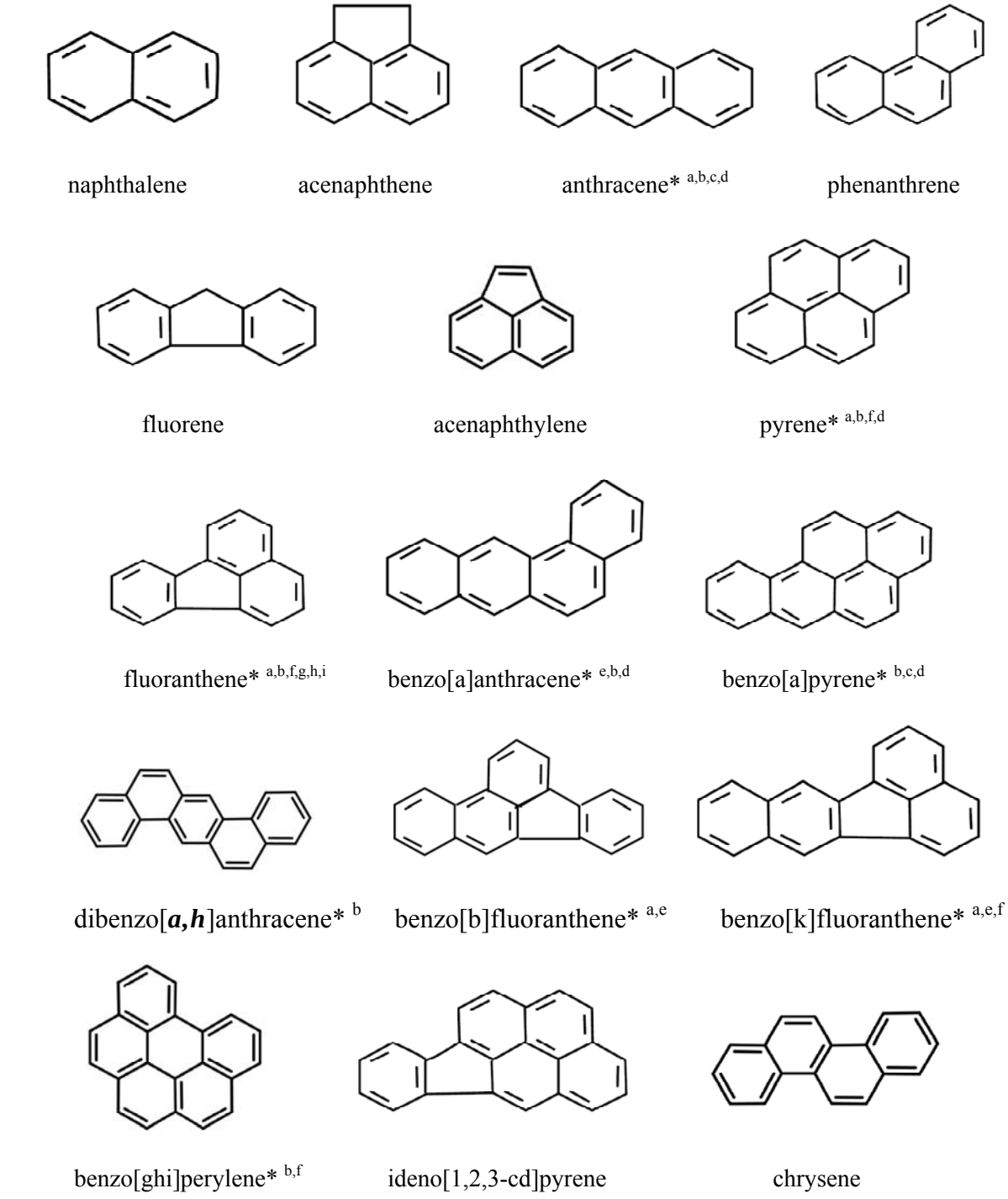

Figure 1. The 16 USEPA priority PAHs. Asterisks indicate potentially phototoxic PAHs (ppPAHs), a designation based on evidence of phototoxicity reported in other studies: [a] Mekenyan et al. 1994; [b] Lampi et al. 2005; [c] Weinstein and Polk 2001; [d] Oris and Giesy 1987; [e] Grote et al. 2005; [f] Peachey 2005; [g] Cho et al. 2003; [h] Spehar et al. 1999; [i] Ankley et al. 1995.

This Study

The purpose of this research was to evaluate the influence of two different lighting treatments on the toxicity of environmental samples containing phototoxic contaminants using WET testing protocols. To make this determination, acute WET tests were conducted on PAH-contaminated groundwater under standard laboratory lighting (LL) and under standard laboratory lighting plus UVR (LL+UVR), and the results were compared.

Groundwater

WET tests were conducted using groundwater samples collected from Little Squalicum Park (48.766103° N latitude and -122.515862° W longitude), Bellingham, WA (Figure 2). Groundwater was evaluated in this project because: 1) lack of UVR and the presence of anoxic conditions in most groundwater environments minimize the chemical, biological, and photodegradation of the PAH parent molecules that are the focus of most phototoxicity studies (Neff, 1979); 2) discharge of PAH-contaminated groundwater to adjacent surface waters represents a potential phototoxic hazard to organisms exposed to UVR in those waters; and 3) groundwater phototoxicity has not been reported in the literature.

Little Squalicum Park (Ecology site #7551533) in Bellingham, WA was chosen as a sampling site because groundwater at the site contains high levels of PAH contaminants relative to surrounding areas and analytical profiles of water collected from wells at the site show the presence of known photoactive PAHs (Table 1) (Integral, 2006). Little Squalicum Park consists of approximately 21 publicly owned acres, most of which are currently zoned for recreational open space. Little Squalicum Creek runs through the park and discharges into Bellingham Bay. Since the early 1900s, land within the park has been used for a variety of purposes, including dairy farming, ranching, conveying sugar-processing wastes, sand and gravel mining, landfill, and conveyance of stormwater. PAH contamination in the park stems primarily from industrial activities at The Oeser Company (hereafter referred to as Oeser), an up-gradient wood treatment facility that is currently designated a USEPA Superfund site. Located adjacent to the park, the

facility has been in operation since the early 1940s. Creosote, which can contain up to 90% PAHs (Environment Canada, 1993), was used to treat wood products at the facility from the 1940s to the early 1980s. Pentachlorophenol (PCP) treatment, a creosote replacement, began at Oeser in the mid 1950s, and is currently used at the facility for wood treatment (Integral, 2006). Since the late 1940s, Oeser has discharged contaminated stormwater and/or processed wastewater to Little Squalicum Creek. These discharges have contained a variety of wastes associated with wood treatment, including creosote, PCP, dioxins, and furans (associated with PCP), and diesel fuels. Soil, groundwater, sediment, and surface water in the park, therefore, have become repositories for wood treatment wastes because of stormwater discharges and from migration of contaminated groundwater from beneath the Oeser site to down-gradient park areas (USEPA, 2002b). Subsurface soils and groundwater in park areas located north of the Marine Drive Bridge contain the highest levels of creosote and PCP-related contaminants.

All range-finding and definitive WET tests in this study were conducted using samples from groundwater well SB-29. Portions of these samples were also analyzed to quantify the concentration of PAHs, PCP, and dibenzofuran. Groundwater well SB-29 was selected for WET testing because historical data show that groundwater collected from the well contains: 1) mixtures of PAHs (Table 1); 2) phototoxic PAHs at levels known to induce phototoxicity in *Daphnia magna* and *Pimephales promelas*, based on the available literature; and 3) lower levels of potential co-contaminants (e.g. PCP) relative to other wells at the site (Integral, 2006; Fortner, 2009).

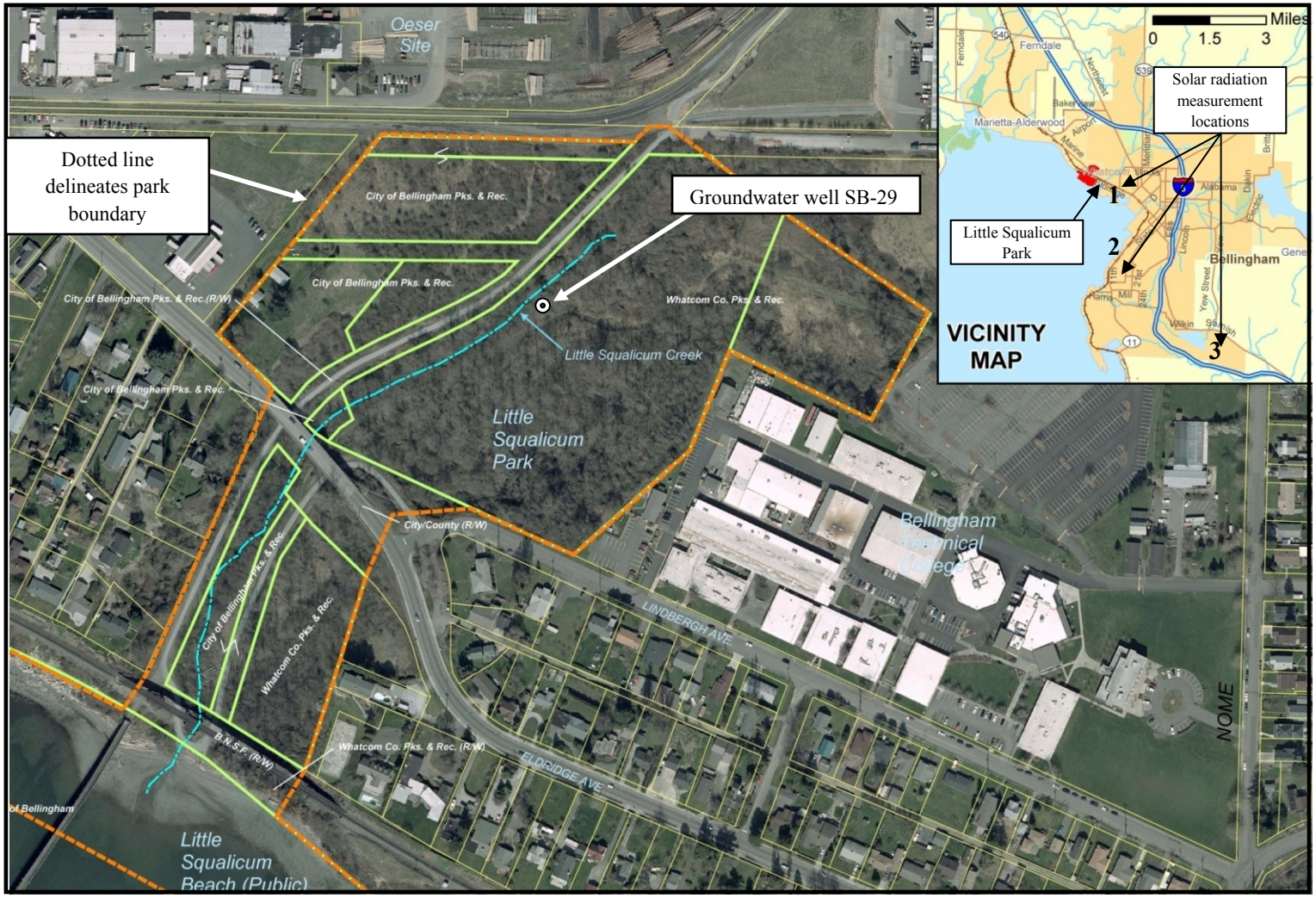

Figure 2. Little Squalicum Park area, ownership, and groundwater well SB-29. Figure obtained and modified from 2006 Draft Little Squalicum Park RI/FS (Integral, 2006). Insert illustrates park location relative to local-habitat solar radiation measurement sites. Upper inset: 1 – Squalicum Creek, 2 – Taylor dock, 3 – Lake Padden.

Table 1. Little Squalicum Park groundwater well SB-29 pentachlorophenol, dibenzofuran, and PAH concentrations measured in previous study (LSP RIFS) and in this study (WWU). All reported values are measured concentrations.

Data Source		LSP RIFS		WWU *D. magna* Tier II		WWU *P. promelas* Tier I		WWU *P. promelas* Tier II	
Groundwater sampling date		5/2/2006		9/13/2007		10/15/2007		11/28/2007	
Toxicity test start date		N/A		9/14/2007		10/16/2007		11/29/2007	
Analyte	Potentially phototoxic	µg/l	MDL (µg/l)	µg/l	MDL (µg/l)	µg/l	MDL (µg/l)	µg/l	MDL (µg/l)
Pentachlorophenol		16	0.91	6.2	0.60	37.	0.50	9.8	0.52
Dibenzofuran		71	0.35	25	2.4	150 D	2.0	140	2.1
Acenaphthene		270 D	23	110	2.4	330 D	2.0	140	2.1
Acenaphthylene		5.8	0.43	ND	2.4	2.9	2.0	2.2	2.1
Anthracene	Yes[a,b,c,d]	14	0.3	5.8	2.4	45.	2.0	3.4	2.1
Fluorene		100 D	22	50.	2.4	220 D	2.0	65.	2.1
Naphthalene		2800 D	23	25	2.4	ND	2.0	ND	2.1
Phenanthrene		180 D	19	49.	2.4	500 D	2.0	40.	2.1
2-Methylnaphthalene		400 D	22	87.	2.4	120 D	2.0	23.	2.1
Benzo(a)anthracene	Yes[e,b,d]	5.5	0.33	2.9	2.4	82. D	2.0	3.2	2.1
Benzo(a)pyrene	Yes[b,c,d]	2.5	0.3	ND	0.60	30.	0.50	ND	0.52
Benzo(b)fluoranthene	Yes[a,e]	2.2	0.25	2.8	2.4	33.	2.0	ND	2.1
Benzo(k)fluoranthene	Yes[a,e,f]	2.5	0.72	ND	2.4	24	2.0	2.5	2.1
Chrysene		4.4	0.4	3.4	2.4	57.	2.0	3.9	2.1
Dibenzo(a,h)anthracene	Yes[b]	0.4 J	0.22	ND	4.8	ND	4.0	ND	4.2
Fluoranthene	Yes[a,b,f,g,h,i]	36	0.34	42	2.4	330 D	2.0	28.	2.1
Indeno(1,2,3-cd)pyrene		0.7 J	0.26	ND	4.8	ND	4.0	ND	4.2
Pyrene	Yes[a,b,f,d]	28	0.34	17.	2.4	320 D	2.0	23.	2.1
Benzo(g,h,i)perylene	Yes[b,f]	0.8 J	0.27	ND	4.8	5.2	4.0	ND	4.2
Total PAH		3853		426.1		2286.1		484	
PAH		92		70.5		869.2		60.1	

LSP RIFS = Data obtained from Integral (2006).

WWU = Analytical results for groundwater samples that were collected for this study. Samples were collected by Herrenkohl Consulting LLC (Bellingham, WA). Undiluted samples were submitted to Am Test, Inc. (Redmond, WA) for analysis. Analysis of the *D.m*

N/A = Not applicable

MDL = Method detection

J = The analyte was positively identified; the associated numerical value is the approximate concentration of the analyte in the sample.

U = The analyte was analyzed for, but was not detected above the reported sample quantitation limit.

D = The reported value is from a dilution.

ND = Not detected.

[a] Mekenyan et al. 1994; [b] Lampi et al. 2005; [c] Weinstein and Polk 2001; [d] Oris and Giesy 1987; [e] Grote et al. 2005; [f] Peachey 2005; [g] Cho et al. 2003; [h] Spehar et al. 1999; [i] Ankley et al. 1995.

Lighting Conditions

According to *The Standard Guide for Use of Laboratory Lighting* (hereafter referred to as the *Lighting Guide*), many factors need to be considered when designing laboratory light treatments, particularly when the goals of testing involve relating light treatment attributes to actual environmental conditions (ASTM, 2002). Among the environmental conditions that are important to consider are differential absorption of wavelengths of solar radiation in the water column, and daily and seasonal fluctuations in spectral irradiance. Organism characteristics, such as presence of protective pigmentation and behaviors that reduce UVR exposure are other environmental conditions of potential interest. In the laboratory, lighting design is limited by the spectral qualities of available lamps, although modifications can be made, such as use of screening materials to filter certain wavelengths. These considerations are discussed further in Fortner (2009).

Given the range of environmental conditions that can influence phototoxic potential and the lack of clear guidance on adequate representation of these conditions in the laboratory, a common recommendation by phototoxicity researchers is that laboratory lighting conditions be based on the spectral and irradiance characteristics of habitat-specific solar radiation. Use of this solar reference condition should include consideration of UVA and UVB radiation in local habitats as well as consideration of levels of photosynthetically active radiation (PAR) (Diamond et al., 2000; Diamond, 2003; Diamond et al., 2003; Weinstein and Diamond, 2006). The application of habitat-specific solar radiation in the lighting design process facilitates the development of light treatment criteria and helps researchers evaluate how closely laboratory lighting conditions approximate local *in situ* conditions.

Despite the recommendation to use a local habitat-specific solar reference, an extensive review of the phototoxicity literature supports that there is not a standard measure with which to evaluate how closely an artificial light source mimics natural sunlight (Fortner, 2009) and terminology used is non-specific. For example, according to Villafañe et al. (2003), "ecologically relevant" studies should provide radiation with a "realistic" spectral composition. The *Lighting Guide* also has non-specific guidance that includes, "the spectral distribution of

radiation sources should mimic sunlight adequately to be considered environmentally relevant" (ASTM, 2002). Comparisons of laboratory conditions to natural conditions, therefore, are subject to individual interpretation and bias, suggesting that researchers should be particularly cautious when extrapolating laboratory results to environmental systems. The proposed need for a habitat-specific solar reference is not easily dismissed, however. To best incorporate these recommendations given the limitations and considerations discussed, at least eight specific elements should be considered when designing or evaluating light sources used to approximate solar conditions in the laboratory:

- Local solar reference data;
- Spectral characteristics;
- Waveband irradiance versus spectral irradiance;
- Ratios of PAR to UVA to UVB waveband irradiance;
- Exposure of organisms to optical radiation;
- Total daily dose and parabolic variations in optical irradiance;
- Photoperiod; and
- Uptake period prior to radiation exposure.

These eight elements were used in conjunction with other resources to develop criteria for designing light treatments used for the groundwater toxicity tests conducted (Fortner, 2009).

The indoor and outdoor light sources were measured in terms of spectral irradiance in this study. Available options for conducting measurements of PAR and UVR, and the advantages and disadvantages of each option are discussed in Fortner (2009). The PAR waveband was defined as wavelengths from 400 to 700 nm. UVR, which is composed of UVC, UVB, and UVA wavebands, was defined as wavelengths from 100 to 400 nm. Since UVC is attenuated by the atmosphere, UVR investigated here included UVB (280 -320 nm) and UVA (320 – 400 nm) radiation only.

WET Testing

WET test methods were used for the development of phototoxicity reference controls and for groundwater toxicity tests. Tier I (range-finding) WET tests were used to establish the concentration series to be used in subsequent Tier II (definitive) WET tests. Point estimates (e.g.

LC25 and LC50), no observed effects concentration (NOEC) values, and lowest observed effects concentration (LOEC) values were calculated when possible for Tier I and Tier II WET tests and were used to compare the toxicity of groundwater samples under LL and LL+UVR light treatments.

Acute 48 h static tests with *D. magna* and acute 96 h static-renewal tests with *P. promelas* were used to evaluate phototoxicity. These test organisms were chosen because they are extensively used in acute and chronic WET testing and other regulatory testing programs and because life-history traits make these organisms particularly vulnerable to UVR-mediated effects in the water column (Rand et al., 2005; Ankley and Villeneuve, 2006). The relative transparency of *D. magna* and *P. promelas* larvae likely contributes to this vulnerability, possibly increasing the potential for phototoxic effects through both photosensitization and photomodification mechanisms (Lampi et al., 2005). These species were also chosen because *D. magna* are among the most sensitive species to aquatic contaminants (Rand et al., 1995; Lampi, 2005), and Dwyer et al. (2005a, 2005b) have shown that *P. promelas* is a reliable estimator of toxic effects to federal and state-listed endangered and threatened fishes. Mortality was used as the effect endpoint for both *D. magna* and *P. promelas*.

Methods

Some of the considerations involved in the design of laboratory lighting used in this research are summarized here. Full explanation of the considerations and the evaluation process are detailed in Fortner (2009).

Lighting Measurements in Field and Laboratory

Laboratory conditions were designed so that toxicity of PAH contaminated groundwater could be determined under typical laboratory lighting (i.e. LL) conditions and under laboratory conditions more representative of lighting scenarios in the environment with UVR included (i.e. LL+UVR). Specific priorities used to guide the design of both light treatments are discussed in detail in Fortner (2009). Briefly, these priorities were: 1) the eight design elements discussed in the *Lighting Conditions* Section, page 12; 2) current WET testing protocols (USEPA, 2002a); 3) the *Lighting Guide* (ASTM, 2002); 4) review of the phototoxicity literature, with an emphasis on proposed mechanisms of phototoxicity, conditions reported to induce phototoxic effects, and laboratory lighting recommendations (Arfsten et al. 1996; Diamond et al. 2000; Weinstein and Diamond, 2006); 5) Ecology's goals; and 6) laboratory logistical considerations.

All light sources were measured radiometrically with a LI-COR 1800UW (LI1800UW) underwater spectroradiometer (LI-COR Biosciences, Lincoln, NE). With a translucent PTFE-dome optical receptor, one monochromator, and a silicon photodiode detector, the LI1800UW has a range of 300 to 850 nm (with selectable scan intervals of 1, 2, 5, or 10 nm), a wavelength accuracy of ± 1.5 nm, and a wavelength repeatability of 0.5 nm (LI-COR, 1990; Kirk et al. 1994). Since the instrument cannot measure wavelengths below 300 nm, it is not possible to directly assess the absence or presence of wavelengths in the low UVB part of the spectrum. It is possible, however, to extrapolate spectral irradiance from 280 to 300 nm using 3-parameter sigmoidal regression (Fortner, 2009). In this study, the instrument was used to characterize the optical wavelengths emitted by indoor and outdoor sources, quantify the irradiance associated with each of these wavelengths, and determine the total irradiance of specific wavebands.

Total irradiance of UVB (300-320 nm), UVA (320-400 nm), and PAR (400-700 nm) were determined by integrating irradiance with respect to wavelength with the trapezoidal rule of numerical integration using the LI-COR 1800UW software (LI-COR, 2004). Prior to the first use, the spectroradiometer was calibrated with a LI-COR 1800-02 optical radiation calibrator using LI-COR calibration procedures. As part of this process, the spectroradiometer was calibrated by transfer calibration to a LI-COR working standard lamp (1000 W quartz halogen lamp traceable to a U.S. National Institute of Standards and Technology (NIST) primary lamp standard) (LI-COR, 1996). An immersion correction effect calibration file from LI-COR was used to transform the absolute calibration of the LI1800UW in air to an absolute calibration in water for underwater measurements.

Noise equivalence irradiance (NEI) was evaluated for wavelengths from 300 to 700 nm. NEI is a measure of instrument noise (dark signal) and is assessed by conducting instrument scans in a completely dark environment. Average NEI from 300 to 320 nm was 5.58E-04 $\mu W/cm^2$ with a maximum value of 2.16E-03 $\mu W/cm^2$ occurring at 303 nm.

Outdoor measurements were conducted to evaluate the habitat-specific spectral and irradiance characteristic of local solar radiation. On 4/03/07 solar measurements were taken in two freshwater environments (Lake Padden - 48.7033°N latitude and -122.4479°W longitude and Squalicum Creek -48.7615°N and -122.5085°W) and one marine environment (Taylor Dock - 48.7257°N and -122.5070°W) (Table 2). All sites are located within 10 km of Little Squalicum Park groundwater sampling well SB-29 (Figure 2). Measurements were taken within 2.5 hours of solar noon (13:13 PDT ± 2.5 h) under a clear sky (approximately 90 to 100% blue sky) with no vegetative or anthropogenic shading at the sampling locations.

Table 2. Solar measurement conditions for Lake Padden, Squalicum Creek, and Taylor Dock.

	Lake Padden	Squalicum Creek	Taylor Dock
Latitude	48.7033°N	48.7615°N	48.7257°N
Longitude	-122.4479°W	-122.5085°W	-122.5070°W
Date	4/3/2008	4/3/2008	4/3/2008
Time (PDT)	15:16	13:36	10:45
Measurement depths (cm)	surface, 10, 50, 100, 175	surface, 10, 50	surface, 10, 50, 100, 250
Depth to bottom (cm)	30, 40, 80, 130, 205	30, 40, 80	30, 40, 80, 130, 280
Air temperature (°C)	15.5	18.3	8.9
Water temperature (°C)	10.6	9.4	8.9
Field Notes	[a]	[b]	[c]

[a] Very light air movement. Greater than 90% blue sky. Instrument placed on bottom (sloping boat ramp) for all measurements.

[b] Calm (no breeze). Greater than 90% blue sky. Rippling of water surface due to stream-flow. Instrument placed on bottom of stream for all measurements. Measurements conducted at least 0.3 m from shoreline.

[c] Slight breeze. Greater than 90% blue sky. Instrument suspended from dock for all measurements. Measurements were performed within 1.5 hours of low tide.

When possible, the detector was oriented parallel to the water surface. Spectral irradiance was measured from 300 to 700 nm in 1 nm increments. Measurements were conducted at the water surface (detector not submerged) and at several depths at each location. Multiple scans (five or ten) were averaged for each depth to reduce wavelength-to-wavelength signal variability. Spectral data were stored and later analyzed using LI1800UW software. Data files were integrated to provide separate measurements of total instantaneous PAR, UVA, and UVB.

In the laboratory, spectral irradiance was assessed using different LL and LL+UVR designs. The lighting variables that were altered in the lighting design were lamp type, combinations of lamps, presence of Nitex screen, and distance from the top of the test chambers to light source. These lighting variables were optimized as described in Fortner (2009). The final configuration for the LL treatment was four Sylvania (Danvers, MA) Cool White Plus (CWP) bulbs with no Nitex screen and a vertical distance of 100 cm between the lamps and the top of the test chamber. Nitex did not decrease overall irradiance without altering spectral character and so was not a neutral density filter in this study. The final LL+UVR configuration was identical to the LL treatment except three Q-lab (Cleveland, OH) UVA-340 lamps were also included (Figure 3). Each lighting treatment was replicated for a total of four WET tests conducted at one time. Hereafter, these replicates are referred to as T1-LL and T2-LL (two replicates for the standard laboratory light treatments), and as T1-LL+UVR and T2-LL+UVR (two replicates for the standard laboratory lighting plus UVR treatments).

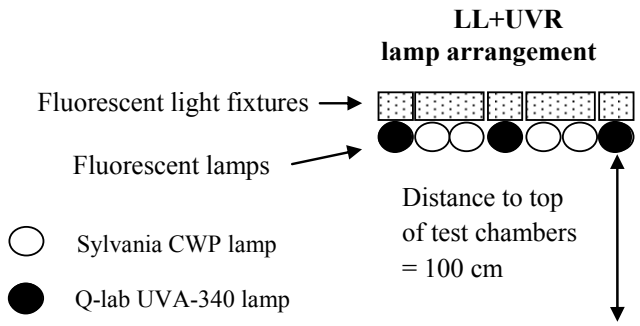

Figure 3. Final LL+UVR lamp arrangement.

The range of spectral irradiance received by the 40 WET test chambers in each of the four light treatments (160 test chamber positions total) was measured from 6/01/07 to 6/12/07 before the first *D. magna* WET tests as described in Fortner (2009) using bulbs that had been seasoned for at least 100 hours. These measurements included wavelengths from 300 nm to 700 nm in 1 nm increments and are hereafter referred to as Round 1 (R1) irradiance measurements. A second set of measurements was conducted from 1/02/08 to 1/10/08 after the final *P. promelas* WET tests; these measurements are the Round 2 (R2) measurements. Total PAR, UVA, and UVB instantaneous irradiance, and illuminance were calculated for R1 and R2 measurements. The spectroradiometer software calculates illuminance (lux) based on the waveband 370 to 790 nm, but R1 and R2 measurements were performed from only 300 to 700 nm. Consequently, all R1 and R2 illuminance values are "short-range" values, calculated from 370 to 700 nm. "Full-range" (370 to 790 nm) illuminance calculations were performed on measurements collected after R2 (post-R2) for T1-LL and T1-LL+UVR treatments. Total daily doses (J/cm^2) of PAR, UVA, and UVB were calculated by multiplying average treatment irradiance by the length of time the lights were on for the LL and LL+UVR daily photoperiods.

Groundwater Sampling

Groundwater samples were collected from Little Squalicum Park well SB-29 by Herrenkohl Consulting LLC (Bellingham, WA) according to standard protocols (Integral, 2005). Samples were collected on 6/29/07, 9/13/07, 10/15/07, and 11/28/07 for Tier I and Tier II *D. magna* WET tests and for Tier I and Tier II *P. promelas* WET tests, respectively. A Waterline (Envirotech, Ltd.) electronic water depth probe was used to measure depth to water during the 6/29/07 and 9/13/07 sampling events. The depth was estimated during the final two sampling events by noting the depth at which a probe first became wet when lowered into the well (10/15/07) and when water first started to move through a tube attached to a peristaltic pump (11/28/07). A minimum of three well volumes was removed prior to sample collection. During the first sampling event, a 0.5" Teflon bailer was used to purge the well and collect the sample. For the remainder of the sampling events, a Teflon-lined low-density polyethylene (LDPE) tube attached to a peristaltic pump was used for well purging and sample collection. The ends of the tube were rinsed with distilled water between sampling events. A Cole-Palmer Masterflex E/S portable

sampler with a 12 volt direct current (DC) power supply was used for sampling on 9/13/08 and 10/15/08 and a Solinst Model 410 peristaltic pump attached to a 12 volt DC power supply was used for sampling on 11/28/08. The sampling tube was positioned 2" to 6" above the well bottom during sampling in all cases.

All samples were collected in new (or pesticide-grade acetone and acid-rinsed) 4-L Level 1 amber-glass containers. When well yield allowed, the sampling containers were filled to minimize the headspace. At the end of sample collection, sample containers were closed, placed in a cooler with ice, and transported to Western Washington University (WWU). Within 2.5 hours of completion of sample collection, the groundwater was removed from ice and stored in the dark at 5^{o}C until needed for test solution preparation. The sample collected for the Tier I *D. magna* WET test (only) was decanted into a new (or pesticide-grade acetone and acid-rinsed amber-glass container) prior to storage to remove most of the sediment that was also collected during this sampling event. A YSI DO200 meter was used to measure dissolved oxygen of groundwater stock solutions prior to test solution preparation. *D. magna* and *P. promelas* Tier I and Tier II WET tests were initiated within 36 hours of each sampling event.

Laboratory Experiments

All glassware was cleaned according to USEPA protocols. Water for synthetic hard freshwater (USEPA, 2002a) and for fluoranthene stock solutions was Barnstead NANOpure®. Unless noted, all chemicals were reagent grade.

Fluoranthene Chemistry

For the groundwater bioassays, fluoranthene was used as the reference control for acute phototoxic effects and is hereafter identified as the phototoxicity control. This PAH was used because: 1) the phototoxic potential of fluoranthene to a variety of aquatic organisms has been thoroughly demonstrated (including to *D. magna* and *P. promelas* (Fortner, 2009)); 2) fluoranthene absorbs radiation across the UVA and UVB portions of the optical spectrum (Jinno Laboratory, 2008); and 3) fluoranthene does not break down as quickly in the laboratory as other phototoxic PAHs (e.g. anthracene). The concentrations used for the phototoxicity controls for

D. magna and *P. promelas* were determined with the fluoranthene reference test. The only objective of the phototoxicity control in the Tier 1 and 2 WET tests was to confirm that the LL+UVR light treatment could induce phototoxic effects.

Saturated aqueous stock solutions of fluoranthene were prepared using a shell-coating procedure based on methods employed by Cho et al. (2003) and Weinstein and Diamond (2006). All of the unfiltered phototoxicity control stock solutions were prepared within 90 hours of the start of exposures with 98% purity fluoranthene (Sigma-Aldrich, MO). Fluoranthene (0.199 -0.201 g) was added to pesticide-grade acetone (50 ml) and stirred until dissolved. The resulting solution was then added to a new 4-L amber-glass sampling bottle. The uncapped bottle was placed horizontally on a roller apparatus and rotated at approximately one revolution per 70 seconds until all the acetone had evaporated. Synthetic, aerated hard water was added to the bottle and the bottle was covered in aluminum foil and capped. Solutions were stirred at approximately 25^{o}C for 24 hours. Based on the work of Weinstein and Diamond (2006) these fluoranthene solutions were considered to be at steady state.

Within 24 hours of the start of the test exposure, the saturated fluoranthene solutions were filtered twice through a glass column (3.6 cm (ID) x 26.5 cm) packed with glass wool (Pyrex® 3950 borosilicate glass fiber with 8 µm pore size). Each filtration elutriate was collected in an amber-glass 4-L sampling bottle and served as the fluoranthene stock solution for the fluoranthene reference test, the phototoxicity controls, and chemical analyses. Filtered stock solutions were stored in the dark and at room temperature.

The filtered stock solutions were used as the highest exposure concentrations in the fluoranthene reference test and were diluted with synthetic hard freshwater for the lower concentrations. Since the fluoranthene preparation resulted in variable saturation concentrations in solution, a sub-sample of the filtered fluoranthene stock solution was analyzed by Am Test, Inc. (Redmond, WA) with USEPA Method 625 (Semivolatile Organic Compounds by Isotope Dilution GC/MS). This sub-sample of each filtered stock solution was collected on the first day of the relevant toxicity test and stored in an amber bottle at 5^{o}C in the dark for three to eleven days before being sent to Am Test, Inc. Chemical concentrations listed in this study were measured in the filtered

stock solutions and calculated for lower test solution concentrations using the measured concentration of the stock solution, unless noted otherwise. These are identified in this study as measured or nominal concentrations, respectively.

WET Testing

The acute toxicity of fluoranthene and Little Squalicum Park groundwater to *D. magna* and *P. promelas* under LL and LL+UVR lighting scenarios was assessed using USEPA WET methods 2021.0 and 2000.0 (USEPA, 2002a). Deviations from USEPA WET methods are noted. Synthetic hard freshwater (USEPA, 2002a) was made within 14 days of each test and used as the dilution water for preparation of all groundwater treatments and all controls for each WET test. Test chambers were 250 ml borosilicate glass beakers. Since glass and plastic can attenuate and filter UVR (ASTM, 2002), the USEPA methods were modified to exclude the use of covers on all test chambers during WET tests, unless noted otherwise.

In all *D. magna* tests, organism mortality counts were performed at 24 and 48 h. Mortality was defined as lack of translational organism movement and lack of organism rotation in the water column for more than 15 seconds after gentle prodding with a plastic pipette tip. In all *P. promelas* test, mortality counts were performed at 24, 48, 72, and 96 h. Fish mortality was defined as lack of organism movement through the water column for more than 10 seconds after gentle prodding with a plastic pipette tip. Deceased organisms were removed from test chambers during mortality counts. Test chambers were removed from the testing room for all water quality, organism mortality measurements, and solution renewals when conducted.

Laboratory Design

The testing room was designed so that each of the four assays could be considered an independent test with the two lighting treatments in duplicate. To accomplish this, a laboratory at WWU was divided into four separate test compartments using 4 mm black polyethylene sheeting (Poly-America, TX) that isolated the light treatments and individual light fixtures of the same light treatment from one another. One large plastic container was placed on a table in each test compartment directly under the appropriate bank of lights. Test chambers were placed in

each plastic container to match the 40 positions measured in R1 and R2 irradiance measurements (*Lighting Measurements in Field and Laboratory* Section, page 15). Water was maintained in these water baths at the same height as the test chamber solutions throughout the WET tests to moderate the effects of room temperature fluctuations on test chamber solutions (Figure 4).

Organism Loading and Exposure

Test chambers were assigned in random order using a stratified randomization approach to position test chambers within irradiance blocks. The R1 irradiance measurements in T1-LL+UVR were used to assign each test chamber position (1-40) to one of four irradiance blocks, based on the level of incident UVA irradiance measured at each position. The assignments were accomplished by first ranking the positions in order from lowest to highest incident UVA irradiance. Blocks were then identified as block A through block D, with block A containing the ten positions with the highest UVA irradiance and block D containing the ten positions with the lowest UVA irradiance. Blocks B and C represented intermediate levels and each included ten positions. The same block assignments were used for T1-LL, T2-LL, and T2-LL+UVR WET tests.

In the *D. magna* and *P. promelas* fluoranthene reference test, fluoranthene test chambers were assigned first, followed by negative control, and then positive control chambers. Fluoranthene chambers were randomly assigned to the lowest nine UVA irradiance positions in block D. Since these chambers occupied almost all of block D, the remaining 31 positions had to be divided into four new blocks of seven or eight contiguous positions to accommodate control chamber replicates. One negative control replicate was assigned to each block and then randomly assigned to a position within that block. The same process was used for positive control assignments; a replicate from each positive control concentration was assigned to a block and then randomly assigned to a position within that block.

Figure 4. Example of one WET test replicate (bottom photo). Two trays with test chamber positions numbered 1-40 (top photo) were used to accommodate test chambers in a static water bath (bottom photo). Colors illustrate different solution types (purple = fluoranthene reference control; green = negative dilution water control; blue = positive reference control; red = groundwater).

Tier I and Tier II WET groundwater test arrangements were similar. When used, the phototoxicity control (fluoranthene) was assigned first. Groundwater test chambers were assigned next, followed by the negative control, and finally positive control chambers. The phototoxicity control chamber was always assigned to the lowest UVA irradiance position in block D. In Tier I tests, a replicate from each groundwater concentration was randomly assigned to a position in the highest and lowest UVA blocks. A negative control and a positive control replicate (when used) were then randomly assigned to one B or C block position. In Tier II tests, a negative control replicate and a replicate from each groundwater concentration were randomly assigned to positions in each of the four UVA blocks. A replicate from each positive control concentration was then randomly assigned to the remaining available positions in each block, starting with the lowest UVA level positions in each block. A true random number generator was used to generate random sequences for all randomization procedures (Haahr, 2008).

Solutions were brought to the desired test temperature, and then organisms were loaded (USEPA, 2002a). Loading occurred one WET test at a time, with T1-LL being completely loaded followed by T1-LL+UVR, T2-LL, and T2-LL+UVR. Organisms were loaded into test chambers for each WET test so that chambers had the same number of organisms ± 10%. Once organism loading began for T1-LL, all the CWP lamps were turned on for T1-LL and T1-LL+UVR WET tests; this was recorded as the exposure start time (time 0 hours) for both T1 tests. For the T2-LL and T2-LL+UVR WET tests, all CWP lamps were turned on when organism loading for T2-LL commenced; this was recorded as the exposure start time (time 0 hours) for both T2 tests. Once chambers had been loaded for a WET test, they were returned to assigned positions in the appropriate test compartment.

A 16:8 h light:dark photoperiod was used for LL and LL+UVR treatments. For the LL treatments, the 16 h light period was CWP only illumination. For the LL+UVR treatments, the light period included 4 hours of LL CWP-only illumination followed by twelve hours of CWP plus UVR-illumination with the UVR-340 lamps turned on. These T1 and T2 light cycles were repeated every 24 hours during testing. The actual duration of UVA-340 lamp illumination varied somewhat during the course of tests due to experimental design constraints. During the Tier II WET tests, for example, *D. magna* had 23.5 hours (T1-LL+UVR) to 24 hours

(T2-LL+UVR) of UVA-340 lamp illumination out of 48 hours total. Out of 96 hours, *P. promelas* had 46 hours (T1-LL+UVR) to 46.75 hours (T2-LL+UVR) of UVA lamp illumination. Daily and total hours of CWP and UVA-340 lamp illumination were recorded for all tests in all experiments (Table 3).

Table 3. Daily lamp illumination for LL and LL+UVR light treatments for fluoranthene reference, Tier I, and Tier II WET tests. Unless noted by shading, illumination conditions met the requirements of the experimental design protocol.

Experiment	Test Day	T1-LL treatment [a]	T2-LL treatment [a]	T1-LL+UVR treatment [a]		T2-LL+UVR treatment [a]	
		CWP [b] lamp hours	CWP [b] lamp hours	CWP [b] lamp hours	UVA-340 [c] lamp hours	CWP [b] lamp hours	UVA-340 [c] lamp hours
D. magna fluoranthene reference test	1	16	16	16	11.5	16	12
	2	16	16	16	12	16	12
	Total	32	32	32	23.5	32	24
P. promelas fluoranthene reference test	1	16	16	16	12	16	12
	2	16	16	16	12	16	12
	3	16	16	16	12	16	12
	4	16	16	16	12	16	12
	Total	64	64	64	48	64	48
D. magna Tier I test	1	16 [d]	16 [e]	16 [d]	12 [f]	16 [e]	12 [g]
	2	16	16	16	12	16	12
	Total	32	32	32	24	32	24
D. magna Tier II test	1	16 [h]	16	16	11.75 [i]	16	12
	2	16	16	16	11.75	16	12
	Total	32	32	32	23.5	32	24
P. promelas Tier I test	1	16	16	16	12	16	12
	2	16	16	16	12	16	12
	3	16	16	16	12	16	12
	4	16	16	16	12	16	0 [j]
	Total	64	64	64	48	64	36
P. promelas Tier II test	1	16	16	16	11.25	16	12
	2	16	16	16	11.25	16	12
	3	16	16	16	11.75	16	10.75
	4	16	16	16	11.75	16	12
	Total	64	64	64	46	64	46.75

[a] T1= test replicate 1; T2 = test replicate 2; LL treatments consisted of four CWP lamps; LL+UVR treatments consisted of four CWP and three UVA-340 lamps.
[b] Sylvania (Danvers, MA) Cool White Plus (CWP) fluorescent lamps.
[c] Q-Lab (Cleveland, OH) UVA-340 fluorescent lamps.
[d] Groundwater chambers were inadvertently covered for up to the first 6 hours of exposure period by clear polystyrene Petri dish covers.
[e] Groundwater chambers were inadvertently covered for up to the first 5 hours of exposure period by clear polystyrene Petri dish covers.
[f] Groundwater chambers were inadvertently covered for up to the first 2 hours of exposure period by clear polystyrene Petri dish covers.
[g] Groundwater chambers were inadvertently covered for up to the first 45 minutes of exposure period by clear polystyrene Petri dish covers.
[h] Groundwater chambers were inadvertently covered for up to the first 2.75 hours of exposure period by clear polystyrene Petri dish covers.
[i] Groundwater chambers were inadvertently covered for up to the first 30 minutes of exposure period by clear polystyrene Petri dish covers.
[j] UVA-340 lamps were not turned on during the last 24 hour photoperiod.

Fluoranthene Reference Tests

The fluoranthene reference tests were used to determine the concentration of fluoranthene to be used for the phototoxicity control. *D. magna* (< 24 hours) and *P. promelas* (6-day) were obtained from Aquatic Biosystems (Fort Collins, CO) and were acclimated to room temperature before being loaded into test chambers. *D. magna* were acclimated in shipping water. Fish were acclimated to test room temperature and synthetic hard freshwater by diluting the shipping solution with 400 ml synthetic freshwater every 15 minutes for 2-3 hours. WET tests were initiated the same day the organisms were received.

The *D. magna* and *P. promelas* fluoranthene reference tests were conducted on 6/19/07 and 7/13/07, respectively. On the first day of both tests, fluoranthene stock solutions were stirred slowly with a stir bar in containers covered with aluminum foil to bring solutions to room temperature. A 1 ml glass pipette attached to an aeration pump was used for 1-1.5 hours to slowly increase dissolved oxygen above 6 mg/L. Stock solutions were then diluted and distributed to appropriate test chambers.

Three fluoranthene solution concentrations were chosen for each experiment, based on phototoxic effects of fluoranthene reported in the literature for these organisms. Fluoranthene exposures were conducted in triplicate with ten organisms per test chamber. Test chamber solution volumes were 100 ml for *D. magna* and 200 ml for *P. promelas*. The positive control for the *D. magna* test was zinc as $ZnSO_4 \cdot 7\ H_2O$ (Sigma Chemical Company, St. Louis, MO). The positive control for the *P. promelas* test was potassium chloride (JT Baker, Phillipsburg, NJ). A summary of the test designs are included as Table 4.

In the *D. magna* test, temperature was measured at time 0, 24, and 48 h in six test chambers in each WET test (one at each corner and two near the middle positions of the test setup) with calibrated mercury thermometers. Temperatures were also recorded throughout the experiment at 15 minute intervals in a surrogate test chamber located in block B or C of test T2-LL. Other water quality measurements were conducted for test solutions at time 0 and 48 h. These tests

included: total hardness as $CaCO_3$ (CHEMetrics K-4520 hardness test kit), pH (Accumet AR25), conductivity (Orion 130), and dissolved oxygen (YSI DO200).

In the *P. promelas* test, temperature was measured at time 0, 24, 48, 72, and 96 h in six test chambers of each WET test (one at each corner and two near the middle positions of the test setup) with a Digi-Sense® DataLogR RTD meter and temperature probe. Temperatures were also recorded at 15 minute intervals in one of the test chambers from block B or C of WET test T2-LL. Other water quality measurements were conducted for test solutions at 0, 24 h, pre-renewal (48 h), post-renewal (48 h), and at 72 and 96 h. These tests included pH (Accumet AR25), conductivity (Orion 130), and dissolved oxygen (YSI DO200). Total hardness as $CaCO_3$ was measured at 0 h, pre-renewal (48 h), post-renewal (48 h) and at 96 h following EPA Method 130.2 (titrimetric) using a Hach Company (Loveland, CO) Hardness Reagent Kit (Hach Method 8226).

Solution renewals for the *P. promelas* tests were conducted with the filtered fluoranthene stock solution, which was removed from 5°C storage, covered, stirred and aerated gently for 1 hour, and then diluted to prepare test solutions. These solutions were prepared within 5 hours of test chamber renewals. Solution renewal was conducted at 48 h for all test chambers in a test compartment (e.g. T1-LL or T1-LL+UVR) before renewing solutions in the next test compartment. Renewals were accomplished by removing 80-90% test chamber solution (approximately 160-180 ml) and then gently pouring approximately 160-180 ml new solution into the test chamber. Two hours before each renewal, fish were fed one to two drops of concentrated *Artemia salina* nauplii in 25 ppt synthetic saltwater (Forty Fathoms Bioassay Mixture, Aquatic Ecosystems, Inc, Apopka, FL).

D. magna Groundwater Tests (48 h Static)

D. magna (< 24 hours) were obtained from Aquatic Biosystems (Fort Collins, CO) and were acclimated to test room temperature in shipping water before being loaded into test chambers. The WET tests were initiated the same day the organisms were received by the laboratory.

The Tier I range finding WET test was conducted on 6/30/07 with the groundwater sample collected on 6/29/07 and the Tier II definitive WET test was conducted on 9/14/07 with the groundwater sample collected on 9/13/07. On the first day of both tests, samples were stirred slowly with a stir bar in containers covered with aluminum foil. A 1 ml glass pipette attached to an aeration pump was used for 1-1.5 hours to slowly increase the dissolved oxygen above 6 mg/L. After aeration, Tier I and Tier II sample solutions were diluted to selected concentrations with synthetic freshwater and distributed to appropriate test chambers. For the Tier II groundwater sample, 1 liter of undiluted, aerated sample was also poured into a 1 L amber-glass sampling bottle and stored in the dark for less than six days at 5°C until it was shipped to Am Test, Inc. for chemical analysis.

For the Tier I test, four groundwater concentrations (100 ml volume) were prepared according to a logarithmic dilution series. The groundwater exposures were conducted in duplicate with five organisms per test chamber. For the Tier II test, five groundwater concentrations (100 ml volume) were used with four replicates per concentration and ten organisms per replicate. A phototoxicity control of 4.5 µg/L fluoranthene (measured) was used as the phototoxicity control in the Tier II test. The positive control for the Tier II test was copper as $CuSO_4 \cdot 5\ H_2O$ (Sigma Chemical Company, St. Louis, MO). The negative control in the Tier I and Tier II tests was synthetic freshwater. A summary of the test design is included as Table 4.

Temperature was measured at time 0, 24, and 48 h in six test chambers in each WET test (one at each corner and two near the middle positions of the test setup) using a Digi-Sense® DataLogR RTD meter and temperature probe. Temperatures were also recorded throughout each experiment at 15 minute intervals in one of the test chambers from block B or C of WET test T2-LL . Water quality measurements were conducted for test solutions at time 0 and 48 h as described for *D. magna* fluoranthene reference tests[1]. The test chambers were removed from the testing room for all water quality and organism mortality measurements.

[1] Tier II pH measurements were conducted using an Accumet AB15. Tier II total hardness measurements were conducted following EPA Method 130.2 (titrimetric) using a Hach Company (Loveland, CO) Hardness Reagent Kit (Hach Method 8226).

During both Tier I and Tier II WET tests, clear polystyrene lids that were used during organism loading to minimize laboratory personnel exposure to test solutions were inadvertently left on some of the test chambers during the beginning of the exposures (Table 3). In the Tier I WET test, covers were left on top of all T1-LL and T1-LL+UVR groundwater chambers for 5-6 hours of the exposure on the first test day. This means that T1-LL and T1-LL+UVR groundwater chambers were covered for the first 5-6 hours of CWP-only illumination and T1-LL+UVR groundwater chambers were covered for 2 hours of UVA-340 illumination. Covers were left on top of all T2-LL and T2-LL+UVR groundwater containers for 4-5 hours of the exposure on the first test day. This means that T2-LL and T2-LL+UVR groundwater chambers were covered during the first 4-5 hours of CWP-only illumination and T2-LL+UVR groundwater chambers were covered for 45 minutes of UVA-340 lamp illumination. In the Tier II WET test, covers were left on top of all T1-LL and T1-LL+UVR groundwater chambers for 1.75-2.75 hours of the exposure on the first test day. This means T1-LL and T1-LL+UVR groundwater chambers were covered during the first 1.75-2.75 hours of CWP-only illumination and T1-LL + UVR groundwater chambers were covered for 30 minutes of UVA-340 lamp illumination.

Table 4. Experimental designs for *D. magna* and *P. promelas* fluoranthene reference, Tier I, and Tier II WET tests.

Experiment	Test solutions	Concentrations [a]	Replicates	Organisms /
D. magna fluoranthene reference test	Fluoranthene	7.00, 78.4, 140.0 [b] µg/L	3	10
	Positive control Total zinc (as $ZnSO_4$)	0.050, 0.10, 0.50, 1.0, 5.0 mg/L	3	10
	Negative control	Synthetic hard water	4	10
P. promelas fluoranthene reference test	Fluoranthene	7.1, 21.3, 57 µg/L	3	10
	Positive control KCl	310, 630, 940, 1300, 1570 mg/L	2	5
	Negative control	Synthetic hard water	4	10
D. magna Tier I test	Groundwater - LL	0.1, 1.0, 10, 100 % (v/v)	2	5
	Groundwater – LL+UVR	0.1, 1.0, 10, 100 % (v/v)	2	5
	Fluoranthene	---	---	---
	Positive control	---	---	---
	Negative control	Synthetic hard water	1	10
D. magna Tier II test	Groundwater - LL	3.1, 6.3, 12.5, 25.0, 50.0% (v/v)	4	10
	Groundwater – LL+UVR	0.047, 0.094, 0.38, 0.75% (v/v)	4	10
	Fluoranthene	4.5 [b] µg/L	1	10
	Positive control Total Cu (as $CuSO_4$ $5H_2O$)	5.7, 11, 23, 46, and 92 µg/L	3	10
	Negative control	Synthetic hard water	4	10
P. promelas Tier I test	Groundwater – LL	0.1, 1.0, 10, 100 % (v/v)	2	5
	Groundwater – LL+UVR	0.1, 1.0, 10, 100 % (v/v)	2	5
	Fluoranthene	18.0 [c] µg/L	1	10
	Positive control - KCl	1.1 g/L	1	10
	Negative control	Synthetic hard water	1	10
P. promelas Tier II test	Groundwater – LL	2.50, 5.0, 10.0, 20.0, 40.0% (v/v)	4	10
	Groundwater – LL+UVR	0.31, 0.63, 1.25, 2.50, 5.00%	4	10
	Fluoranthene	16.0 [b] µg/L	1	10
	Positive control -KCl	0.088, 1.10, 1.3, 1.5, and 1.8 g/L	3	10

[a] All concentrations are nominal unless noted otherwise.
[b] Concentration was measured.
[c] Estimated based analytical results from *P. promelas* fluoranthene reference test.

P. promelas Groundwater Tests (96 h Static-Renewal)

P. promelas (7 and 8 days old for Tier I and Tier II test, respectively) were obtained from Aquatic Biosystems (Fort Collins, CO) and were acclimated to test room temperature and synthetic hard freshwater by diluting the shipping solution with 400 ml synthetic hard freshwater every 15 minutes for 1.5 hours before being loaded into the test chambers. The WET tests were initiated the same day the organisms were received by the laboratory.

The Tier I range finding WET test was conducted on 10/16/07 with the groundwater sample collected on 10/15/07 and the Tier II definitive WET test was conducted on 11/29/07 with the groundwater sample collected on 11/28/07. For the Tier I test, four groundwater concentrations (200 ml volume) were prepared according to a logarithmic dilution series. The groundwater exposures were conducted in duplicate with five organisms per test chamber. A phototoxicity control of 18.0 µg/L fluoranthene (nominal) was included.

For the Tier II experiment, five groundwater concentrations (200 ml volume) were used with four replicates per concentration and ten organisms per replicate. A phototoxicity control of 16 µg/L fluoranthene (measured) was used as the phototoxicity control. The positive control for the Tier II test was KCl (JT Baker, Phillipsburg, NJ). The negative control in the Tier I and Tier II tests was synthetic hard freshwater. A summary of the test designs are included as Table 4. Temperature, and water quality measurements were performed as described for the *P. promelas* fluoranthene tests[2] (*Fluoranthene Reference Tests* Section, page 28).

Groundwater sample bottles were removed from 5°C storage on the first experiment day, covered in aluminum foil, and slowly stirred for 1-2 hours to allow the solutions to warm to room temperature. In the Tier I test, dissolved oxygen was measured before the sample was stirred (3.76 mg/L). A 1 ml glass pipette attached to an aeration pump was used for approximately 45 minutes to slowly increase the dissolved oxygen above 6 mg/L. After Tier II samples had been

[2] Tier I and II pH measurements were conducted using an Accumet AB15 meter.

stirred, half of the solution from each of two sample containers was poured into one new sample container and stirred again. A dissolved oxygen reading was taken (8.84 mg/L). Though this initial reading was above 6.0 mg/l, subsequent readings were erratic; this was a problem later attributed to fouling of the DO probe membrane. Since past groundwater samples had low D.O., the stock solution was gently aerated for 15 minutes. In Tier I and Tier II tests, one liter of stock solution was poured into a 1-L amber-glass sampling bottle and stored in the dark (for less than 14 days for the Tier I experiment, and less than 6 days for the Tier II experiment) at 5°C until it was shipped to Am Test, Inc. for chemical analysis. The remaining portions of groundwater in the original Tier II sample containers were combined into a new container, stored in the dark at 5°C, and used to prepare renewal solutions at 48 h. On renewal day, samples were removed from 5°C storage, covered, stirred for 1.5 hours, aerated gently for 15 minutes, and then diluted to prepare test solutions. Solutions were prepared within 5 hours of test chamber renewals. Renewals were performed as described in the *Fluoranthene Reference Tests* Section, page 28.

Statistical Analyses

Statistical analyses of biological effects data collected from groundwater samples in Tier I and Tier II WET tests and from positive controls in Tier II WET tests were analyzed using CETIS *v. 1.6.4* (Tidepool Scientific, McKinleyville, CA) for *D. magna* (48 h survival) and for *P. promelas* (96 h survival). NOEC and LOEC estimates were determined using the software's automated decision-tree feature for comparisons, which is based on recent USEPA methodologies (CETIS, 2007). A significance level of $\alpha = 0.05$ was used. Comparisons were made to the negative controls. Percent minimum significant differences (pMSD) were calculated for all Tier II comparison tests, using software defaults, and compared to the recommendations for toxicity test sensitivity (USEPA, 2000).

Point estimates and associated confidence intervals were determined based on the USEPA (2002a) statistical guidance, unless noted otherwise. Based on the goals of Ecology, it was decided that the study would focus on phototoxicity occurring at low effects levels, so LC25

point estimates were used to compare groundwater toxicity between light treatments in all Tier II tests. For statistical reasons, LC50 point estimates were used for comparisons in Tier I tests.

When Abbott's correction was applied by the software, it was applied using the empirical response observed in the negative control. An iterative Maximum Likelihood Estimate (MLE) linear regression method, using the Probit model, was used when two or more partial mortalities occurred. When using this model, concentrations were log10 transformed, and the threshold response was optimized (modeled). Regressions were weighted based on the number of individuals within a replicate, the specific probability density and the expected variance. The chi-square goodness of fit alpha value was set to $\alpha = 0.05$. Though the USEPA (2002a) guidance infers pooling replicate concentrations within a WET test when calculating point estimates, the guidance does not implicitly require or recommend pooling. Since important residual information may be lost when replicates are pooled (Freund, 1971; CETIS, 2007), replicates were not pooled when calculating point estimates. The Spearman-Karber method was used for data analysis: 1) when there were two or more partial responses in the test solutions, but the Probit model was a poor fit for the data as determined by the chi-square goodness of fit test (significant heterogeneity); and 2) when there was only one partial response in test solutions. The software automatically minimized the trim level for all calculations. Results with trim values of zero percent, and greater than zero percent, were generated using the standard Spearman-Karber and the trimmed Spearman-Karber calculations, respectively. When there were no partial responses, but responses occurred on both sides of the median effect level (0 and 100%), point estimates were determined with the Binomial method. When it was not possible to analyze a data set using one of the one of methods described above, the data were analyzed using Linear Interpolation (using standard software defaults).

Results

The results of the evaluation of laboratory lighting options are detailed in Fortner (2009). Final laboratory lighting choices are presented here along with outdoor radiation measurements. Results of the fluoranthene reference tests and groundwater exposures for *D. magna* and *P. promelas* are also detailed here.

Habitat-Specific Local Solar Measurements

Wavelengths from 300 to 700 nm were detected at the water surface and spectral irradiance decreased with depth at all locations (Figure 5). Lower UVR wavelengths were not detectable at greater depths. Attenuation of PAR, UVA, and UVB with depth was greatest in Squalicum Creek. The spectral distribution of sunlight was similar at all sites at the water surface. Irradiance at the water surface was greater at the Squalicum site than at the other two sites (up to a 27% difference in PAR, a 33% difference in UVA, and a 50% difference in UVB compared to the other two sites) (Table 5). Including all sites and depths, PAR to UVA ratios ranged from 10:1 to 4800:1; UVA to UVB ratios ranged from 17:1 to 965:1.

The Lake Padden site was chosen as the solar reference for light treatment design. This site was selected because: 1) in general, irradiance levels were intermediate compared to levels detected at the other two locations; 2) differences in radiation with depth in the lake are chiefly dependent upon vertical stratification parameters (in contrast to lotic systems in which there may be significant longitudinal stratification as well); and 3) the lake represents the type of environment naturally inhabited by the species used in this study. Measurements collected at the site showed little to no UVR at 50, 100, and 250 cm depths (Figure 5). Since the focus of this study is on PAH phototoxicity resulting from organism exposure to UVR, water surface and 10 cm depths were chosen as habitat-specific bounding conditions for lighting in this study.

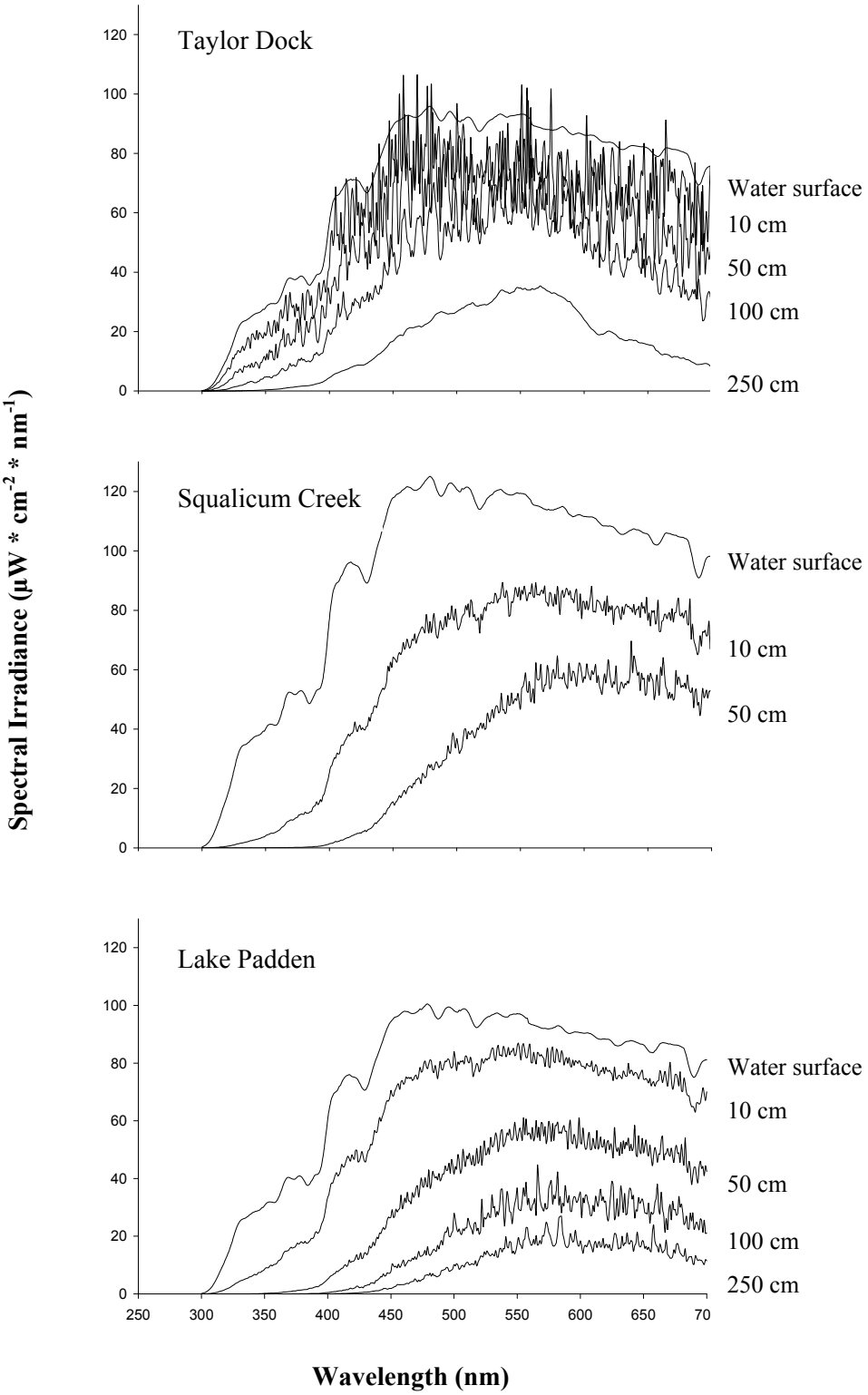

Figure 5. Spectral irradiance with depth for Taylor Dock, Squalicum Creek, and Lake Padden, Bellingham, WA on April 3, 2007.

Table 5. Summary of habitat-specific PAR, UVA, and UVB irradiance ($\mu W/cm^2$) at Taylor Dock, Squalicum Creek, and Lake Padden field sites.

	PAR (400-700nm)	UVA (320-400 nm)	UVB (300-320 nm)	Ratio PAR	: UVA	: UVB
Taylor dock						
water surface	25310	2512	82.11	308	31	1
10 cm	21430	1851	48.94	438	38	1
50 cm	18520	1184	19.55	947	61	1
100 cm	14300	550.9	4.161	3437	132	1
250 cm	6249	85.85	0.1484	42109	579	1
Squalicum Creek						
water surface	33030	3519	136.5	242	26	1
10 cm	21860	603.1	3.529	6194	171	1
50 cm	12090	14.57	0.2808	43056	52	1
Lake Padden						
water surface	26730	2700	94.26	284	29	1
10 cm	21940	990.0	10.90	2013	91	1
50 cm	12770	74.57	0.07726	165286	965	1
100 cm	6785	5.323	0.06242	108699	85	1
175 cm	3508	0.7309	0.04279	81982	17	1

Laboratory Lighting Characteristics

The R1 and R2 measurements were used to determine the lighting characteristics of the final LL and LL+UVR treatments. These measurements represent the quality and quantity of radiation incident at the top of the test chambers during the WET tests. They are compared to the habitat-specific solar radiation at Lake Padden.

Final LL Treatment Characteristics

Both LL treatments (T1-LL and T2-LL) emitted wavelengths from 300 to 700 nm. The lowest irradiance levels occurred from about 300 to 400 nm and from about 650 to 700 nm (Figure 6). At 300 nm, irradiance values of the LL treatments ranged from 0.00958 to 0.01841 $\mu W/cm^2$ 17 to 32-fold higher than the average NEI from 300 to 320 nm). Overall irradiance averaged 1.650 $\mu W/cm^2$ for UVB (ranging from 1.259 to 2.090 $\mu W/cm^2$), 7.768 $\mu W/cm^2$ for UVA (ranging from 6.336 to 9.013 $\mu W/cm^2$) and 356.3 $\mu W/cm^2$ for PAR (ranging from 299.8 to 393.2 $\mu W/cm^2$) (Table 6). Based on these values, the average PAR to UVA to UVB ratio for LL treatments was 216 to 4.7 to 1. Illuminance averaged 1248 lux and ranged from 1054 to 1380 lux. Average PAR, UVA, and UVB irradiance, and illuminance levels all decreased between R1 and R2 (Table 6). Average daily doses of UVB, UVA, PAR under LL treatments were 0.095, 0.45, and 20.51 J/cm^2, respectively, under a 16:8-h light:dark photoperiod (Table 7).

The spectral distribution was characterized by low overall irradiance levels in the LL treatment compared to Lake Padden field conditions (Figure 6). On average, UVB irradiance was 57-fold less than water surface UVB and 7-fold less than UVB at 10 cm. UVA was 348-fold less than water surface UVA and 127-fold less than UVA at 10 cm. PAR was 75-fold less than water surface UVA and 62-fold less than PAR at 10 cm (Table 7).

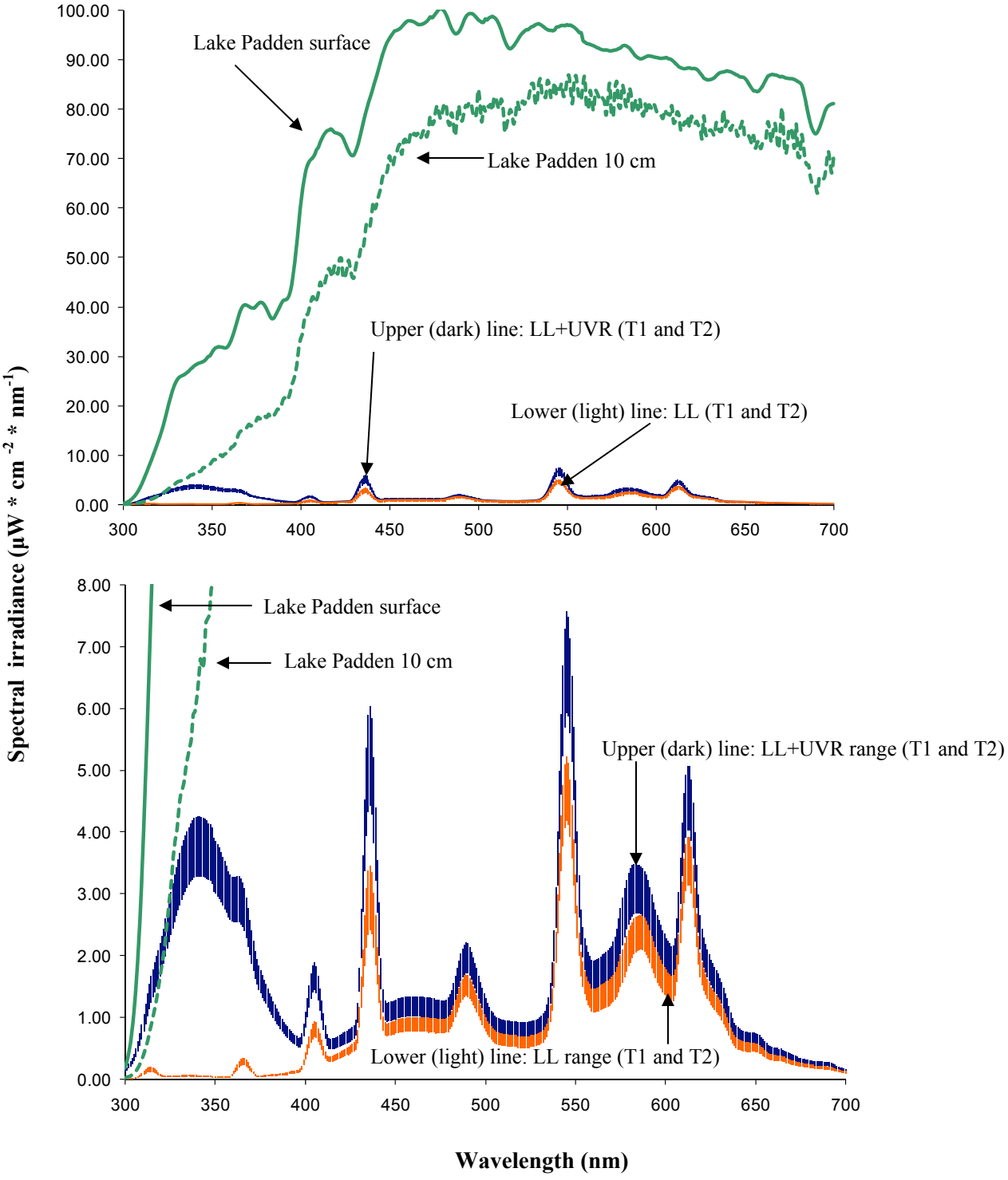

Figure 6. Comparison of the spectral irradiance of habitat-specific radiation (Lake Padden, WA) with all LL and LL+UVR light treatment replicates (i.e. T1-LL, T2-LL, T1-LL+UVR, T2-LL+UVR) at two different scales. Bands in lower graph represent the range of irradiance measured at each wavelength during R1 and R2 for all test chambers in all test replicates.

Table 6. Summary of UVB (300-320nm), UVA (320-400nm), PAR (400-700nm) instantaneous irradiance in μW/cm^2, and illuminance levels in lux for LL and LL+UVR test replicates, measured before (R1) and after (R2) all WET testing, and after R2 measurements (post R2).

Measurement parameters [a]		T1-LL [b]				T1-LL+UVR [b]				T1-LL and T1-LL+UVR
		R1 [c]	R2 [c]	Average R1 and R2	Percent change R1 and R2	R1 [c]	R2 [c]	Average R1 and R2	Percent change R1 and R2	Percent difference
UVB	Ave	1.697	1.490	1.593	-12.3%	20.39	19.32	19.85	-5.26%	170.3%
	Max	1.895	1.707	1.766	-4.2%	22.43	21.08	21.72	-3.87%	169.9%
	Min	1.449	1.259	1.365	-16.1%	17.46	16.68	17.07	-10.92%	170.4%
UVA	Ave	7.825	7.185	7.505	-8.2%	196.5	192.1	194.3	-2.23%	185.1%
	Max	8.485	7.940	8.208	-6.1%	215.0	209.0	212.0	-1.26%	185.1%
	Min	6.936	6.336	6.655	-10.2%	169.6	167.2	168.4	-3.29%	184.8%
PAR	Ave	362.2	340.9	351.6	-5.9%	492.0	472.9	482.5	-3.87%	31.4%
	Max	392.9	372.1	382.3	-4.2%	536.2	512.4	524.3	-2.47%	31.3%
	Min	316.0	299.8	307.9	-9.0%	428.9	418.3	423.6	-4.80%	31.6%
LUX [d]	Ave	1271	1199	1235	-5.7%	1670	1613	1641	-3.35%	28.2%
	Max	1380	1307	1343	-4.1%	1817	1749	1783	-1.85%	28.1%
	Min	1108	1054	1081	-9.3%	1457	1430	1444	-4.27%	28.7%

Measurement parameters [a]		Post-R2 measurements [f]	Post-R2 measurements [f]	Percent difference
LUX [e]	Max	1345	1803	29.1%
	Min	1110	1492	29.4%

Measurement parameters [a]		T2-LL [b]				T2-LL+UVR [b]				T2-LL and T2-LL+UVR
		R1 [c]	R2 [c]	Average R1 and R2	Percent change R1 and R2	R1 [c]	R2 [c]	Average R1 and R2	Percent change R1 and R2	Percent difference
UVB	Ave	1.895	1.516	1.706	-20.0%	19.99	19.39	19.69	-3.02%	168.1%
	Max	2.090	1.667	1.878	-16.6%	21.72	21.15	21.43	4.02%	167.8%
	Min	1.590	1.294	1.458	-23.6%	17.44	16.80	17.12	-5.51%	168.6%
UVA	Ave	8.279	7.783	8.031	-6.0%	194.1	191.8	192.9	-1.19%	184.0%
	Max	9.013	8.447	8.702	-4.9%	211.0	208.4	209.6	4.33%	184.1%
	Min	7.268	6.826	7.047	-7.9%	170.9	167.4	169.2	-3.43%	184.0%
PAR	Ave	365.0	357.2	361.1	-2.1%	499.3	482.3	490.8	-3.43%	30.5%
	Max	393.2	387.1	389.9	-1.2%	542.0	523.2	532.2	-2.22%	30.9%
	Min	323.5	315.6	319.6	-4.2%	437.3	422.4	429.9	-4.79%	29.4%
LUX [d]	Ave	1268	1253	1260	-1.2%	1688	1638	1663	-3.00%	27.6%
	Max	1365	1357	1360	-0.2%	1833	1775	1803	-1.75%	28.0%
	Min	1124	1107	1116	-3.5%	1474	1433	1454	-4.33%	26.3%

[a] Calculated from test chamber positions 1-40 for each test replicate.
[b] T1 = test replicate 1; T2 = test replicate 2.
[c] R1 = Round 1 measurements; R2 = Round 2 measurements.
[d] "Short-range" illuminance calculated from 370 to 700 nm.
[e] "Full-range" illuminance calculated from 370 to 790 nm.
[f] "Full-range" illuminance measurements were conducted after R2 measurements.

Table 7. Comparison of irradiance and total daily dose levels for Lake Padden solar reference and LL and LL+UVR laboratory light treatments.

	UVB (300-320nm)	UVA (320-400nm)	PAR (400-700nm)
Lake Padden surface			
Irradiance ($\mu W/cm^2$)	94.26	2700	26730
Daily dose (J/cm^2)	NM	NM	NM
Lake Padden 10 cm			
Irradiance ($\mu W/cm^2$)	10.90	990.0	21940
Daily dose (J/cm^2)	NM	NM	NM
LL (T1 and T2)			
Average Irradiance[a] ($\mu W/cm^2$)	1.650	7.768	356.1
Range of irradiance[b] ($\mu W/cm^2$)	1.675 ± 0.4155	7.675 ± 1.339	346.5 ± 46.70
Average daily dose[c] (J/cm^2)	0.09504	0.4474	20.51
Range daily dose[d] (J/cm^2)	0.09648 ± 0.02393	0.4421 ± 0.07713	19.96 ± 2.690
LL+UVR (T1 and T2)			
Average Irradiance[e] ($\mu W/cm^2$)	19.77	193.6	486.7
Range of irradiance[f] ($\mu W/cm^2$)	19.20 ± 2.520	189.6 ± 22.40	480.2 ± 61.58
Average daily dose[g] (J/cm^2)	0.9491	8.811	41.54
Range of daily dose[h] (J/cm^2)	0.9259 ± 0.1328	8.633 ± 1.045	40.70 ± 5.350

NM = not measured

[a] Average of all LL test chamber position irradiance in T1 and T2 (i.e. mean of T1 Average R1 and R2 and T2 Average R1 and R2 (Table 6)).

[b] Calculated from LL T1 and T2 Max and Min for both R1 and R2 (Table 6).

[c] Average irradiance of LL (T1 and T2) (in W/cm^2) times light portion of the LL photoperiod (16 hours) in seconds.

[d] Range of irradiance of LL (T1 and T2) (in W/cm^2) times light portion of LL photoperiod (16 hours) in seconds.

[e] Average of all LL+UVR test chamber position irradiance in T1 and T2 (i.e. mean of T1 Average R1 and R2 and T2 Average R1 and R2 (Table 6)).

[f] Calculated from LL+UVR T1 and T2 Max and Min for both R1 and R2 (Table 6).

[g] Average irradiance of LL+UVR (T1 and T2) (in W/cm^2) times daily UVR lamp illumination (12 hours) in seconds, plus average irradiance of LL (T1 and T2) (in W/cm^2) times light portion of photoperiod (16 hours) in seconds.

[h] Range of irradiance of LL+UVR (T1 and T2) (in W/cm^2) times daily UVR lamp illumination (12 hours) in seconds, plus range of irradiance of LL (T1 and T2) (in W/cm^2) times light portion of photoperiod (16 hours) in seconds.

Both of the LL light treatments met the design priorities identified in Fortner (2009). Although the LL treatments were within or exceeded USEPA WET testing illuminance recommendations by up to 28%, the LL treatment was otherwise representative of WET laboratory lighting recommendations where UVR is not specifically considered. The treatment delivered levels of illuminance and PAR, UVA, and UVB irradiance that were considerably less than levels measured at the surface and just below the surface in local aquatic environments (Figures 7 and 8).

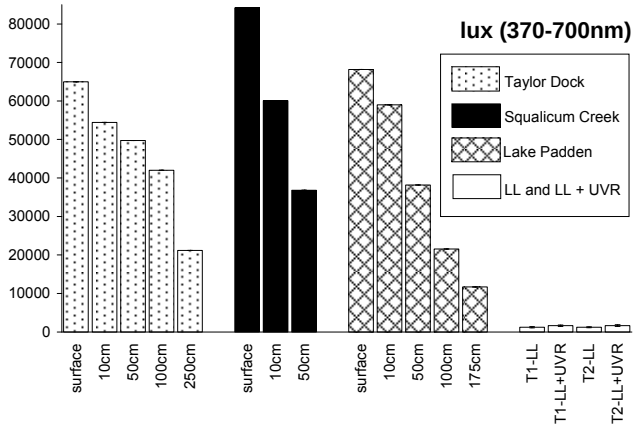

Figure 7. Comparison of illuminance levels at different water depths in three local aquatic environments (measured on April 3, 2007, 13:13 PDT ± 2.5h) with LL treatment illuminance levels. Levels are based on "short-range" illuminance, measured from 370 to 700 nm (instead of from 370 to 790 nm). Bars associated with light treatments represent the range of measurements collected during R1 and R2 measurements.

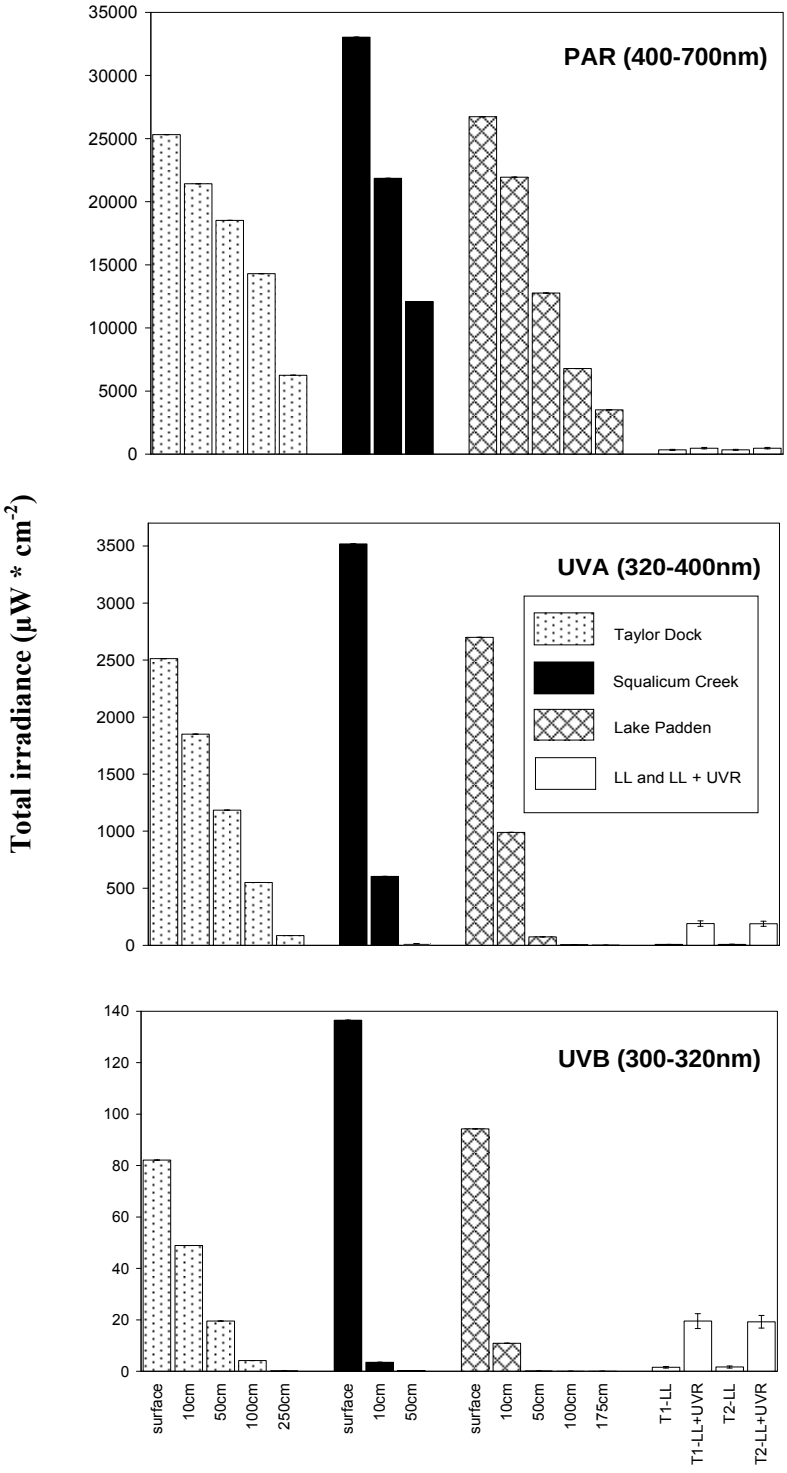

Figure 8. Comparison of PAR, UVA, and UVB irradiance at different water depths in three local aquatic environments (measured on April 3, 2007, 13:13 PDT ± 2.5h) with LL and LL+UVR light treatment irradiance levels. Bars associated with light treatments represent the range of measurements collected during R1 and R2 measurements.

Final LL+UVR Treatment Characteristics

The two LL+UVR treatments (T1-LL+UVR and T2-LL+UVR) were also evaluated before (R1) and after (R2) all WET testing. Both replicates emitted wavelengths from 300 to 700 nm (Figure 6). The lowest irradiance levels occurred from about 650 to 700 nm. The lowest LL+UVR irradiance values occurred around 300 nm, a point at which values ranged from 0.118 to 0.116 $\mu W/cm^2$ (211 to 298 times the average NEI occurring from 300 to 320 nm). Average R1 and R2 values presented in Table 6 for T1-LL+UVR and T2-LL+UVR were used to determine overall average irradiance and the daily dose for both LL+UVR treatments (Table 7). Overall irradiance averaged 19.77 $\mu W/cm^2$ for UVB (ranging from 16.68 to 21.72 $\mu W/cm^2$), 193.6 $\mu W/cm^2$ for UVA (ranging from 167.2 to 212.0 $\mu W/cm^2$) and 486.7 $\mu W/cm^2$ for PAR (ranging from 418.3 to 542.0 $\mu W/cm^2$). Based on these values, the average PAR to UVA to UVB ratio for LL treatments was 25 to 9.8 to 1. Illuminance averaged 1652 lux with a range of 1430 to 1833. As illustrated in Table 6, average PAR, UVA, and UVB irradiance, and illuminance levels all decreased between R1 and R2. Average daily doses of UVB, UVA, and PAR under LL+UVR treatments were 0.9491, 8.811, and 41.54 J/cm^2, respectively, under a 16:8-h light:dark photoperiod supplemented with 12 hours of UVR (Table 7).

LL+UVR spectral distribution was characterized by low overall irradiance levels compared to field conditions. All wavelength irradiance values measured from 325 to 700 nm for the LL+UVR treatment replicates were lower than at the surface of Lake Padden and at 10 cm depth in Lake Padden. From 300 to 325 nm, irradiance was greater in the laboratory than in Lake Padden at 10 cm depth (Figure 6). On average, UVB irradiance in the laboratory was 5-fold less than water surface UVB and 2-fold greater than UVB at a depth of 10 cm. UVA in the laboratory was 14-fold less than water surface UVA and 5-fold less than UVA at 10 cm. PAR in the laboratory was 55-fold less than water surface UVA and 45-fold less than PAR at 10 cm (Table 7).

The LL+UVR light treatments met the design priorities, which included incorporating spectral characteristics of the environment in the laboratory (Fortner, 2009). The LL+UVR treatments

exceeded USEPA WET testing illuminance recommendations by 33 to 70%. It was not possible to design laboratory lighting with illuminance levels that met the EPA recommendations and included UVR, so one of the lighting design priorities was to have illuminance values less than two times the EPA recommended maximum and less than habitat-specific values measured locally, both of which were achieved (Figure 7). PAR and UVA irradiance levels were considerably lower than levels measured at Lake Padden at the water surface and at a 10 cm depth, and lower than most levels measured at Taylor Dock and Squalicum Creek (Figure 8). UVB irradiance levels in the laboratory were not lower than those measured underwater at Lake Padden, but they were lower than levels measured underwater at Taylor Dock. UVB, UVA, and PAR irradiance were within the range of levels reported in many phototoxicity laboratory studies (Fortner, 2009) and of levels reported in a survey of spectral irradiance in open water marshes at a depth of 10 cm (Barron et al., 2000).

WET Test Results

Analytical results for groundwater samples used in Tier I and Tier II tests are provided in Table 1. Water-quality data for all WET tests are summarized in Appendix A.

Lighting

Under the 16:8-h light:dark photoperiod used for LL treatments, total daily doses of PAR, UVA, and UVB, based on R1 and R2 instantaneous irradiance averages for LL treatments, were 20.51, 0.4474, and, 0.09504 J/cm^2, respectively (Table 7). Under the 16:8-h light:dark (plus 12 hours UVR) photoperiod used for LL+UVR treatments, total daily doses of PAR, UVA, and UVB were 41.54, 8.811, and 0.09504 J/cm^2, respectively. In some cases, UVA-340 lamps were not illuminated for 12 full hours during a 24 hour exposure period (Table 3). The resultant daily doses that occurred during those periods are presented in Table 8.

Table 8. Daily doses of PAR (400-700 nm), UVA (320-400 nm), and UVB (300-320 nm), for *D. magna* and *P. promelas* WET tests, based on number of hours of daily lamp illumination when experimental design conditions were not met (see Table 3).

Light treatments	Illumination (hours / day)			Total daily dose[a] (J/cm^2)		
LL+UVR exceptions [b]	16	+	11.75 [c]	41.10	8.637	0.9313
	16	+	11.5 [d]	40.66	8.463	0.9135
	16	+	11.25 [e]	40.22	8.289	0.8957
	16	+	10.75 [f]	39.35	7.940	0.8601
	16	+	0 [g]	20.51	0.4474	0.09504

[a] Average daily irradiance (Table 7) in W/cm^2 times daily lamp illumination in seconds.
[b] Excludes covered test chamber exceptions noted in Table 3.
[c] *D. magna* Tier II test, T1-LL+UVR, UVA-340 lamps, first and second 24 hour exposure periods, and *P. promelas* Tier II test, T1-LL+UVR, UVA-340 lamps, third and fourth 24 hour exposure periods (Table 3).
[d] *D. magna* fluoranthene reference test, T1-LL+UVR, UVA-340 lamps, first 24 hour exposure period (Table 3).
[e] *P. promelas* Tier II test, T1-LL+UVR, UVA-340 lamps, first and second 24 hour exposure periods (Table 3).
[f] *P. promelas* Tier II test, T2-LL+UVR, UVA-340 lamps, third 24 hour exposure period.
[g] *P. promelas* Tier I test, T2-LL+UVR, UVA-340 lamps, fourth 24 hour exposure period.

Fluoranthene Reference Tests: *D. magna* and *P. promelas*

For both *D. magna* and *P. promelas*, fluoranthene toxicity was greater under light treatments supplemented with UVR than under standard laboratory lighting alone (Figures 9 and 10). In the *D. magna* test, a fluoranthene concentration of 7.00 µg/L resulted in 90-100% mortality under LL+UVR treatments but no mortality when used under LL treatments. In the *P. promelas* test, a concentration of 21.3 µg/L fluoranthene resulted in 100% *P. promelas* mortality under LL+UVR treatments and 0-30% mortality under LL treatments. *D. magna* and *P. promelas* LC50 values were 12.1 to 12.8-fold and 3.4 to 3.6-fold less, respectively, under LL+UVR treatments than under LL treatments (Table 9).

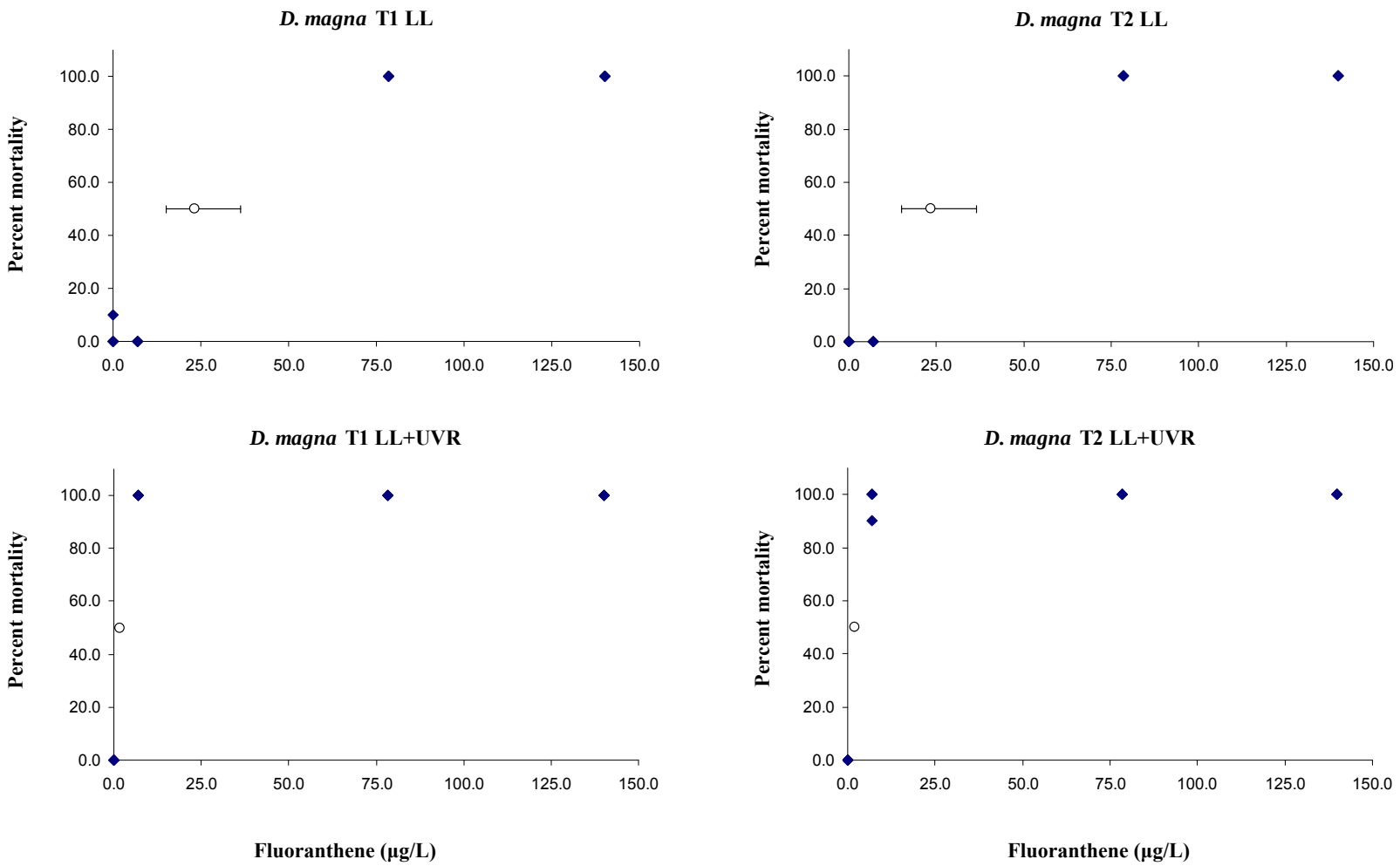

Figure 9. 48 h concentration response results for *D. magna* fluoranthene reference experiments. ○ = LC50 with associated 95% confidence intervals as bars (when available); ♦ = test chamber replicate.

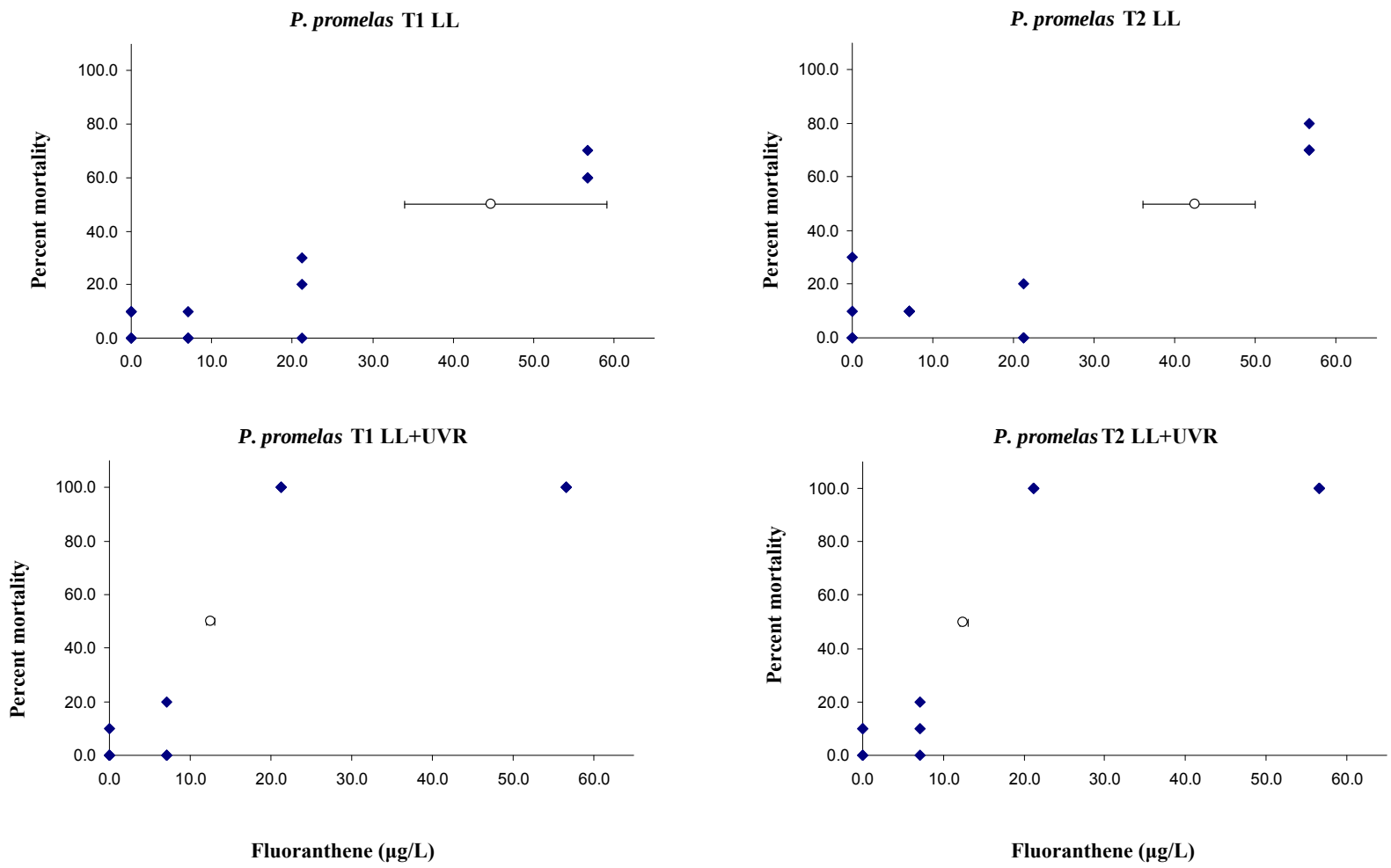

Figure 10. 96 h concentration response results for *P. promelas* fluoranthene reference experiments. ○ = LC50 with associated 95% confidence intervals as bars (when available); ♦ = test chamber replicate.

Table 9**.** 48 h *D. magna* and 96 h *P. promelas* fluoranthene reference test LC50s.

	Test replicate	Light treatment	Fluoranthene concentrations (µg/L)[a]	LC50 (µg/L) (95% CL)	Statistical test
D. magna	T1	LL	7.00, 78.4, 140[b]	23.4 (15.1-36.4)	Spearman Karber
	T1	LL+UVR	7.00, 78.4, 140[b]	1.83 (NA)	Linear interpolation
	T2	LL	7.00, 78.4, 140[b]	23.4 (15.1-36.4)	Spearman Karber
	T2	LL+UVR	7.00, 78.4, 140[b]	1.93 (1.71-2.17)	Linear interpolation
P. promelas	T1	LL	7.1, 21.3, 57	44.7 (33.9-59.1)	Spearman Karber [c]
	T1	LL+UVR	7.1, 21.3, 57	12.6 (12.1-13.1)	Spearman Karber [c]
	T2	LL	7.1, 21.3, 57	42.5 (36.1-50)	Spearman Karber [c]
	T2	LL+UVR	7.1, 21.3, 57	12.5 (12-13.1)	Spearman Karber [c]

NA = Not available
[a] Nominal unless noted otherwise.
[b] Concentration was measured.
[c] Trimmed Spearman Karber

Analytical chemistry

Undiluted groundwater samples collected for *D. magna* Tier II, *P. promelas* Tier I, and *P. promelas* Tier II tests were analyzed. Of the nine ppPAHs, fluoranthene was present at the highest concentration in all three samples (Table 1 and Figure 11). The *D. magna* Tier II sample contained ten of the sixteen priority compounds (total PAH level of 426 μg/L). It contained five ppPAHs with a total concentration of 71 μg/L. The *P. promelas* Tier I sample contained 13 of the 16 priority PAHs and had the highest total PAH level (2286 μg/L) of the three samples. This sample contained at least eight potentially phototoxic PAH (ppPAH) compounds with a total concentration of 869 μg/L. Though the *P. promelas* Tier II groundwater sample contained ten of the sixteen priority compounds and five ppPAH compounds, it had the lowest total PAH level (484 μg/L) and the lowest total concentration of ppPAHs (60.1 μg/L).

In the *D. magna* Tier II tests, nominal concentrations of total ppPAHs in test solution were 2.2, 4.4, 8.8, 17.6, and 35.3 μg/L and 0.039, 0.078, 0.158, 0.315, and 0.623 μg/L under LL and LL+UVR treatments, respectively. In the *P. promelas* Tier I tests, nominal concentrations of total ppPAHs were 0.870, 8.70, 87.0, and 870 μg/L. In the *P. promelas* Tier II tests, nominal concentrations of total ppPAHs were 1.50, 3.0, 6.0, 12.0, and 24.0 μg/L and 0.22, 0.45, 0.89, 1.78, and 3.55 μg/L under LL and LL+UVR treatments, respectively (Table 1).

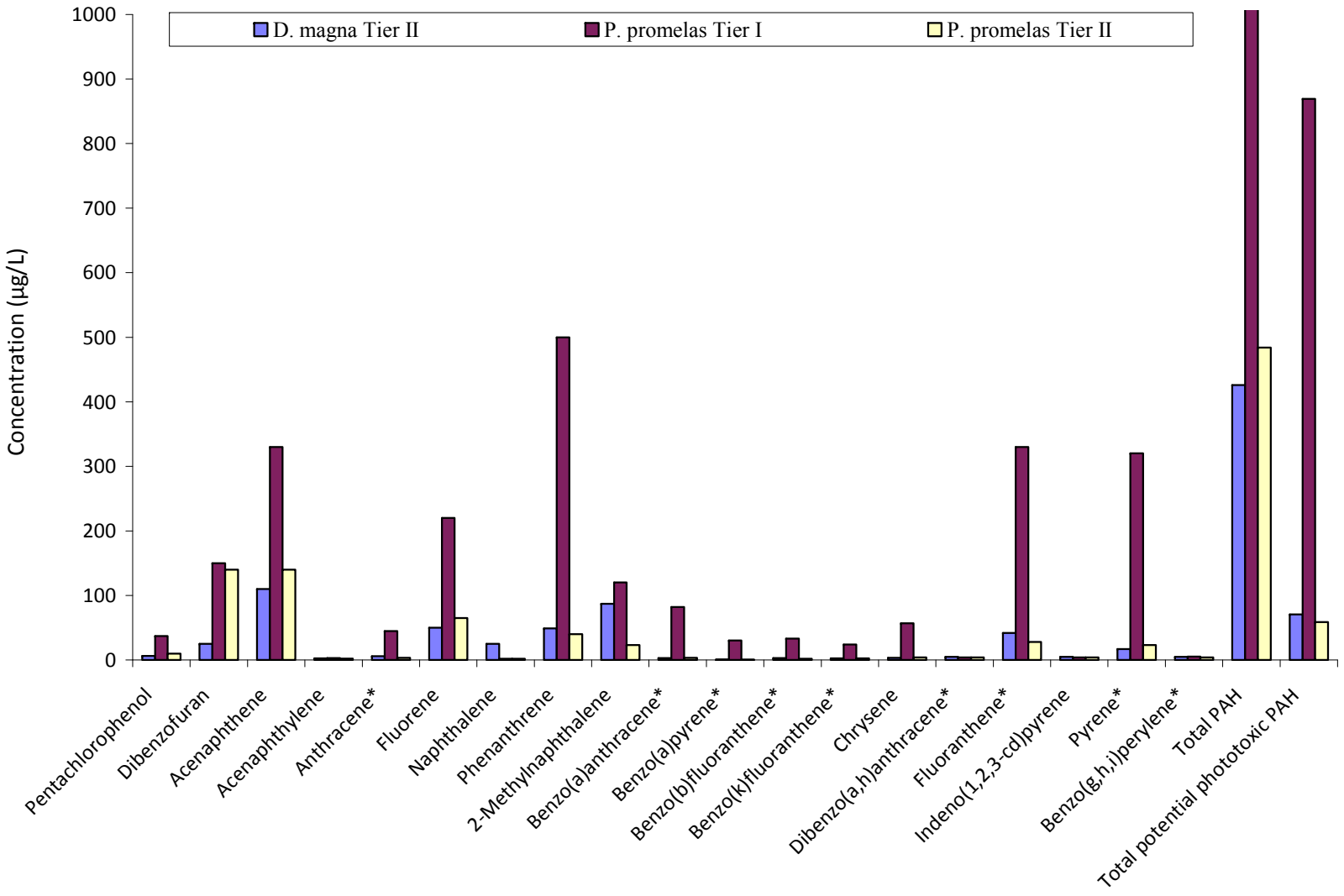

Figure 11. Analytical results for groundwater samples collected from Little Squalicum Park groundwater well SB-29. Asterisks indicate ppPAHs, a designation based on phototoxicity reported in other studies (Figure 1 and Fortner, 2009).

Groundwater Toxicity to *D. magna:* Tier I and Tier II Tests

Tier I and Tier II experiments were conducted to assess the 48 h acute toxicity of groundwater samples to *D. magna* under LL and LL+UVR light treatments. In Tier II WET tests, 4.5 µg/L fluoranthene phototoxicity controls resulted in 0% mortality under LL treatments and 100% mortality under LL+UVR treatments (Table 10).

In all Tier I and Tier II tests, percent mortality increased with increasing groundwater concentration (Figures 12 and 13). Mortality was greater under light treatments supplemented with UVR than under standard laboratory lighting alone. In Tier I tests, at least 100% groundwater was required to produce 100% mortality at 48 h under LL treatments. In contrast, under LL+UVR treatments a 1.0% solution of groundwater achieved the same level of effect. In Tier II tests, a solution of a least 12.5% groundwater was required to produce a mean response of 42.5% mortality under LL treatments at 48 h, whereas a 0.75% groundwater solution produced approximately the same effect level (40% mortality) under LL+UVR treatments (Figure 13). Statistical analyses of Tier I and Tier II mortality data are presented in Table 11. NOEC and LOEC values for both Tier I test replicates were 100-fold less under LL+UVR treatments than under LL treatments. NOEC and LOEC values for both Tier II test replicates were 17-fold less under LL+UVR treatments than under LL treatments. Tier I LC50 values and Tier II LC25 values were 81 to 94-fold and 34 to 44-fold less, respectively, under LL treatments than under LL+UVR treatments and it was determined that the LC25 and LC50 point estimates of the LL treatments did not overlap those of the LL+UVR treatments (Table 11). Percent minimum significant difference (pMSD) values for Tier II comparison tests ranged from 6.74 to 12.3%, all lower than the USEPA recommended maximum value of 23% for *D. magna* acute toxicity tests (USEPA, 2000).

Table 10. 48 h *D. magna* and 96 h *P. promelas* fluoranthene reference control toxicity results for Tier I and Tier II groundwater tests.

	Test replicate	Light treatment	Fluoranthene (µg/L)	Mortality (%)
D. magna				
	T1	LL	N/A	N/A
Tier I	T1	LL+UVR	N/A	N/A
(range-finding test)	T2	LL	N/A	N/A
	T2	LL+UVR	N/A	N/A
	T1	LL	4.5 [a]	0
Tier II	T1	LL+UVR	4.5 [a]	100
(definitive test)	T2	LL	4.5 [a]	0
	T2	LL+UVR	4.5 [a]	100
P. promelas				
	T1	LL	18 [b]	0
Tier I	T1	LL+UVR	18 [b]	100
(range-finding test)	T2	LL	18 [b]	0
	T2	LL+UVR	18 [b]	100
	T1	LL	16.0 [a]	0
Tier II	T1	LL+UVR	16.0 [a]	100
(definitive test)	T2	LL	16.0 [a]	10
	T2	LL+UVR	16.0 [a]	100

N/A = Not applicable
[a] Concentration measured.
[b] Nominal concentraions.

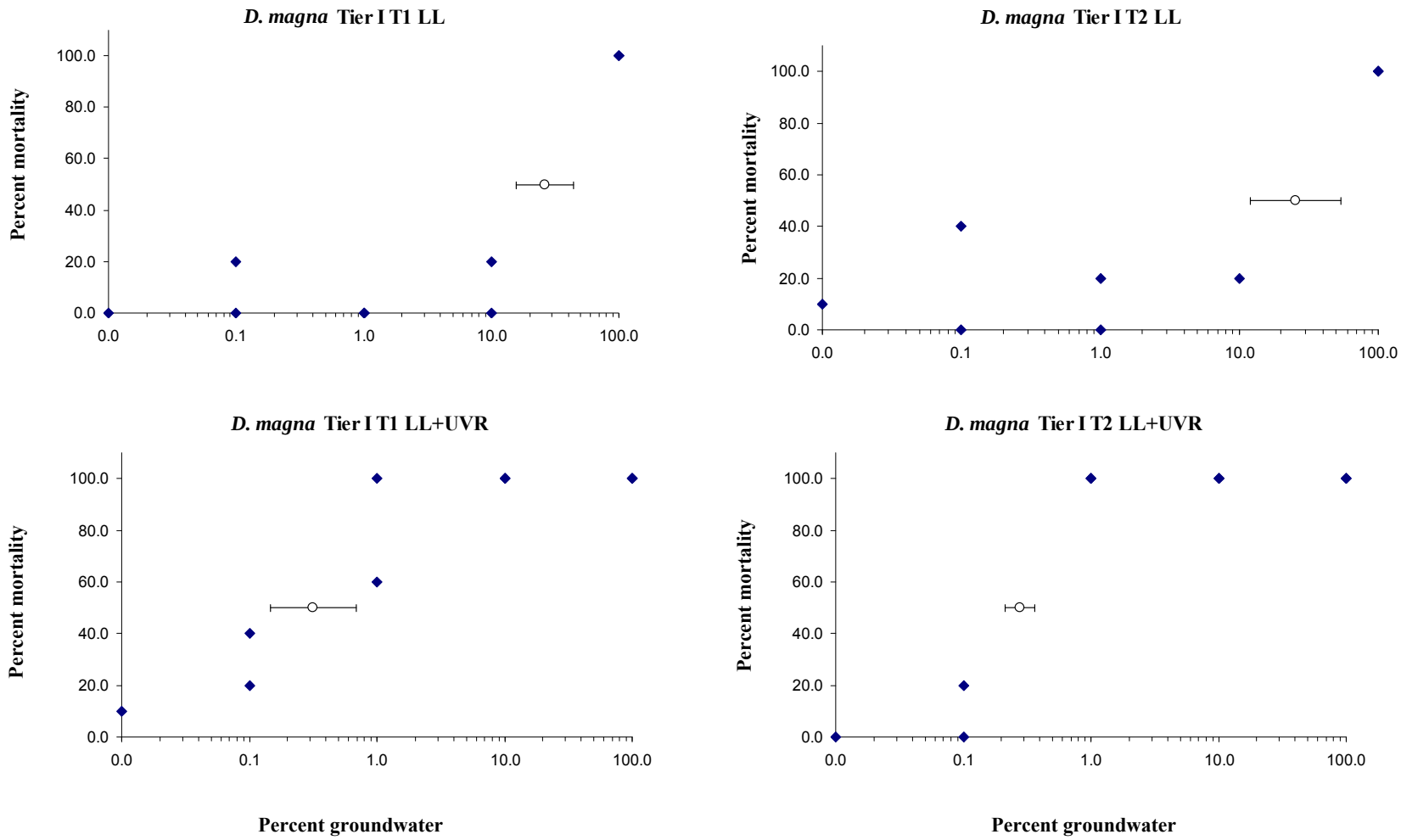

Figure 12. 48 h concentration response results for *D. magna* Tier I groundwater experiments. ○ = LC50 with associated 95% confidence intervals as bars; ♦ = test chamber replicate).

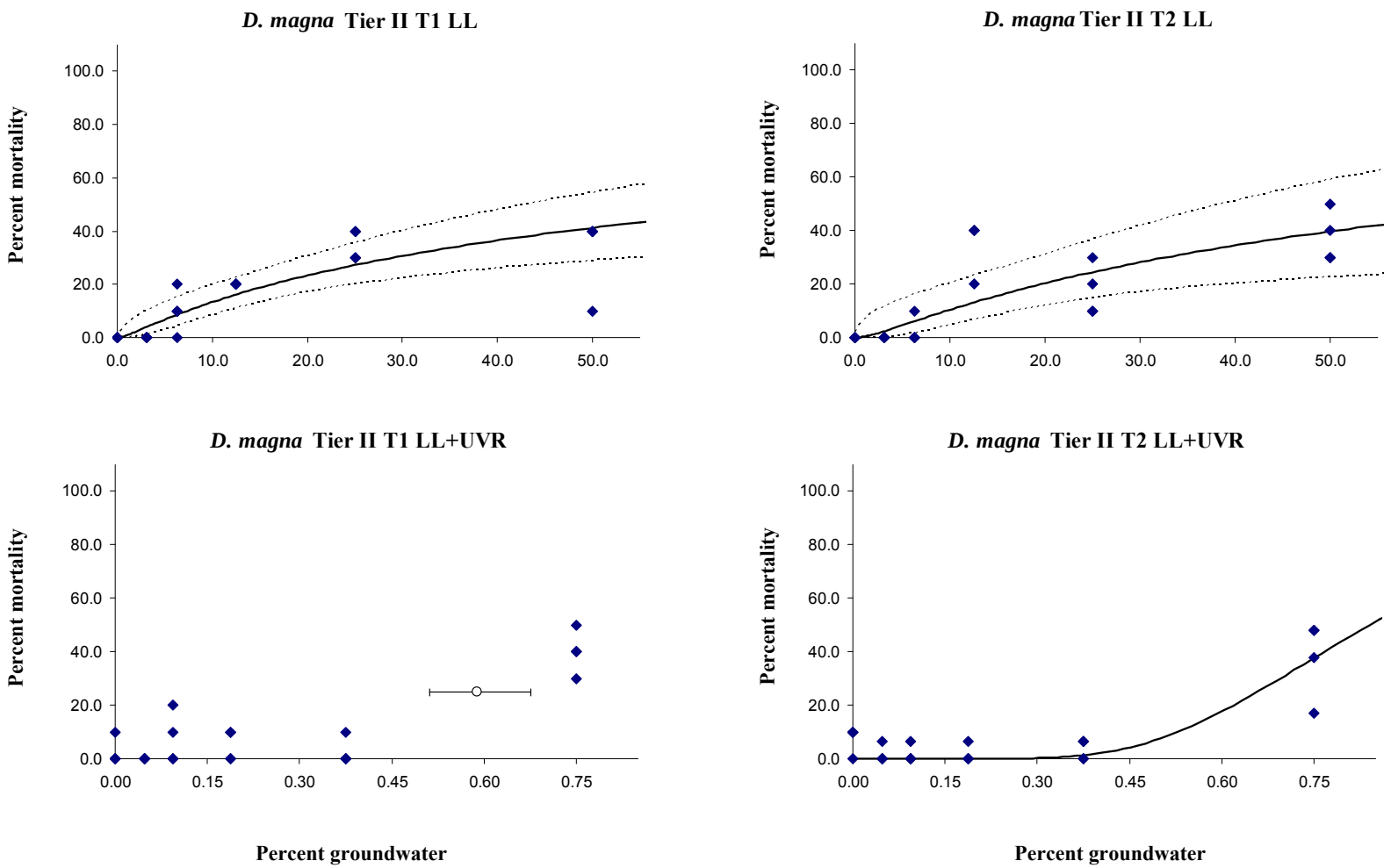

Figure 13. 48 h concentration response results for *D. magna* Tier II groundwater experiments. Solid line is model concentration response prediction, with dashed lines or bars representing associated 95% confidence intervals, when available. ○ = LC25 with associated 95% confidence intervals; ♦ = test chamber replicate.

Table 11. *D. magna* Tier I and Tier II WET test statistical analyses. 48 h LC50s are reported for Tier I (range-finding tests) and 48 h LC25s are reported for Tier II (definitive) tests.

	Test replicate	Light treatment	Groundwater Concentrations (%)	NOEC (%)	LOEC (%)	pMSD (%)	LC50 (%) (95% CL)	Statistical test
Tier I - range finding tests	T1	LL	0.10, 1.0, 10.0, 100	10	100	NA	26.2 (15.6-43.9)	Spearman Karber[a]
	T1	LL+UVR	0.10, 1.0, 10.0, 100	0.1	1	NA	0.316 (.146-.683)	Spearman Karber[a]
	T2	LL	0.10, 1.0, 10.0, 100	10	100	NA	25.6 (12.1-54.2)	Spearman Karber[a]
	T2	LL+UVR	0.10, 1.0, 10.0, 100	0.1	1	NA	0.278 (.213-.364)	Spearman Karber[a]
	Test replicate	Light treatment	Groundwater Concentrations (%)	NOEC (%)	LOEC (%)	pMSD (%)	LC25 (%) (95% CL)	Statistical test
Tier II - definitive tests	T1	LL	3.1, 6.3, 12.5, 25.0, 50.0	6.25	12.5	6.74	22.1 (14.9-35.9)	Probit
	T1	LL+UVR	0.047, 0.094, 0.19, 0.38, 0.75	0.375	0.75	9.9	0.588 (.511-.675)	Linear Interpolation
	T2	LL	3.1, 6.3, 12.5, 25.0, 50.0	6.25	12.5	9.69	25.7 (17.8-41.5)	Probit
	T2	LL+UVR	0.047, 0.094, 0.19, 0.38, 0.75	0.375	0.75	12.3	0.658 (NA)	Probit

NA = Not available
[a] Trimmed Spearman Karber

Groundwater Toxicity to *P. promelas:* Tier I and Tier II Tests

Tier I and Tier II experiments were conducted to assess the 96 h acute toxicity of groundwater samples to *P. promelas* under LL and LL+UVR light treatments. In Tier I tests, 18 µg/L fluoranthene controls resulted in 0% mortality under LL treatments and 100% mortality under LL+UVR treatments (Table 10). In Tier II WET tests, 16 µg/L fluoranthene controls resulted in 0-10 % mortality under LL treatments and 100% mortality under LL+UVR treatments.

In the Tier I test, high levels of mortality occurred in groundwater solutions in the first 24 hours under both light treatments. As a result, an abbreviated (17.5 h) range finding test was conducted prior to the Tier II test to refine the final concentration series (Fortner, 2009). In Tier I and Tier II tests, *P. promelas* mortality increased with increasing groundwater concentration (Figures 14 and 15). In Tier I tests, mean percent mortality at 96 h under LL+UVR treatments was consistently higher or the same at each test concentration (0.10, 1.0, 10.0, and 100% groundwater) than for the same concentrations under LL treatments (Figure 14). A similar trend was observed in Tier II results. Although two different concentration series were used for LL and LL+UVR treatments in Tier II tests, both series included 2.5 and 5.0% groundwater solutions, making it possible to compare effects levels at these concentrations. Mean percent mortality in Tier II tests at 96 h was consistently higher for 2.5 and 5.0% groundwater under LL+UVR treatments than under LL treatments. The only exception was observed in the T2-LL+UVR test in which 2.5% groundwater resulted in 7.8% mean mortality, compared to 10% mean mortality in the T1-LL 2.5% groundwater test (Figure 15).

Statistical analyses of Tier I and Tier II tests are presented in Table 12. In Tier I tests, NOEC and LOEC values for groundwater were the same for all LL and LL+UVR treatments (NOEC = 1% and LOEC = 10%). In Tier II tests, NOEC and LOEC values varied between treatments and between test replicates for the same treatment type. Under LL treatments, Test Replicate 1 values were: NOEC = 5% and LOEC = 10%. Test Replicate 2 values were: NOEC = 20% and LOEC = 40%. Under LL+UVR treatments, Test Replicate 1 values were: NOEC = 5% and LOEC > 5%. Test Replicate 2 values were: NOEC = 2.5% and LOEC = 5%. Percent minimum

significant difference (pMSD) values for Tier II comparison tests ranged from 12.8 to 26%, all lower than the USEPA (2000) recommended maximum value of 30% for *P. promelas* acute toxicity tests. Tier I LC50 values were 3.3 to 5.4-fold lower for LL+UVR than LL treatments. There was very little to no overlap in confidence intervals for LC50 values between Tier I LL and LL+UVR treatments (Table 12). Tier II LC25 values were 1.5 to 3.7-fold lower for LL+UVR than LL treatments, but there was a high degree of overlap in LC25 confidence intervals between treatments.

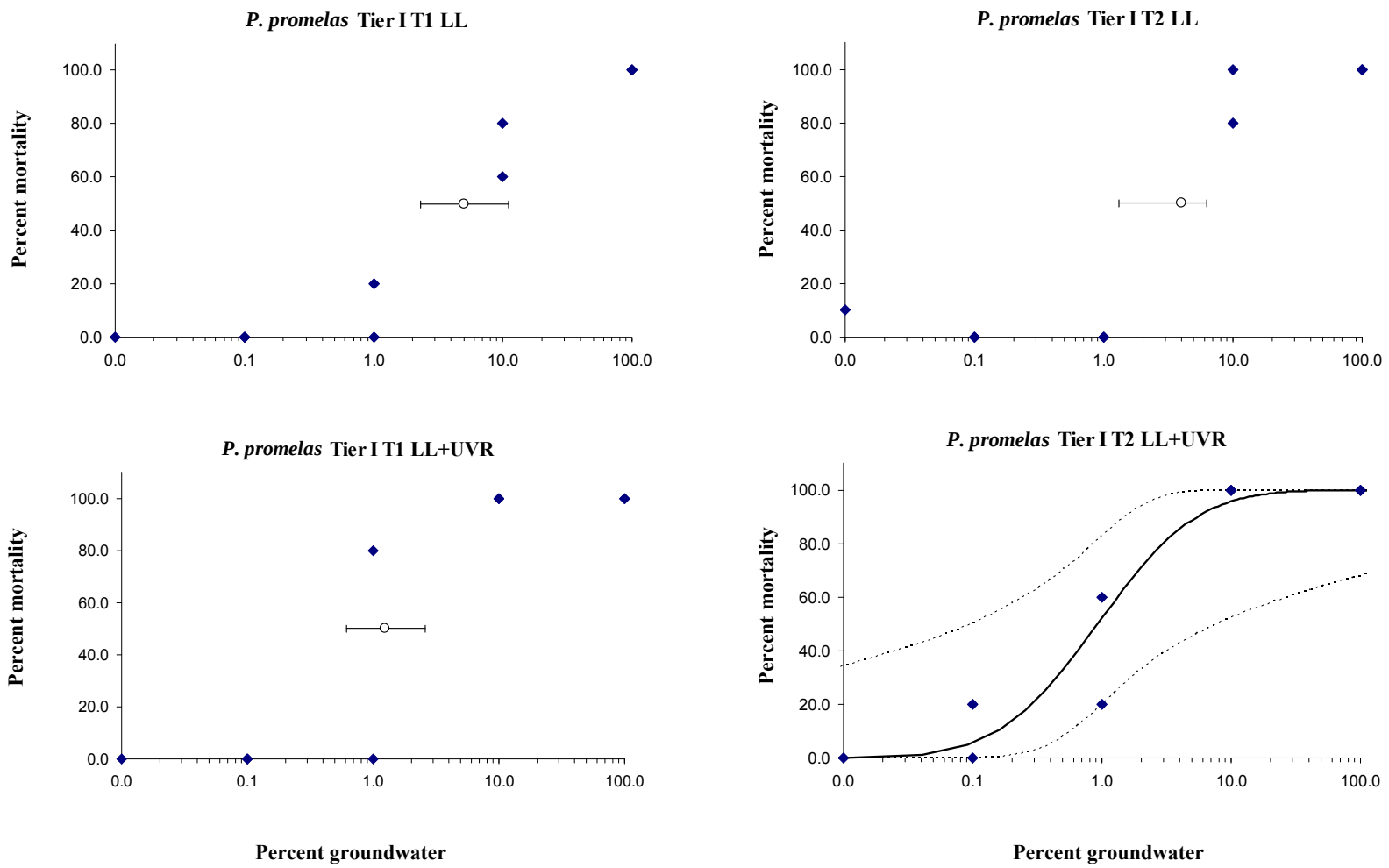

Figure 14. 96 h concentration response results for *P. promelas* Tier I groundwater experiments. Solid line is model concentration response prediction, with dashed lines or bars representing associated 95% confidence intervals. ○ = LC50 with associated 95% confidence intervals; ♦ = test chamber replicate.

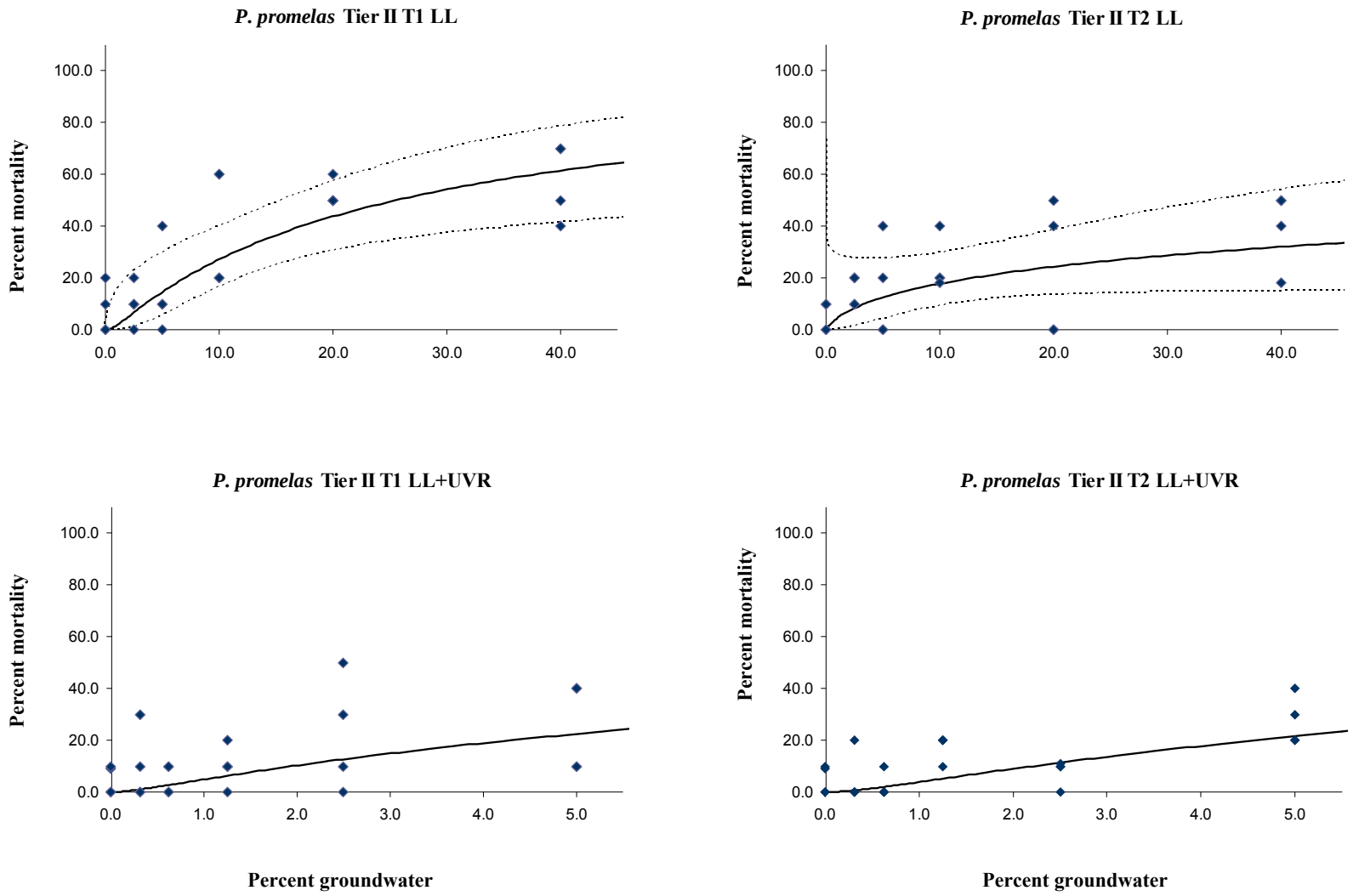

Figure 15. 96 h concentration response results for *P. promelas* Tier II groundwater experiments. Solid line is model concentration response prediction, with dashed lines representing associated 95% confidence intervals, when available.

Table 12. *P. promelas* Tier I and Tier II WET test statistical analyses. 96 h LC50s are reported for Tier I (range-finding tests) and 96 h LC25s are reported for Tier II (definitive) tests.

	Test replicate	Light treatment	Groundwater Concentrations (%)	NOEC (%)	LOEC (%)	pMSD (%)	LC50 (%) (95% CL)	Statistical test
Tier I - range finding tests	T1	LL	0.10, 1.0, 10.0, 100	1	10	NA	5.0 (2.3-11)	Spearman Karber
	T1	LL+UVR	0.10, 1.0, 10.0, 100	1	10	NA	1.23 (0.62-2.6)	Spearman Karber
	T2	LL	0.10, 1.0, 10.0, 100	1	10	NA	4.01 (2.6-6.3)	Spearman Karber
	T2	LL+UVR	0.10, 1.0, 10.0, 100	1	10	NA	0.930 (0.34-2.5)	Probit

	Test replicate	Light treatment	Groundwater Concentrations (%)	NOEC (%)	LOEC (%)	pMSD (%)	LC25 (%) (95% CL)	Statistical test
Tier II - definitive tests	T1	LL	2.50, 5.0, 10.0, 20.0, 40.0	5	10	22.8	9.00 (3.91-14.0)	Probit
	T1	LL+UVR	0.31, 0.63, 1.25, 2.50, 5.00	5	>5	22.9	5.7 (NA)	Probit[a]
	T2	LL	2.50, 5.0, 10.0, 20.0, 40.0	20	40	26.0	21.3 (5.27 ->100)	Probit
	T2	LL+UVR	0.31, 0.63, 1.25, 2.50, 5.00	2.5	5	12.8	6.0 (NA)	Probit[b]

NA = Not available.

[a] T2-LL+UVR control responses were used for T1-LL+UVR Probit analysis.

[b] Groundwater replicates were pooled for Probit analysis.

WET Test Criteria and Quality Assurance

The USEPA (2002a) acute WET testing manual outlines specific protocols for conducting acute WET tests. Among other quality assurance elements, the manual includes recommendations and requirements related to chemical and physical characteristics of test solutions and the use of reference toxicants (positive controls) and dilution water (negative) controls. These recommendations and requirements were followed as closely as possible for this study.

Negative controls were used in all tests. WET guidelines require 90% or greater mean survival in each test. This requirement was met in each of the 24 WET tests conducted in this study, with the exception of the *P. promelas* Tier II T1-LL+UVR test. During solution renewals for this test, two negative control replicates were inadvertently combined so that after renewals one replicate contained two fish and the other contained 16 fish. Fish were not redistributed. Mean negative control survival at 96 h in this test was 85%. Statistical analysis did not correct for negative control mortality.

Positive (reference toxicant) controls were used in all experiments except *D. magna* Tier I tests. To evaluate positive control performance, responses were compared to effects levels reported by other laboratories. Copper (II) sulfate was used as a positive control in *D. magna* Tier II tests. In these tests, total copper LC50 values ranged from 52-60 µg/L. These values are in close agreement with the total copper LC50 value of 60 µg/L reported by Villavicencio et al. (2005) for *D. magna* tests conducted using hard synthetic water (DOC = 0.1 mg/L). In *P. promelas* Tier II tests, LC50s ranged from 0.98 to 1.1 g KCl/L (Fortner, 2009), which are similar to the results from other tests. In a summary of inter-laboratory organism responses to KCl, the USEPA (2002a) reports 96 h LC50s for *P. promelas* that range from 0.83 to 0.94 g KCl/L in tests using moderately hard water, and a local WET laboratory has consistently observed LC50 values of about 1.13 g KCl/L for 96 h acute tests conducted with *P. promelas* (Gerald Irissarri, Northwestern Aquatic Sciences, Newport, OR, personal communication).

The WET manual lists requirements related to dissolved oxygen and temperature and recommendations for other water quality characteristics. In every experiment, dissolved oxygen

levels were near saturation for dilution water and greater than 4.0 mg/L, the USEPA (2002a) WET test criterion, for samples in all tests. Though dissolved oxygen readings in the *P. promelas* Tier II groundwater sample were erratic after the initial reading (8.84 mg/L) because of membrane fouling, the initial reading was stable, indicating that dissolved oxygen levels were near saturation. Dilution water pH and total hardness levels ranged from 7.30 to 8.46 and 150 to 180 mg/L as $CaCO_3$, respectively (Appendix A). These levels closely approximated the WET manual recommendations for dilution water pH and hardness (7.6-8.0 and 160-180 mg/L, respectively). The pH of groundwater test solutions ranged from 7.28 to 8.80, satisfying the USEPA pH recommendation of 6.0 to 9.0 for test solutions.

The USEPA (2002a) presents 20 and 25°C test temperatures as options for conducting *D. magna* and *P. promelas* tests; 20°C was the target temperature for all tests in this study. The temperatures of six test solutions in each WET test were recorded daily (point measurements) during tests and used to evaluate compliance with WET criteria. WET criteria recommend no more than a ± 1°C average daily deviation in test chambers from the temperature at which the test is conducted and require no more than a 3°C deviation (maximum minus minimum temperature) during each test. The point measurements taken during each experiment in test chambers demonstrate that both criteria were met in all tests, with the exception of all *P. promelas* Tier I tests. During the last 24 hours of this experiment, extremely low outside temperatures resulted in lower than normal test solution temperatures. During the first 72 hours of the experiment, average daily temperatures ranged from 17.9 to 19.6°C. During the last 24 hours, temperatures ranged from 14.7 to 16.5°C.

Discussion

The primary goal of this project was to determine if supplementing standard laboratory lighting with UVR during WET tests could modify the toxicity of PAH-contaminated environmental samples to aquatic organisms. The complex nature of solar radiation in aquatic environments is reviewed in Fortner (2009) along with the many assumptions and considerations required in the design of artificial light sources. An important conclusion from this review is that there is inconsistency in how phototoxicity tests are conducted and in how lighting conditions used in those tests are reported. Furthermore, most regulatory test protocols do not require the use of UVR during testing. Depending on the management goals of toxicity tests, this omission may have significant consequences as is demonstrated in this study by the increase in toxicity to *D. magna* when UVR is present. The importance of linking testing goals to light source characteristics and of reporting both the spectral distribution and irradiance characteristics of light sources, therefore, is clear and should be carefully considered in future studies. In support of the primary goal of this study, these characteristics were considered as part of the evaluation and finalization of the artificial light treatments with an emphasis on approximating optical radiation in local aquatic environments. The framework used for that evaluation is included in Fortner (2009).

The evaluation of the characteristics of the final light treatments used here supports that the LL treatments approximated standard WET test lighting conditions, producing PAR but very little to no UVR. In contrast, the final LL+UVR treatments were much more representative of local solar conditions, emitting all UVA and UVB wavelengths detected in three local aquatic systems. Although instantaneous PAR, UVA, and UVB irradiance levels were less under LL+UVR treatments than at the water surface in all three local systems, UVA and UVB laboratory levels exceeded levels measured at some depths at each of the field sites. This is significant because it means that LL+UVR treatments best approximated irradiance to a depth of approximately 50 cm at Taylor Dock and to depths less than 10 cm at Lake Padden. Since aquatic organisms may occupy a variety of depths in the water column, these exposure levels apply to organisms spending the majority of their time at these depths. These findings

underscore the importance of evaluating radiation exposure with depth in relation to organism behavior and life history strategies.

Reference tests were conducted to determine if fluoranthene could be used as a phototoxicity control for the laboratory light treatments designed for this study. Results of these tests demonstrated that specific fluoranthene concentrations could be used that would cause minimal to no acute toxicity under LL treatments and 100% acute effects under LL+UVR treatments for both *D. magna* and *P. promelas*. Based on these results, it was concluded that fluoranthene is an appropriate phototoxicity control for this type of study. When fluoranthene controls were used in the Tier I and Tier II toxicity tests conducted on Little Squalicum Park groundwater, the controls demonstrated that the light treatments functioned as designed with enhanced toxicity in the LL+UVR treatments compared to the LL treatments.

Groundwater test chambers were inadvertently covered with clear polystyrene covers during portions of the *D. magna* Tier I and Tier II tests (Table 3). Given that polystyrene filters out all wavelengths below 288 nm and reduces irradiance transmission by 50% at 300 nm (ASTM, 2002), the presence of covers during testing could have influenced the quality and quantity of radiation entering the chambers. Analysis of the laboratory lighting conditions showed, however, that little to no irradiance was present at or below 300 nm without covers under both treatment types (Fortner, 2009). Irradiance modification by the covers, therefore, was likely minimal and had little to no influence on sample toxicity. Indeed, no trends were identified in the effects data that suggest the presence of covers significantly influenced organism mortality.

Analysis of *D. magna* Tier I and Tier II toxicity results revealed substantial differences in groundwater toxicity between light treatments. In the absence of significant UVR, LC25s for the Tier II tests were 22.1 and 25.7%, compared to 0.59 and 0.68% when UVR was present. There were no overlaps in point estimate confidence intervals between LL and LL+UVR treatments. The 17-fold difference in toxicity between LL and LL+UVR light treatments, based on the NOEC and LOEC values, also supports a greater toxicity under laboratory lighting augmented with UVR. In all replicates of the Tier II tests, NOEC values were 6.25% under LL treatments and 0.38% under LL+UVR treatments and the LOEC values were 12.5% and 0.75% under LL

and LL+UVR treatments, respectively. Since water quality parameters and negative and positive control responses in all tests met WET testing criteria, the enhanced groundwater toxicity can be attributed to exposure of test organisms to LL+UVR light treatments. Previous research has demonstrated that UVR can significantly augment the toxicity of photoactive molecules, and further supports that differences in acute toxicity in this study are most likely the result of elevated levels of UVR under the LL+UVR treatments. Total daily doses of UVA and UVB under LL+UVR treatments were 20, and 10-fold greater, respectively, under LL+UVR treatments than under LL treatments (Table 7). In contrast, total daily doses of PAR were only 2-fold greater under LL+UVR treatments.

The analytical profile of PAH constituents in the Tier II groundwater sample suggests that phototoxicity to *D. magna* may have resulted in part from the presence of photoactive PAHs in the sample. Research has shown that at least nine of the sixteen USEPA PAH priority pollutants can cause phototoxic effects in some aquatic organisms and analysis of the *D. magna* Tier II groundwater sample shows the sample contained at least five of the nine potentially phototoxic PAHs. These nine ppPAHs represent commonly studied PAHs. Less is known about the phototoxic potential of the many congeners of these compounds and other environmental contaminants. Since groundwater samples were analyzed only for select chemicals, it is possible that other substances present in site groundwater contributed to the phototoxicity that was observed in these tests (Table 1).

In contrast to the enhanced toxicity observed in the *D. magna* tests, analysis revealed no clear differences in groundwater toxicity between treatments in the *P. promelas* Tier I and Tier II tests. In Tier I tests, NOEC and LOEC values were the same under both light treatments. The 3.3 to 5.4-fold lower LC50 estimates for LL+UVR than LL treatments in Tier I tests are suggestive of a difference in toxicity, but since the confidence intervals of these estimates are so close or overlap each other (depending on which replicates are being compared), there is not a statistically significant difference in toxicity between treatments. This is expected in a range-finding test, however, where the dilution series covers a broad range of concentrations and less replicates are used, resulting in more variability and larger confidence intervals. Analysis of Tier II *P. promelas* results indicate that, while trends in the data suggest differences in mortality

between treatments, these differences are inconsistent between test replicates (NOEC and LOEC comparisons) and statistically insignificant between treatments (LC25 estimates) (Table 12). For example, when T2-LL+UVR values (NOEC = 2.5% and LOEC = 5%) are compared to T1-LL values (NOEC = 5% and LOEC = 10%) or to T2-LL values (NOEC = 20% and LOEC = 40%), groundwater toxicity appears to be greater under LL+UVR treatments. Groundwater also appears to be more toxic under LL+UVR treatments when T1-LL+UVR values (NOEC = 5% and LOEC > 5%) are compared to T2-LL values. When T1-LL-UVR values are compared to T1-LL values, however, toxicity appears to be the same between treatments. Due to the inconsistency of NOEC and LOEC values between test replicates, analysis using this hypothesis testing approach is inconclusive. Analysis of test replicate data using a point estimate approach, however, is not. Despite the 1.5 to 3.7-fold higher LC25 estimate for LL than LL+UVR treatments, there are substantial overlaps in LC25 confidence intervals. This means there was not a statistically significant difference in toxicity between light treatments in *P. promelas* Tier II tests.

To better understand the inconsistent results of the *P. promelas* tests, Tier I and Tier II tests were evaluated in relation to WET test acceptability criteria, study lighting protocols, test replicate reproducibility, test sensitivity, and analytical chemistry results. The fluoranthene controls confirmed that Tier I and Tier II LL+UVR treatment lighting was of sufficient quality and quantity to elicit phototoxic effects, so that was eliminated from further evaluation.

WET test acceptability criteria were met in *P. promelas* tests with two exceptions: 1) temperatures were lower during the last 24 hour period of Tier I tests than permitted under WET guidelines; and 2) control mortality was greater than 10% in one of the Tier II test replicates. It is unlikely that Tier I temperature deviations contributed to the absence of toxicity differences between LL and LL+UVR treatments because lower temperatures occurred in all test chambers, not just in chambers under one type of light treatment. Also, the majority of organism mortality in Tier I tests occurred prior to the final 24 hours of the test, suggesting that lower temperatures had little influence on 96 h mortality results. Finally, despite the lower temperatures, there were still significant differences in organism mortality between treatments in the fluoranthene phototoxicity controls.

Fifteen percent mean negative control mortality was observed in the Tier II T1-LL+UVR test, but control mortality in the other Tier II tests met the 10% WET test negative control mortality criterion. It appears the inadvertent combining of two T1-LL+UVR control chambers during solution renewals contributed to the higher than acceptable level of mortality in this test, with increased mortality in these chambers possibly resulting from organism injury during renewals or a higher than normal loading density. Therefore, the 5% difference between observed and acceptable mean mortality for this test is probably more attributable to issues that occurred with these two test chambers than to overall test conditions. For this reason, it was concluded that failure to meet the WET test negative control criterion in this test does not explain the absence of detectable differences between LL and LL+UVR treatments. Even if negative control mortality had been indicative of overall T1-LL+UVR test conditions, greater organism mortality in groundwater chambers would have contributed to a difference between LL and LL+UVR treatments, not the absence of a difference between treatments.

An evaluation of the study lighting protocol for the *P. promelas* tests, shows that there were several deviations from the protocol for UVR-source (UVA-340 lamp) illumination (Table 3). The design protocol called for 12 hours of UVR-source illumination during each 24 hour photoperiod for all LL+UVR treatments. During Tier I testing, UVR lamps were not turned on during the last 24 hours of testing in the T2-LL+UVR test. It is possible that mortality would have been greater at 96 hours for this test, as a result of phototoxic effects, if the UVR lamps had been turned on; however, mortality levels and the LC50 estimate for this test are consistent with those of the T1-LL+UVR test that did have UVR during the last 24 hours of testing. During Tier II testing, UVR lamps were illuminated each day for 11.25 to 11.75 hours in the T1-LL+UVR test, and for 10.75 hours during the third day of testing in the T2-LL+UVR test. These abbreviated UVR durations resulted in smaller daily doses of UVR than what the study originally targeted, which might have resulted in lower mortality because of decreased UVR-induced phototoxicity. In these cases, however, durations of UVR lamp illumination were only slightly less than the 12 hour per day illumination target, and NOEC, LOEC, and LC25 estimates were consistent between LL+UVR test replicates. Therefore, deviations in illumination durations for

UVR lamps do not explain the absence of detectable differences in toxicity between LL and LL+UVR treatments.

The evaluation of test replicate repeatability shows that there was the lack of consistency between *P. promelas* results for the two Tier II LL treatments (Table 12). NOEC and LOEC values were three-fold higher for the Tier II T2-LL test than the Tier II T1-LL test, and the LC25 estimate for the T2-LL test was 2.4-fold greater than that of the T1-LL test. These differences between treatment replicates, in conjunction with the large confidence intervals for the T2-LL LC25 estimate and no confidence intervals for the LL+UVR tests, made it difficult to determine if toxicity differences existed between LL and LL+UVR treatments. Though there was not a statistically significant difference in toxicity between treatments, mean mortality was generally higher under increased UVR when the same concentrations were compared between LL and LL+UVR treatments (Figure 15). Trends in the data, therefore, suggest there may have been a small difference in toxicity between treatments. If LL treatment results had been more consistent and confidence limits had been available for LL+UVR results, it would have been possible to evaluate the significance of these trends.

The results of the test sensitivity evaluation show that some of the Tier II tests may not have been sufficiently sensitive to permit the detection of small differences in mortality between groundwater solutions and negative controls. Since pMSD serves as an approximate index of test sensitivity, USEPA recommends evaluating test sensitivity by calculating pMSD values and comparing these values to pMSD ranges established for specific tests (USEPA, 2000). The pMSD values calculated for Tier II T1-LL, T2-LL, T2-LL+UVR, and T2-LL tests (12.8, 22.8, 22.9, and 26.0%, respectively) all fall within the pMSD range of 4.2 to 30% established for acute *P. promelas* tests, but three of these values approach the 30% upper limit, beyond which a test would be considered insufficiently sensitive by the USEPA. A visual inspection of the Tier II concentration response curves (Figure 15) shows maximum effects near the LC25 and a high degree of variability between replicates within any single test. This supports that the *P. promelas* tests may not have been sensitive enough to identify significant results at the chosen level of effect.

An evaluation of analytical chemistry results was prompted by the greater groundwater sample toxicity observed in the Tier I tests compared to the Tier II tests, with high levels of mortality in the Tier I tests occurring within the first 24 hours of testing. For example, under Tier I LL treatments, mean percent mortality ranged from 70 to 90% at a concentration of 10% groundwater. In contrast, under Tier II LL treatments, mean percent mortality ranged from 25 to 40% for the same concentration of groundwater. Under Tier I LL+UVR treatments mean percent mortality was 40% at a concentration of 1% groundwater. In contrast, under Tier II LL+UVR treatments, mean percent mortality ranged from 10 to 18% for 1.25% groundwater. These differences in groundwater toxicity are best explained by changes in the chemical profile of Little Squalicum Park groundwater over time, which may have been the result of groundwater dilution caused by precipitation events that occurred between the groundwater sampling times. Chemical analysis of Tier I and Tier II groundwater samples did reveal decreases in all measured analytes between Tier I and Tier II sampling events (Table 1). Chemical analysis also revealed that the total concentration of ppPAHs in the *P. promelas* Tier II groundwater sample was 14.5-fold lower than total ppPAHs in the *P. promelas* Tier I sample. Based on groundwater sample analysis, *P. promelas* were exposed to 869.2 µg/L total ppPAHs in the highest concentrations in Tier I LL and LL+UVR treatments (Table 1). Fluoranthene constituted 330 µg/L of this total. In contrast, organisms in the highest concentration after dilutions in Tier II LL and LL+UVR tests were exposed to only 24.04 and 3.55 µg/L total ppPAHs, respectively, with fluoranthene comprising 11.2 and 1.4 µg/L of these totals (Fortner, 2009). These differences suggest that phototoxic effects, if present, would have been greater under Tier I test conditions than under Tier II conditions, but they do not explain the absence of significant differences in mortality between LL and LL+UVR treatments in Tier I tests. As discussed, however, since the Tier I tests were designed as range-finding tests, it is likely that the low statistical power associated with the tests did not allow for the detection of small differences in toxicity between replicates and treatments in these tests. Overall, the chemical analysis suggests the relatively low ppPAH concentrations in the Tier II LL+UVR test solutions may not have been high enough to induce phototoxic effects to *P. promelas* under Tier II test conditions.

The study results are important for many reasons. First, the results demonstrate that PAH-contaminated field samples can be phototoxic to *D. magna*. Previous studies have focused

primarily on the phototoxicity of single substances or mixtures created in the laboratory. This study contributes a better understanding of phototoxic hazards associated with field samples. In particular, the findings demonstrate that a phototoxic hazard can exist in samples with multiple co-contaminants. The findings also suggest the presence of known phototoxic compounds in samples can be indicative of phototoxic hazard. Finally, the results show that phototoxicity can occur under WET testing conditions when UVR is present. This is significant because WET protocols do not incorporate a contaminant uptake period prior to test initiation, something that is usually incorporated into the design of phototoxicity studies. Since contaminant uptake is required for photosensitization-driven phototoxicity, it was not clear at the beginning of this study if the relatively short exposure period of acute WET tests could provide enough time for phototoxic effects to occur. The results of the fluoranthene reference tests and the *D. magna* groundwater tests demonstrate that WET test acute exposure durations were long enough to induce phototoxic effects. This suggests that WET tests can be used to effectively evaluate phototoxic hazard with a consistent approach.

Conclusions

In fluoranthene reference and groundwater tests, the presence of UVR substantially increased the toxicity of fluoranthene test solutions to both *D. magna* and *P. promelas*. The toxicity of PAH-contaminated groundwater samples increased substantially to *D. magna* under laboratory lighting supplemented with UVR. There was not, however, a consistently significant increase in groundwater toxicity to *P. promelas* under UVR lighting conditions.

These test results are significant on many levels. The results of the fluoranthene reference tests show that fluoranthene is an effective phototoxicity control for the UVR treatment design used in this study and demonstrate that phototoxic effects can be induced under WET testing conditions. The *D. magna* results are significant because they demonstrate that: 1) PAH-contaminated field samples can be phototoxic to aquatic organisms; 2) WET testing conditions can induce phototoxicity when laboratory lighting is supplemented with UVR; 3) WET tests can be used to measure the magnitude of acute phototoxic effects; and 4) current WET testing lighting recommendations can lead to underestimation of sample hazard when phototoxic contaminants are present.

In contrast to the enhanced toxicity observed in *D. magna* tests, there was not a consistent or statistically significant difference in groundwater toxicity between LL and LL+UVR light treatments in *P. promelas* Tier II tests. After evaluating these test results in the context of WET test acceptability criteria, adherence to study lighting protocols, test replicate reproducibility, test replicate sensitivity, and analytical chemistry results, the best explanation for the absence of toxicity differences between LL and LL+UVR treatments in these tests is the absence of detectable phototoxic effects. This means one or more of the following: 1) none of the constituents of the groundwater solutions were phototoxic to *P. promelas* under Tier II testing conditions; 2) groundwater constituents were phototoxic but did not occur at high enough levels to significantly impact organism survival, possibly masked by co-contaminants; or 3) test conditions were not sufficiently sensitive to allow for the detection of low level differences in toxicity between treatments. Although there was not a significant and consistent difference in groundwater toxicity between light treatments, trends in *P. promelas* effects data do indicate that

higher toxicity may have occurred to *P. promelas* exposed to groundwater under LL+UVR treatments. Consequently, the *P. promelas* portion of this study should be repeated to better define the significance of these trends. Specifically, since the concentration of ppPAHs in groundwater samples decreased by more than an order of magnitude during the six weeks between Tier I and II experiments, there is a need to assess the phototoxic potential of samples containing higher levels of ppPAHs than those observed in the Tier II portion of this study since this likely impacted the Tier II *P. promelas* toxicity tests.

A major implication of this study from a regulatory context is that current USEPA WET test lighting recommendations may allow for dramatically different estimates of sample toxicity when phototoxic contaminants are present compared to the toxicity that may occur in the field. WET methods do not clearly stipulate specific testing conditions related to test lighting. Not only are the current recommendations somewhat ambiguous, but they also lack important information concerning the quality of radiation (e.g. presence or absence of UVR) that should be present during testing. This lack of specific guidance allows for great flexibility in lighting choices, something that can lead to considerable variability in toxicity test results. Managers who use WET tests to aid in decision making may, therefore, find it useful to consider the influence lighting choices may have on toxicity estimates, on a case-by-case basis. For example, if a test involves evaluating the toxicity of ground water that is hydrologically connected to surface waters in which organisms are concomitantly exposed to high levels of UVR and known phototoxic PAHs, managers may choose to incorporate spectral characteristics in laboratory lighting, specifically wavelengths of UVR that are present in the environment but greatly reduced in the output of standard laboratory light sources.

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Appendix A. Water Quality Data

Table A-1. Summary of *D. magna* and *P. promelas* fluoranthene reference test water quality data and WET criteria.

	T1 LL Min	T1 LL Max	T1 LL+UVR Min	T1 LL+UVR Max	T2 LL Min	T2 LL Max	T2 LL+UVR Min	T2 LL+UVR Max	WET criteria[a]	
Daphnia magna										
Dissolved oxygen (mg/L)										
dilution water	7.18	8.22	7.29	8.22	8.22	8.75	8.18	8.22	~ saturation	S
groundwater	6.75	8.15	6.75	8.33	6.75	8.59	6.75	8.32	> 4.0mg/L	R
pH										
dilution water	7.91	8.39	7.91	7.95	7.10	7.91	7.60	7.91	7.6-8.0	S
groundwater	8.10	8.46	8.18	8.50	8.00	8.60	8.18	8.70	6.0-9.0	S
Temperature (°C)										
point measurements										
min max range	18.25	19.5	19.50	20.5	18.94	19.5	19.00	20.1		S
min max difference	1.25		0.98		0.56		1.08		< 3°C[b]	R
test temp[c]	18.88		19.99		19.22		19.54			
deviation from test temp[d]	0.20	0.54	0.06	0.40	0.03	0.11	0.03	0.41	20°C (± 1°C)[e]	S
continuous measurement					19.18 ± 0.93					
Conductivity (µS/cm)										
dilution water	596	604	579	604	589	604	585	604		
groundwater	510	589	510	588	510	603	510	607		
Hardness (mg/L as ($CaCO_3$))										
Dilution water	180	NM	180	180	180	180	180	180		
Highest groundwater	180	NM	180	180	180	180	180	180	160-180	S
Pimephales promelas										
Dissolved oxygen (mg/L)										
dilution water	6.76	7.62	6.74	7.62	7.02	8.27	6.67	7.53	~ saturation	S
groundwater	4.69	7.88	5.30	7.88	5.88	7.55	5.47	7.37	> 4.0mg/L	R
pH										
dilution water	7.40	8.46	7.97	8.46	7.89	8.40	7.73	8.40	7.6-8.0	S
groundwater	7.82	8.60	7.91	8.60	8.00	8.70	7.88	8.70	6.0-9.0	S
Temperature (°C)										
point measurements										
min max range	21.04	22.96	22.06	23.28	22.13	23.41	22.08	23.63		S
min max difference	1.92		1.22		1.28		1.55		< 3°C[b]	R
test temp[c]	22.00		22.67		22.77		22.86			
deviation from test temp[d]	0.06	0.95	0.06	0.51	0.16	0.58	0.02	0.66	20°C (± 1°C)[e]	S
continuous measurement					23.33 ± 1.64					
Conductivity (µS/cm)										
dilution water	548	583	548	575	544	573	544	577		
groundwater	549	571	549	604	547	578	547	613		
Hardness (mg/L as ($CaCO_3$))										
Dilution water	160	164	160	170	164	176	164	176	160-180	S
Highest groundwater	160	164	160	168	164	166	164	172		

[a] WET test criteria are from USEPA-821-R-02-012. S = values that are recommended. R = required criteria.
[b] WET criteria require less than a 3 °C difference between maximum and minimum temperature values measured during each test.
[c] The temperature at which the test was conducted, calculated as the average of the endpoints of the max min range.
[d] Range of daily average deviation from test temperature. Deviation was calculated as the difference between average daily temperature values and the temperature at which the test was conducted.
[e] WET criteria require no more than a ± 1 °C average daily deviation from the temperature at which the test was conducted. Though 20 °C was the target test temperature for all tests, it was not possible to conduct tests centered at exactly 20 °C. Test temperature deviations are therefore evaluated with respect to the temperature at which the test was actually conducted.

Table A-2. Summary of *D. magna* Tier I and Tier II water quality data and WET criteria.

	T1 LL		T1 LL+UVR		T2 LL		T2 LL+UVR		WET criteria[a]	
	Min	Max	Min	Max	Min	Max	Min	Max		
Groundwater Tier I										
Dissolved oxygen (mg/L)										
dilution water	6.79	8.59	8.48	8.59	8.17	8.59	8.47	8.59	~ saturation	S
groundwater	7.67	8.79	8.15	8.79	8.18	8.87	8.14	8.79	> 4.0mg/L	R
pH										
dilution water	7.30	8.30	8.20	8.30	8.10	8.30	8.30	8.40	7.6-8.0	S
groundwater	7.70	8.60	8.30	8.60	7.70	8.50	7.70	8.70	6.0-9.0	S
Temperature (°C)										
point measurements										
min max range	18.59	20.1	19.42	20.6	18.89	20.0	19.06	20.4		
min max difference	1.51		1.18		1.14		1.37		< 3°C[b]	R
test temp[c]	19.35		20.01		19.46		19.75			
deviation from test temp[d]	0.10	0.58	0.09	0.57	0.23	0.28	0.02	0.56	20°C (± 1°C)[e]	S
continuous measurement					20.43 ± 1.31					
Conductivity (µS/cm)										
dilution water	546	556	546	548	546	555	541	546		
groundwater	387	543	387	541	387	537	387	544		
Hardness (mg/L as ($CaCO_3$))										
Dilution water	168	172	168	174	167	178	168	172	160-180	S
Highest groundwater	202	224	204	232	204	228	204	220		
Groundwater Tier II										
Dissolved oxygen (mg/L)										
dilution water	8.32	8.32	7.92	8.32	8.32	9.08	8.02	8.32	~ saturation	S
groundwater	7.80	9.09	7.32	9.23	8.56	9.09	8.12	9.23	> 4.0mg/L	R
pH										
dilution water	7.40	8.50	7.40	8.30	7.40	8.30	7.40	8.40	7.6-8.0	S
groundwater	8.00	8.80	7.90	8.70	8.00	8.60	7.90	8.60	6.0-9.0	S
Temperature (°C)										
point measurements										
min max range	19.53	20.5	19.32	20.7	19.04	20.5	19.67	20.4		S
min max difference	0.96		1.37		1.46		0.71		< 3°C[b]	R
test temp[c]	20.01		20.01		19.77		20.03			
deviation from test temp[d]	0.07	0.33	0.25	0.40	0.09	0.22	0.02	0.20	20°C (± 1°C)[e]	S
continuous measurement					20.99 ± 1.23					
Conductivity (µS/cm)										
dilution water	552	577	552	591	552	559	552	584		
groundwater	432	571	542	579	432	560	542	576		
Hardness (mg/L as ($CaCO_3$))										
Dilution water	156	172	156	172	156	168	156	166	160-180	S
Highest groundwater	152	164	160	174	152	162	160	172		

[a] WET test criteria are from USEPA-821-R-02-012. S = values that are recommended. R = required criteria.
[b] WET criteria require less than a 3 °C difference between maximum and minimum temperature values measured during each test.
[c] The temperature at which the test was conducted, calculated as the average of the endpoints of the max min range.
[d] Range of daily average deviation from test temperature. Deviation was calculated as the difference between average daily temperature values and the temperature at which the test was conducted.
[e] WET criteria require no more than a ± 1 °C average daily deviation from the temperature at which the test was conducted. Though 20 °C was the target test temperature for all tests, it was not possible to conduct tests centered at exactly 20 °C. Test temperature deviations are therefore evaluated with respect to the temperature at which the test was actually conducted.

Table A-3. Summary of *P. promelas* Tier I and Tier II water quality data and WET criteria.

	T1 LL	T1 LL+UVR	T2 LL	T2 LL+UVR	WET criteria[a]	
	Min Max	Min Max	Min Max	Min Max		
Groundwater Tier I						
Dissolved oxygen (mg/L)						
dilution water	7.53 - 8.24	7.29 - 8.24	7.36 - 8.24	8.12 - 8.25	~ saturation	S
groundwater	7.40 - 8.68	7.33 - 9.00	7.68 - 8.72	7.13 - 9.20	> 4.0mg/L	R
pH						
dilution water	7.73 - 8.28	7.73 - 8.28	7.73 - 8.28	7.73 - 8.28	7.6-8.0	S
groundwater	7.28 - 8.26	7.28 - 8.26	7.28 - 8.26	7.28 - 8.26	6.0-9.0	S
Temperature (°C)						
point measurements						
min max range	13.92 - 19.30	16.37 - 19.96	14.58 - 19.90	15.44 - 19.67		S
min max difference	5.38	3.59	5.32	4.23	< 3°C[b]	R
test temp[c]	16.61	18.17	17.24	17.56		
deviation from test temp[d]	1.33 - 2.62	0.59 - 1.71	1.34 - 2.58	1.39 - 2.05	20°C (± 1°C)[e]	S
continuous measurement			17.12 ± 3.65			
Conductivity (µS/cm)						
dilution water	552 - 582	552 - 604	552 - 572	552 - 573		
groundwater	369 - 586	369 - 571	369 - 587	369 - 587		
Hardness (mg/L as ($CaCO_3$))						
Dilution water	150 - 171	160 - 168	160 - 164	157 - 162		
Highest groundwater	178 - NM	178 - NM	178 - NM	178 - NM	160-180	S
Groundwater Tier II						
Dissolved oxygen (mg/L)						
dilution water	7.36 - 8.53	6.50 - 8.43	7.16 - 8.53	7.16 - 8.43	~ saturation	S
groundwater	7.05 - 9.67	5.70 - 9.28	7.05 - 8.72	7.07 - 9.14	> 4.0mg/L	R
pH						
dilution water	8.08 - 8.28	8.08 - 8.35	8.08 - 8.43	8.08 - 8.43	7.6-8.0	S
groundwater	7.62 - 8.33	7.80 - 8.56	7.62 - 8.42	7.84 - 8.56	6.0-9.0	S
Temperature (°C)						
point measurements						
min max range	19.50 - 20.42	19.52 - 20.82	19.39 - 20.52	19.45 - 20.93		S
min max difference	0.92	1.30	1.13	1.48	< 3°C[b]	R
test temp[c]	19.96	20.17	19.96	20.19		
deviation from test temp[d]	0.11 - 0.28	0.00 - 0.13	0.11 - 0.31	0.00 - 0.27	20°C (± 1°C)[e]	S
continuous measurement			19.48 ± 2.14			
Conductivity (µS/cm)						
dilution water	552 - 568	543 - 579	552 - 568	543 - 574		
groundwater	450 - 555	528 - 839	450 - 556	533 - 839		
Hardness (mg/L as ($CaCO_3$))						
Dilution water	164 - 170	162 - 173	164 - 191	162 - 172	160-180	S
Highest groundwater	160 - 166	170 - 172	160 - 171	170 - 170		

[a] WET test criteria are from USEPA-821-R-02-012. S = values that are recommended. R = required criteria.

[b] WET criteria require less than a 3 °C difference between maximum and minimum temperature values measured during each test.

[c] The temperature at which the test was conducted, calculated as the average of the endpoints of the max min range.

[d] Range of daily average deviation from test temperature. Deviation was calculated as the difference between average daily temperature values and the temperature at which the test was conducted.

[e] WET criteria require no more than a ± 1 °C average daily deviation from the temperature at which the test was conducted. Though 20 °C was the target test temperature for all tests, it was not possible to conduct tests centered at exactly 20 °C. Test temperature deviations are therefore evaluated with respect to the temperature at which the test was actually conducted.