

ABSTRACT

Aquatic Toxicology of Salinity and Silver in *Daphnia magna*: A Comparative Study of Standardized Chronic Endpoints and Progeny Phototaxis

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Behavioral bioassays with the model freshwater cladoceran *Daphnia magna* have the potential to serve as nontraditional but sensitive endpoints of sublethal stress in the field of ecotoxicology. However, few studies have examined the comparative sensitivity of phototaxis perturbations to standardized endpoints in chronic toxicity testing protocols. *D. magna* 21 d chronic toxicity studies were conducted with model stressors of salinity (NaCl) and dissolved silver (Ag⁺). Phototaxis assays of progeny response to relative light changes in small water columns were conducted of each brood. Results indicated predominantly decreased phototactic behavior in comparison to control brood performance at common ecotoxicological thresholds. Phototaxis assays showed effects at treatment levels below the 21-d adult reproduction LOEC value in broods 2, 3, 5 and 6 in the salinity study and broods 2, 5, and 6 in the silver study. Future refinement of robust and potentially highly ecologically relevant behavioral assays such as phototaxis in combination with traditional and nontraditional endpoints may prove useful in characterization of assessment factors.

Aquatic Toxicology of Salinity and Silver in *Daphnia magna*:
A Comparative Study of Standardized Chronic Endpoints and Progeny Phototaxis

by

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LIST OF ABBREVIATIONS

ANOVA	analysis of variance
CCC	criterion continuous concentration
DO	dissolved oxygen
EC10	10% effects concentration
LC50	50% lethal concentration
LOEC	lowest observable effects concentration
LOAEC	lowest observable adverse effects concentration
MATC	maximum acceptable treatment concentration
MCPA	2-methyl-4-chlorophenoxyacetic acid
NOEC	no observable effects concentration
OPPTS	Office of Prevention, Pesticides and Toxic Substances
PPB	parts per billion
PPT	parts per thousand
SSM	species sensitivity model
TBTO	bis(tri- <i>n</i> -butyltin)oxide
USEPA	United States Environmental Protection Agency
UV	ultra violet

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DEDICATION

To my parents

CHAPTER ONE

Introduction

Behavior ecotoxicology of aquatic organisms is a developing field that offers sensitive, rapid, and ecologically relevant approaches to linking sensitive organism level responses (Gerhardt 2007, Amiard-Triquet 2009). Aquatic vertebrate and invertebrate behavior involving predator-prey interactions, avoidance, and spatial orientation have been shown to be impacted by chemicals at sublethal levels and have great potential as ecologically relevant endpoints for contributing to weight of evidence approaches in ecological risk assessment (Gerhardt 2007, Dodson and Hanazato 1995, Boyd et al. 2002, Zhou et al. 2008, Stanley et al. 2007, Valenti et al. 2009, Berninger et al. 2011). Consequently, further examination of behavioral effects of chemicals on representative model organisms is warranted in order to advance an understanding of ecological effects caused by environmental stressors (Gerhardt 2007).

The freshwater cladoceran *Daphnia magna* (*D. magna*) is utilized as a model aquatic invertebrate for toxicity testing of effluents and ambient water bodies, for chemical safety assessments around the world, and to support development of water quality criteria for the protection of aquatic life (USEPA 1996). Cohorts of *D. magna* are particularly vital to ecotoxicology as females reproduce via parthenogenesis, yielding genetically identical clones under suitable conditions whereby consistent cohort responses to the effects of stressors may be observed (USEPA 1996). Behavioral endpoints in *D. magna*, such as reduced feeding rates and swimming behavior, have been

effectively employed as assessment tools in ecotoxicology assays in combination with traditional standardized endpoints such as growth, mortality and reproduction (Flickringer et al. 1982, Michels et al. 1999, Allan et al. 2006). In particular, *D. magna* phototaxis behavior as a proximate cause of diel vertical migration (DVM) has been linked to life history and fitness parameters, and phototaxis inhibition or perturbation has increasingly been utilized as an effective indicator of toxicant exposure and organism stress (Rinke et al. 2008, Ohman 1990, Fiksen et al. 1997, Loose and Dawidowicz 1994, Michels et al. 1999, Ren et al. 2007, Wu et al. 2008, Martins et al. 2007). Both positive and negative phototaxis (light attraction or avoidance) have been shown to be highly reproducible behaviors in strains of *D. magna* clones and have thus been recognized as potential tools for biomonitoring purposes of environmental integrity and water quality (Allan et al. 2006, Martins et al. 2007, Gerhardt et al. 2007).

In aquatic systems, *D. magna* regularly perform DVM as a predator avoidance mechanism (Lampert 1989, Ringelberg 2003). Predator avoidance is thought to be the primary reason for DVM (Lampert 1989, Cousyn et al. 2001). DVM is driven by phototaxis, whereby a locomotion response to changes in light intensity within the water column occurs for migratory purposes (Gool and Ringelberg 2003). The sensitivity and response of *D. magna* to light depends on many biotic and abiotic factors, including the genetic adaption of the clone, the timing and intensity of light, temperature, age of *D. magna*, predator kairomones, UV intensity, and food availability (Lampert 1989, Loose and Dawidowicz 1994, Ringelberg 1999, Gool and Ringelberg 2003).

The vertical magnitude and sensitivity of *D. magna* phototaxis in the water column is foremost determined by genetic adaptation (De Meester 1993, Cousyn et al.

2001). A study by Cousyn et al. (2001) compared the genetic adaptation of *D. magna* as a function of phototactic response and neutral genetic markers over 30 years from resting eggs collected from sediment cores of Oud Heverlee Pond, Belgium. Cousyn et al. (2001) found that negative phototactic response behavior increased in prevalence according to high fish presence, which was correlated as a function of historical fish stocking practices. The authors concluded that *D. magna* that are positively phototactic (attracted to light) adapted under low fish predation pressure, whereas highly negatively phototactic (light avoiding) *D. magna* adapted under high fish predation environments where DVM is useful to avoid predators.

Loose and Dawidowicz (1994) showed that *D. magna* DVM as an adapted and environmentally integrated migration behavior may be simulated utilizing sophisticated environmental and biotic gradients in laboratory water columns. However, phototaxis migration, as opposed to rigorous simulation of DVM, can be easily reproduced and characterized in small isothermal water columns with clones adapted to any magnitude of positive or negative phototaxis (Cousyn et al. 2001). Phototactic responses of negatively or positively phototactic clones under regulated light and observation have been shown to be uniform with relatively low variation (De Meester 1993). Repeated observations of *D. magna* migration in response to light stimuli seemingly support a balanced migration decision mechanism as proposed by Gool and Ringelberg (2003), whereby the stimulus threshold (light intensity change) necessary to cause upwards or downwards phototaxis migration is of similar strength (Ringelberg 1999). Gerhardt et al. (2006) observed a regular sinusoidal pattern of *D. magna* column height position when negatively phototactic clones were exposed to light and dark conditions over a 20 hour period,

resulting in upwards and downwards migration within laboratory water columns. Szulkin et al. (2006) found that *D. pulicaria* behave uniformly on an individual basis (e.g., a single *D. magna* is comparable to another single *D. magna* of the same clone type) in response to light and fish kairomones in small water columns, and augment their swimming behavior in the presence of light, fish and invertebrate predator info-chemical stimuli.

Phototaxis responses are subject to a relative light change (RLC) threshold (Gool and Ringelberg 1995, 1998). RLC refers to a change in light intensity in comparison to a previous light intensity value over a specific time period described as a rate quotient:

$$RLC = \frac{1}{I} * \frac{dI}{dt}$$
 where I is light intensity, and t is time (Ringelberg 1964, Gool and

Ringelberg 1998). Therefore, the level of light intensity is not always critical, but the percentage of intensity change over time relative to the previous light intensity is critical for stimulation of phototaxis in *D. magna*. RLC may be positive or negative in the environment, depending on whether the sun is rising or setting. Negatively phototactic clones in the lake environment have been observed to migrate upwards in the water column during sunsets, and migrate downwards during sun rises (Ringelberg and Flik 1994).

RLC is required to functionally stimulate phototaxis orientation and migration of negatively phototactic clones of *D. magna* (Gool and Ringelberg 2003). Other factors may influence this migration; for example, Gool and Ringelberg (1997) found that RLC combined with fish kairomones elicits the greatest vertical displacement velocity in a *D. galeata x hyalina* hybrid. The phototaxis behavior systems governing light related migration behavior are differentiated by Gool and Ringelberg (2003) into the

photobehavior systems 1 and 2. Photobehavior system 1 is governed primarily by RLC changes at dawn and dusks (Gool and Ringelberg 2003), whereas photobehavior system 2 is governed by acceleration of change in light intensity within water columns, which is also associated with dawn and dusk. In Lake Maarsseveen, The Netherlands, RLC generally does not exceed 15% of light intensity change min^{-1} , whether the sun is rising or setting at this location (Ringelberg and Flik 1994). Therefore, at particular times of the day there are peak and minimal rates of light intensity change in environmental water columns. Gool and Ringelberg (1997) reported a minimum stimulus threshold for migration response of a *D. galeata x hyalina* hybrid clone to be 4% min^{-1} of light intensity change and for *D. magna* to be 10% min^{-1} of light intensity change. Artificial light intensity changes above 15% min^{-1} tested with *D. magna* in laboratory columns resulted in reduced latency periods of response and increased *D. magna* swimming velocity (Gool and Ringelberg 1997). Artificially high rates of light intensity change, while never found in nature (in accordance with light irradiance of sunrise and sunsets over the water column), have been shown to maximally induce phototaxis, regardless of the presence of predator kairomones (Gool and Ringelberg 1997). With regards to the use of stimulating phototaxis for assessment purposes, negatively phototactic *D. magna* may be sufficiently stimulated by RLC to migrate over distances of 1-2 meters (Ringelberg 1964, Gool and Ringelberg 1998, Gool and Ringelberg 2003).

Whereas *D. magna* magnitudes of response (migration) to RLC may depend on the genetic adaption of clones (De Meester 1993, Cousyn et al. 1994), study of *D. magna* response to RLC in the field may be utilized to predict DVM in cladoceran populations. Attempts to model phototaxis and DVM are subject to limitations concerning estimation

of prolonged migration behavior, which may be impacted by parameters such as ambient light intensity accelerations (e.g., nonlinear changes in the percentage rate of light intensity change) and kairomones (Gool and Ringelberg 1997, 1998, Gool 1997, Rinke 2008). However, modeling DVM and the functional role of phototaxis in predator avoidance as an important fitness parameter demonstrates potential (Rinke 2008). The success of quantifying the phototactic behavior of specific clones of *D. magna* has allowed for numerous studies characterizing the effects of toxicants under controlled temperature and light regimes. Inhibition of phototaxis behavior as a sublethal endpoint has drawn particular attention. Ecotoxicological assays relying on both positively phototactic (e.g., Michels et al. 2000, Martins et al. 2007, Wu et al. 2008) and negatively phototactic behaviors (Flickringer et al. 1982, Brausch et al. 2011) have been reported. Phototaxis assays of positively or negatively phototactic *D. magna* typically utilize a static light intensity gradient vertically over a small water column (less than a meter in height) (Flickringer 1982, Gerhardt 2006, Brausch et al. 2011). However, phototaxis of negatively phototactic adapted *D. magna* may only be maximally stimulated by method of RLC previously mentioned (Ringelberg and Gool 2003).

Impairment of normal phototaxis behavior of *D. magna* associated with exposure to toxicants has been typically observed to be 10 times more sensitive than lethality (LC50, EC50; Martins et al. 2007, Michels et al. 2000, Kieu et al. 2001). According to the stepwise sensitivity model (SSM) proposed by Gerhardt (2005), decreased locomotion is the first and most sensitive step in detecting pollution as part of a pollution early warning system. Kieu et al. (2001) observed positively phototactic adult *D. magna* exposed to PCP (pentachlorophenol) and copper (Cu²⁺) and reported detection limits of

phototaxis impairment within literature reported NOECs values determined by chronic toxicity testing. Martins et al. (2007) assessed the effects of 11 toxicants on phototactic performance using a similar short term behavioral assay with positively phototactic 7-8 day old *D. magna* and observed phototaxis to be typically 16-32 times more sensitive an endpoint than acute lethality over a 48 hour exposure period. Many phototaxis endpoint assays performed in acute (<48 hour) laboratory studies have been observed to be comparable to chronic endpoint values reported from other sources (Table 1). Though the sensitivity of *D. magna* phototaxis behavior as an endpoint of toxicant exposure over acute magnitudes and durations (<48h) has been well established, the use of phototaxis to characterize the effects of chronic durations and low levels of exposure to toxicants has been less frequently studied. Flickringer et al. (1982) investigated the capacity of *D. magna* phototaxis and filtration to predict chronic toxicity in a standardized 21 day chronic toxicity test, and found behavior endpoints may indicate copper exposure and effects at levels below reproduction NOEC values determined by concurrent chronic toxicity testing of *D. magna*. Studies thus far have frequently reported LOECs using phototaxis as an endpoint often fall near or below LOECs characterized by more traditional chronic endpoints of selected chemicals and metals (Table 1). Comparison of available literature on determination of phototaxis endpoint concentrations of cadmium and copper exposure levels have been observed to be within 1-2 magnitudes of literature reported chronic values (Table 1, Semsari et al. 2007, Flickringer et al. 1982, Michels et al. 1999, Kieu et al. 2001). However, direct comparison to standardized and traditional ecotoxicology endpoints may be inappropriate

due to conditions that may affect the sensitivity of For example, the majority of phototaxis behavioral assays utilized adult *D. magna*, where adult *D. magna* are exposed

Table 1. Literature reported *Daphnia magna* lowest observable effects concentrations (LOECs) determined by phototaxis behavior for selected chemicals and metals, with literature reported chronic values (growth, reproduction) from chronic toxicity tests for comparative purposes.

Author	Date	Toxicant	Exposure Duration (h)	Phototaxis LOEC (µg/L)	Chronic Endpoint (µg/L)	Chronic Endpoint/ Value	Duration	Author
Martins et al.	2007	Benzo(b) fluoranthene	48	267.81	-	-	-	-
Martins et al.	2007	Mercury (II) chloride	24,48	1.88	18	LOAEC	21 d	De Coen and Janssen 1997
Martins et al.	2007	Dimethoate	48	187.5	40-100	MATC	-	USEPA ¹ De Coen and Janssen 1997
Martins et al.	2007	Lindane	0.25	218.75	100	LOAEC	21 d	Janssen 1997
Martins et al.	2007	Linuron	3.5	93.75	130-240	MATC	-	USEPA ²
Martins et al.	2007	2-methyl-4-chlorophenoxyacetic acid (MCPA)	48	5625	22000	LOAEC	-	USEPA ³
Martins et al.	2007	Tributyltin oxide (TBTO)	24,48	20	0.34	CCC	-	USEPA ⁴
Martins et al.	2007	Carbon tetrachloride	24	2343.75	3100	NOEC	21 d	Thompson et al. 1997
Martins et al.	2007	Thiram	0.25	9.38	170.6	NOAEC	-	USEPA ⁵
Martins et al.	2007	2,4,6-trichlorophenol	24,48	7500	500	NOEC	21 d	Radix et al. 1999
Martins et al.	2007	Arsenic trioxide	3.5	937.5	188.9	CCC	-	-
Whitman and Miller	1989	Napthelene	2	500-1000	620	CCC	-	USEPA ⁶
Sensari et al.	2007	Cadmium	0.25-6	30	4.3	CCC	-	USEPA ⁷
Flickringer et al.	1982	Cu ²⁺ (CuSO ₄)	96	10	20	LOEC	21 d	Flickringer et al. 1982
Michels et al.	1999	Cu ²⁺ (CuSO ₄)	3.3	36	30	LOEC	14 d	Blaylock et al. 1985
Kieu et al.	2001	Cu ²⁺ (CuSO ₄)	3	20 [†]	30	LOEC	14 d	Blaylock et al. 1985
Michels et al.	1999	Pentachlorophenol (PCP)	3.3	800	340	NOEC	21 d	Radix et al. 1999
Kieu et al.	2001	Pentachlorophenol (PCP)	3	400	340	NOEC	21 d	Radix et al. 1999

† Only concentration tested in study; ‡ Determined by ACR Method with Ziram; CCC=Criterion Continuous Concentration; NOEC= No Observable Effect Concentration; LOAEC=Lowest Observable Adverse Effects Concentration; MATC=Maximum Acceptable Treatment Concentration

and assessed (Table 1), whereas neonates typically <24 hours in age are utilized for standardized acute and chronic toxicity tests (Martins et al. 2007, Michels et al. 1999, Kieu et al. 2001, USEPA 1994).

It is clear, however, that even with use of non-standard life stages, *D. magna* phototaxis assays have reported responses to toxicants in less than 24 h (Table 1). Sublethal effects characterized by *D. magna* phototaxis assays have been observed in less than 4 hours of exposure to organic herbicides, metal, petroleum products and other organics (Martins et al. 2007, Whitman and Miller 1989, Semsari et al. 2007, Michels et al. 1999, Kieu et al. 2001). Relatively simple comparison of reported phototaxis LOEC values to literature reported chronic values shows that phototaxis may be a highly sensitive endpoint at chronic levels of exposure for effects characterization of chemicals including linuron, MCPA, carbon tetrachloride, thiram, and copper; thus, the utility of phototaxis as a sublethal endpoint appears robust based on previous reports in the peer-reviewed literature.

The sensitivity of phototaxis behavior to toxicant exposure at chronic magnitudes is of interest from both an ecological (Dodson and Hananzato 1995) and bio-monitoring (Gerhardt 2007) perspective. Assessment of *D. magna* progeny phototaxis from a standardized test such as the 21 day chronic test may serve as the most direct approach for determining the sensitivity of phototaxis behavior to chronic levels and trace levels of exposure in comparison to standardized endpoints (mortality, reproduction, growth) (Flickringer et al. 1982). However, no studies have comprehensively examined the comparative sensitivity of *neonate* phototaxis behavior responses to *D. magna* mortality and reproduction thresholds over a chronic study period.

It is proposed here that neonates present a more sensitive life stage for phototaxis assessment purposes. Sarma et al. (2007) found that when conducting acute toxicity testing with exposure to methyl parathion and mercury across 7 species of Cladocera,

including 3 *Daphnia spp.*, neonates were more sensitive to toxicants at the neonate life stage than at the adult life stage, probably due to kinetic differences associated with differences in body size. Differences in adult and neonate *D. magna* may be of high importance concerning toxicants that disrupt ionoregulatory homeostasis of *D. magna*. Bianchini and Wood (2008) observed that *D. magna* neonates and adults possess different ionoregulatory kinetic mechanisms, where at the adult life stage a $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter replaces the Na^+/Cl^- exchanger that exists at the neonate life stage. In terms of *D. magna* body size and mass differences of neonates and adults, Na^+ uptake under higher salinity conditions was found to be inversely related to *D. magna* body masses across 0.050-3.700 mg. Neonates were observed to have a lower affinity for Na^+ , but have a higher maximum capacity of Na^+ uptake in comparison to the adult life stage (Bianchini and Wood 2008). Uptake kinetic differences suggest that neonates are more sensitive to effects of toxicants or stressors that induce ionoregulatory disturbances. For example, these disturbances may be caused by elevated salinity levels or an inhibition of Na^+ uptake caused by the disposition of metals such as Ag^+ on *D. magna* gill sites (Bianchini and Wood 2008). Therefore the sensitivity of the neonate life stage rather than the adult life stage of *D. magna* may be of higher interest concerning the effects of ionoregulatory stressors, and inclusion of neonates into behavioral assays, including phototaxis assessments, seems logical.

Changes in environmental salinity, and the potential for effects on aquatic organisms, are a concern in many parts of the world (Hassel et al. 2006, Kaushal et al. 2005). However, variable levels of sensitivity among strains of *D. magna* strains to changes salinity levels have been observed (Martínez-Jeronimo and Martínez-Jeronimo,

2007, Gonçalves et al. 2007). Salinization of inland freshwater environments (e.g., de-icers on roadways, climate change, interbasin water transfers, water reuse) is predicted to increase heavily in the near future (Kaushal et al. 2005). Therefore, the availability of sensitive indicators of salinity stress may prove vital to characterizing the environmental integrity of critical freshwater resources (Kaushal et al. 2005).

Dissolved ionic silver (Ag^+) remains a contaminant of concern in freshwater environments (Kramer et al. 2002). Particularly ionic silver associated with nanoparticles used to treat commercial products has been increasingly recognized as a point of concern to freshwater ecosystems (Blaser et al. 2008). In fact, ionic silver has been recently considered as a significant component of silver nanoparticle ecological toxicity in aquatic invertebrates and fish (Park and Choi 2010, Powers et al. 2011). *D. magna* are highly sensitive to ionic silver, with Naddy et al. most recently reporting a chronic *D. magna* MATC of 3.00 $\mu\text{g/L}$ dissolved Ag^+ (2007). Ionic silver affects *D. magna* at trace levels, and phototaxis as a behavioral assessment tool may be valuable to effects characterization and biomonitoring of metal contaminants (Zhou et al. 2008).

The objective of this study was to characterize the effects of aquatic invertebrate stressors including salinity (NaCl) and dissolved silver (Ag^+) on phototaxis of *D. magna* neonates (F1 generation) during standardized 21 day chronic experiments in order to compare the sensitivity of phototaxis as an endpoint at low and trace levels of exposure to traditional acute and chronic ecotoxicological endpoints (survival, fecundity, clutch size). My primary hypothesis is that neonate phototaxis will provide a more sensitive threshold of toxicity than the standardized endpoints employed in *D. magna* bioassays. The results of this study may contribute to further efforts to develop ecologically relevant behavior

assays as part of a weight of evidence approach to ecological risk assessments of stressors.

CHAPTER TWO

Materials and Methods

Culture Methods

A single *D. magna* clone type BU1 was acquired from Aquatic Eco-Systems, Inc. and maintained in laboratory cultures at Baylor University's Ecotoxicology and Aquatic Research Laboratory (Waco, TX, USA) for 6 months prior to this study. *D. magna* were cultured in 500 ml beakers containing 400 ml of reconstituted hard water (RHW). Culture beakers did not contain more than 10 adult *D. magna* to avoid crowding. *D. magna* cultures were renewed with fresh RHW every other day and fed once every 24 hours at a regular time (USEPA 2002). Cultures were fed a mixture of algae (*Pseudokirchneriella subcapitata*) and Ceriophyll[®] grass extract Knight and Waller (1992) with suggested modification by Hemming et al. (2002). *D. magna* cultures were maintained in a climate controlled room at $25 \pm 1^\circ \text{C}$ on a 16:8 h light (200 lux):dark photoperiod.

Water Quality

RHW was produced with Nanopure[™] water (18 m Ω) according to standardized methods (USEPA 1996) and then used for culturing *D. magna* and creation of experiment media (USEPA 2002). RHW was constituted in large (50L) Nalgene[®] carboys and aerated for 24 hours, then allowed to equilibrate for 24 hours. RHW salt and ion concentrations were 1.04 mmol/L MgSO₄, 2.38 mmol/L NaHCO₃, 0.918 mmol/L CaSO₄, and 0.112 mmol/L KCl (USEPA 2002). Readings of dissolved oxygen were taking

using an YSI Model 55 handheld dissolved oxygen and temperature meter (VWR, Suwanee, GA). An Accumet combination pH probe (Thermo Scientific, Fair Lawn, NJ) with Orion meter model 720 was used to determine pH. Alkalinity (CaCO₃ mg/L) and hardness were determined by titration according to USEPA protocol (2002).

Chemical Analysis

Sodium chloride levels were confirmed with a calibrated YSI Model 30 salinity, conductivity and temperature probe. Free ionic silver concentrations were determined by an Accumet combination Ag/S Ion Selective Electrode (Thermo Scientific, Fair Lawn, NJ), which was calibrated daily. During chronic testing on days 0, 7, 14, and 21 (USEPA 1996) silver treatments were measured before and after 3 hour equilibrium in food and treatment media as suggested by Naddy et al. for *D. magna* silver 21-d chronic toxicity testing (2007).

21-d Chronic Toxicity Testing Methods

Salinity concentrations were created from reagent grade ($\geq 99.5\%$) sodium chloride (NaCl) (Omnipur®, EMD Chemicals Inc, Gibbstown, NJ) and diluted with RHW to create stock solutions maintained in amber bottles. Target chronic toxicity test concentrations were created by serial dilution of stock solutions with RHW and treatment levels were 8.0, 4.0, 2.0, 1.0 and 0.5 g NaCl/L. Reagent grade silver nitrate (AgNO₃; Ricca Chemical Company, Arlington, TX) was serial diluted with RHW to create targeted dissolved silver treatment levels of 10, 5, 2.5, 1.25, 0.625 $\mu\text{g Ag}^+/\text{L}$ on treatment renewal days.

Range finding studies of treatments concentrations of NaCl and Ag⁺ were initially conducted with BU1 according to USEPA guidelines (1996). Subsequently, *D. magna* Ag⁺ and NaCl 21 day static renewal chronic experiments were conducted according to EPA Ecological Effects Test Guidelines (USEPA 1996). *D. magna* neonates <24 hrs old from the 3rd brood were randomly transferred to 10 replicates per treatment levels and controls ($n=10$). Each replicate consisted of one *D. magna* per 30 ml of treatment media in a 40 ml container. Replicates were fed daily, and solutions were renewed every other day at a regular time (US EPA 1996). *D. magna* experiments were covered by a translucent light plastic sheet to prevent loss of media (USEPA 1996). Standard *D. magna* endpoints of mortality and reproduction were assessed over the 21 day chronic test period according to USEPA guidelines (1996).

Phototaxis Assessment Method

From each chronic toxicity experiment (NaCl and Ag⁺) during the 21d exposure period, *D. magna* neonate progeny (F1) were selected for phototactic analysis. Neonates released from adult *D. magna* were left in their original experimental unit and adult *D. magna* were transferred to new containers with appropriate treatment level concentrations. Adult *D. magna* were further cultured according to USEPA protocol (1996) until release of the next brood, where transfer of the adult out of the experimental unit and analysis of neonate experimental units was performed again. Over the course of 21 days the process was repeated for the amount of broods produced in each experiment (6 broods). The phototaxis assessment may be summarized by collection of neonates from experimental units and transfer into glass columns for assessment within the phototaxis assessment apparatus (Figure 1).

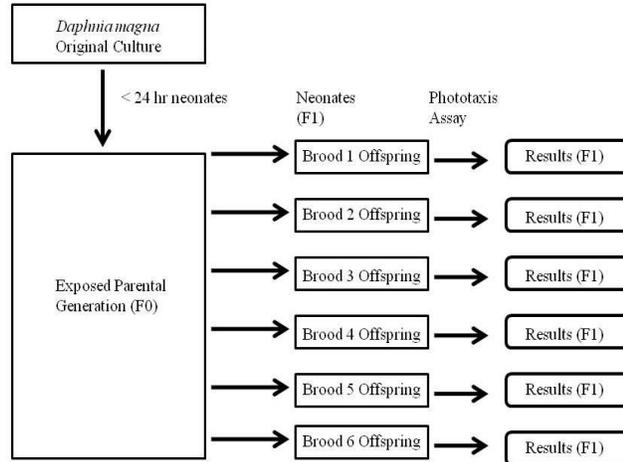


Figure 1. Phototactic assay scheme of *Daphnia magna* progeny during 21 d chronic toxicity experiments.

Neonates remaining in their experimental units were then placed in a randomized block design. In random order, 10 neonates from experimental units (typically clutch sizes of clone BU1 are greater than 10 neonates per female) were transferred by plastic pipette to small glass tubes for phototactic testing using recommended literature methodology (Michels et al. 2000, Martins et al. 2007). Neonate broods were typically released during mornings of testing days, and release time was monitored closely such that neonates were allowed to acclimate within their containers for 3 hours. After 3 hours neonates and media from experimental units were transferred to glass phototaxis columns. Neonates from experimental units were allowed a further 1 hour to acclimate after transfer to glass phototactic assessment columns. Glass tubes were used as phototaxis chambers, with a column height of 12cm, and inner diameter of 1.5cm, filled with experimental treatment media to a height of 10 cm. Column dimensions of this size have been successful in the majority of phototaxis studies (Wu et al. 2008, Michels et al. 2000, Kieu et al. 2001). *D. magna* columns were secured vertically so that the bottom of the column was above a fixed light source (Figure 2). The light source was a 60W

Philips light bulb and diffused with a thin white plastic sheet similar to methods used by Gool and Ringelberg (1997). Luminance at the bottom of *D. magna* columns was 6,700 lux, measured by an Extech Instruments light meter (Figure 1).

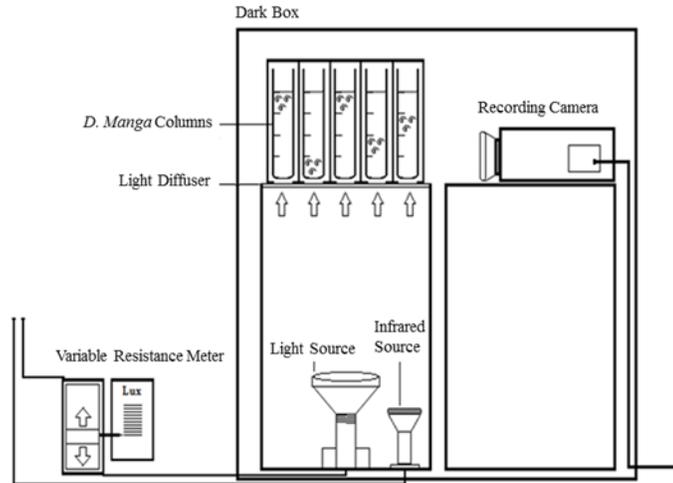


Figure 2. Experimental apparatus used for assessment of *Daphnia magna* phototaxis behavior.

D. magna were illuminated from below to encourage swimming activity as neonates were observed to assemble near the bottom of water columns during daytime hours when the study was conducted, possibly due to ambient light exposure and circadian rhythms of *D. magna* (Gerhardt 2006). Water temperature was monitored to ensure assay conditions were isothermal within the climate controlled room, as temperature effects may influence migration behavior (Loose and Dawidowicz 1994).

Illumination from below of small scale water columns may create a low light intensity refuge at the top of the columns, encouraging *D. magna* to maintain position at the top of the water column. Work conducted by Storz and Paul (1998) studying the effect of light on *D. magna* found that light attenuation in a small water column of 0-11 cm in height may occur by a factor of 5. Storz and Paul (1998) concluded that

attenuation through water occurs in a logarithmic relationship. Regardless of the direction of the light source, light intensity change (RLC) is the stimulus for upwards and downwards migration of negatively phototactic *D. magna* (Fig 3).

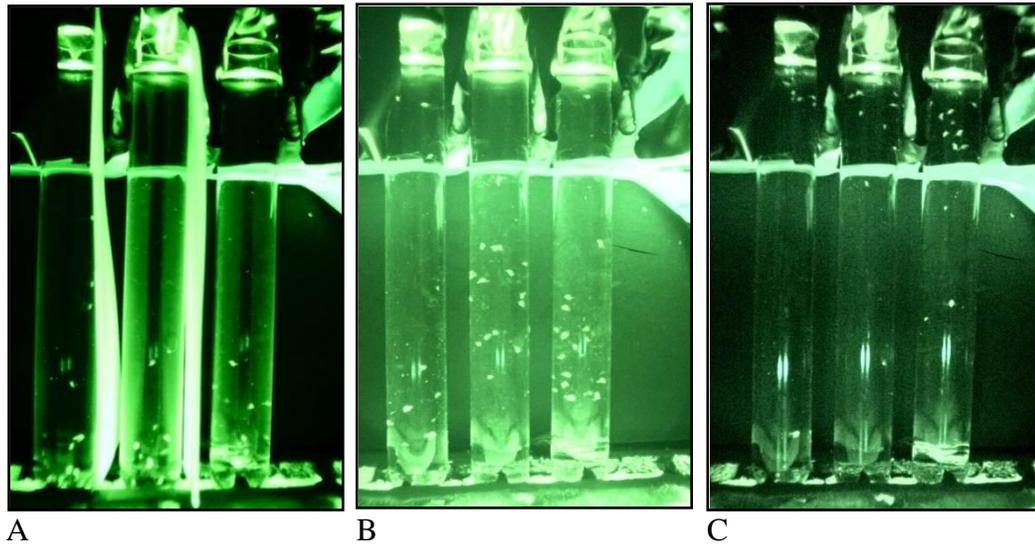


Figure 3. *Daphnia magna* phototaxis columns with illumination from below. BU1 neonates position at the bottom of water columns during the daytime under dark conditions, potentially due to endogenous rhythms associated with ambient conditions and diel vertical migration (A). After illumination from below *D. magna* negatively phototactic behavior occurs as reflex like behavior (B). After a decrease in light intensity *D. magna* migrate upwards and typically remain near the top section of the water column (C).

Study of *D. magna* migration in small, compressed water columns is generally regarded as irrelevant with regards to correlation to dynamic environmental systems that *D. magna* conduct DVM within (Ringelberg 1999). More realistic water columns heights of 1-2 meters in height have been utilized to understand phototaxis mechanisms and magnitudes, and other response disintegration relationships (Gool and Ringelberg 1997). Arguably these larger columns may be more suitable for environmentally relevant approaches to study of DVM proximate mechanisms and potential effects of stressors

(Loose and Dawidowicz 1994). However the purpose of this study was to examine the effects of stressors on a primary mechanism of *D. magna* phototaxis migration for negatively phototactic clones. In this study *D. magna* were illuminated from below, an 180° inversion of normal light direction. With interest in assessing the effects of stressors to negatively phototactic *D. magna*, only two studies have utilized negatively phototactic clones, with one study (Whitman and Miller 1982) utilizing both above and below light positioning. Currently no studies have incorporated changes in light intensity as a mechanism of phototaxis for purposes of the assessment of stressors to *D. magna* swimming or migration behavior.

Positioning of the light from below affords more advantageous observation of the RLC mechanisms of phototaxis by limiting deleterious responses associated with primary phototaxis described by Ringelberg as a more simple “hop-and-sink” swimming in response to light intensity alone (1999). Here it is believed that light intensity changes employed (50%) are of such suprathreshold-response within a very small distance that the behavior (RLC mediated phototaxis) may be elicited to appropriate standardization and magnitude for observation (Ringelberg 1964, Ringelberg 1999). Positioning the light from below does affect *D. magna* orientation behavior, which between positively and negatively phototactic *D. magna* may aid in interpreting and identifying contrast edges (Snell’s window) of the water column used migration (Ringelberg 1964). However, *D. magna* without eyes migrate primarily based on decreases and increases in light intensity, suggesting the dominate role of RLC used as the mechanism in this study. In this study no “somersaulting” or other delays of the *D. magna* response to light cues were observed, further suggesting that the direction of the light source had limited impact on *D. magna* in

very small scale water columns (Harris and Mason 1956). Study of secondary phototaxis (RLC) by illumination from below suggests that secondary phototaxis swimming direction is anchored in geotaxis (Clarke 1930). Thereby it is assumed regardless of the direction of light at small water column scales secondary phototaxis (RLC) was the dominate response mechanism, and phototaxis associated with static direction light may be of negligible contribution to *D. magna* capabilities to position themselves in the water column. Gool and Ringelberg (2003) describe the behavior initiated by RLC as photobehavior system 1, whereas photobehavior system 2 utilized for deep migration may require more complex cues such as fish kairomones.

In order to facilitate use of RLC to stimulate phototaxis, a manual variable resistance meter was used to calibrate the light source, so that light levels may be lowered at step-wise at five discrete light intensity intervals (6700, 3350, 1675, 840, 420, 210 lux). Discontinuous, step-wise relative light changes have been previously shown to repeatedly stimulate phototaxis and orientation migration behavior (Ringelberg 1964, Gool and Ringelberg 1997, 1998, 2003). Preliminary tests with clone BU1 indicated that, at 50% RLC steps, the latency to response of *D. magna* was typically <10 seconds, similar to response times reported by Gool and Ringelberg (1997) of <1 min under artificially high rates of RLC. *D. magna* clone BU1 is a highly negatively phototactic clone, comparable to negatively phototactic clones observed in other studies using columns of identical size (Cousyn et al. 2001).

After the acclimation periods previously described, phototaxis columns were immediately transferred to the phototaxis apparatus. *D. magna* columns were acclimated in darkness for 10 minutes. This length of time is generally regarded as a sufficient for

D. magna acclimation to dark conditions (Cousyn et al. 2001, Michels et al. 2000, Martins et al. 2007). The dark acclimation periods was followed by 5 minutes of illumination which triggers a reflex like but limited phototactic response (Gool and Ringelberg 1997, Ringelberg 1999, 6700 lux, Fig. 2).

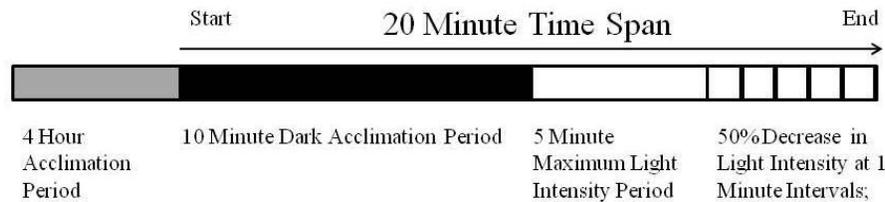


Figure 4. Phototactic assessment period of *Daphnia magna* columns. Neonate position is recorded before each step decrease in light intensity, except the first step (total of 5 steps).

After 5 minutes of illumination, 5 step-wise 50% relative light change steps were performed at 1 minute intervals (Fig. 4). Light change steps were performed over approximately 5 seconds, and vertical neonate position was recorded at one minute intervals, immediately before the next light change. Interval recording of *D. magna* position has been shown in previous phototaxis assays to be a simple but relatively robust method of determining mean phototactic response as a function of depth positioning within the water columns (Cousyn et al. 2001, Michels et al. 2000, Martins et al. 2007).

D. magna position was recorded by an infrared camera recorder with an infrared background light source at low intensity (20 lux), because infrared light has been shown to not significantly affect *D. magna* behavior (Gool and Ringelberg 1997). For each chronic toxicity experiment treatment level 5 experimental units were performed of each brood ($n=5$). *D. magna* columns were divided into 4 vertical chambers according to methods conducted by De Meester (1993), with 4 zones each 2.5 cm in depth composed

of the “Upper” (U, 10-7.5 cm), “Middle 1” (M1, 7.5-5.0 cm), “Middle 2” (M2, 5.0-2.5cm), and “Lower” (L, 2.5-0.0 cm) zones. In order to analyze neonate position due to phototaxis in a relatively holistic manner, the phototactic index developed by De Meester (1993) for positively phototactic *D. magna* was utilized by the formula $U - L / (U + M1 + M2 + L)$, with index values ranging from 1.0 to -1.0. The index was used to integrate the recorded neonate positions (5) after each step decrease in light intensity.

Statistical Analysis

A one-way analysis of variance (ANOVA) was used in Sigma Plot version 11.0 (Systat Software, Inc., San Jose, CA, USA) to identify differences between treatment levels and control ($\alpha = 0.05$). ANOVA assumptions of residual normality and homogenous variances were assessed and confirmed using the Shapiro-Wilk test. Thereafter, Dunnett’s test ($\alpha = 0.05$) was used to compare means of each treatment to respective control means. Each treatment level of the 21 d chronic toxicity experiments of NaCl and Ag⁺ were compared to the control ($n=10$) for the endpoints of lethality, reproduction, and mean clutch size. For phototaxis experiments, mean index values were calculated from each observation (5 observation points). Control phototaxis index values from each brood (broods 1-6, $n=5$ per brood) were compared to their respective treatment level (NaCl, Ag⁺).

CHAPTER THREE

Results

Survival and Reproduction Responses to NaCl and Ag+

NaCl Study

Control survival of *D. magna* for the NaCl test was 90%, which is acceptable (US EPA 1996). Mean hardness was 149.6 (± 13.3) mg/L and mean alkalinity was 102.5 (± 10.1) mg/L, and pH range was 8.01 to 8.59. The median pH level was 8.26. Mean DO was 8.16(± 0.21) mg/L. Sodium chloride concentrations were within 10% of target nominal values according to regression agreement with conductivity measurements (Table 2). The 21 d EC50 was 4.04 (SD ± 0.58) g/L NaCl. The fecundity 21 d LOEC and NOEC values of the NaCl experiment were 1.0 and 0.5 g/L, respectively (Table 2).

Table 2. NaCl salinity concentrations, survival, fecundity and clutch size of *Daphnia magna* females over 21 d toxicity experiment

Nominal Concentration NaCl (g/L)	Estimated NaCl Concentration (g/L)	<i>D. magna</i> Monoculture Survival (%)	Mean Fecundity Reproduction Neonates Female ⁻¹ (N=10, \pm SD)	Mean Clutch Size Neonates Female ⁻¹ (N=10, \pm SD)
0	0.01	90	84.6 (9.8)	14.1 (3.5)
0.5	0.49	100	74.3 (9.2)	12.4 (3.5)
1	1.01	90	68.6 (7.3)*	11.4 (3.0)
2	2.03	90	61.2 (7.4)*	10.2 (3.8)
4	4.13	60	59.4 (5.3)*	9.9 (3.7)
8	8.14	0	0	0

Standard deviations are reported in parenthesis. ^aConcentrations were measured in each monoculture days 0, 7, 14, and 21 (USEPA 1996). *: $p < 0.05$

Ag+ Study

Control survival of *D. magna* for the Ag+ experiment was 100%. For the Ag+ study mean hardness was 150.1 (± 14.1) mg/L, mean alkalinity was 112.3 (± 9.2) mg Ca/L and the pH range was 8.11 to 8.65. Mean DO was 8.53 (± 0.21) mg/L. Dissolved silver (Ag+) measurements were within 10% of target nominal values before addition of food media. After addition of food media to experimental units and 3 hour equilibrium on renewal days a significant drop in measureable ionic silver was observed in all silver concentrations with on average a 42% loss in dissolved silver (Table 3). Loss in measureable ionic silver in test media after food addition in chronic experiments was observed by Naddy et al. (2007), most likely due to complex formation with food media. The measured dissolved Ag+ 21 d EC50 value was 0.81(SD \pm 0.11) $\mu\text{g Ag}^+/\text{L}$. The LOEC and NOEC values of the Ag+ study were 0.63 $\mu\text{g/L}$ and 0.32 $\mu\text{g Ag}^+/\text{L}$, respectively (Table 3).

Table 3. Dissolved silver (Ag+) concentrations, survival, fecundity, and clutch size of *Daphnia magna* females over a 21 d toxicity experiment

Nominal Concentration Dissolved Ag+ ($\mu\text{g/L}$)	Mean Dissolved $\mu\text{g Ag}^+/\text{L}$ ^a	<i>D. magna</i> Monoculture Survival (%)	Mean Fecundity Neonates Female ⁻¹ (N=10, \pm SD)	Mean Clutch Size Neonates Female ⁻¹ (N=10, \pm SD)
0	<0.1	100	75.6 (7.7)	12.6 (2.5)
0.625	0.32	90	75.3 (8.0)	12.5 (2.1)
1.25	0.63	70	65.4 (8.0)*	10.9 (2.7)
2.5	1.53	10	61 (7.1)*	10.2 (3.8)
5	3.15	0	0	0
10	6.67	0	0	0

Standard deviations are reported in parenthesis. ^aConcentrations were measured in each monoculture days 0, 7, 14, and 21 (USEPA 1996) *: $p < 0.05$

Mean Clutch Size Responses to NaCl and Ag

Analysis of 21-d mean clutch indicated no significant differences from the control for both the NaCl and Ag+ experiments ($p<0.05$). Analysis of clutch size has been used as a sensitive endpoint of *D. magna* chronic toxicity test studies (Clubbs and Brooks 2007, Schamphelaere et al. 2007). In general, by brood analysis of clutch size was found to be less sensitive than fecundity over all 6 broods (Table 4). The most sensitive broods for the NaCl by clutch size were broods 2 and 5, where a significant difference from the respective control mean clutch size occurred at 1.0 g/L of NaCl exposure (Fig. 5). While a general dose response relationship as determined by mean clutch size was apparent across brood treatments of Ag+ (Fig 6.), no broods were found to be significantly different from each other or controls ($p<0.05$). Cumulative as well as brood specific data with mean clutch sizes are presented in table 4, and figures 5 and 6.

Table 4. Brood specific mean clutch size over 21-d chronic toxicity experiments of NaCl and Ag+ treatments

Brood	NaCl Treatments (g/L)					Ag+ Treatments ($\mu\text{g/L}$)		
	Control	0.5	1	2	4	Control	0.32	0.63
1	11.6 (1.0)	11.9 (1.2)	12 (1.3)	13.3 (1.0)	12.7 (1.2)	11.6 (1.0)	10.2 (1.3)	8.9 (1.9)
2	19.9 (0.5)	18.5 (1.0)	16.7 (1.0)*	16.1 (0.8)*	13.0 (1.0)*	16.7 (1.6)	15.3 (1.0)	14.7 (2.2)
3	14.4 (1.2)	12.1 (1.0)	10.0 (1.6)	8.8 (0.6)*	12.4 (2.1)	14.6 (1.2)	13.1 (0.6)	13.3 (2.4)
4	10.2 (1.1)	10.8 (0.7)	9.0 (1.1)	6.5 (0.7)*	6.6 (1.7)	10.3 (0.8)	14.1 (1.1)	9.3 (1.1)
5	13.9 (0.9)	12.3 (1.4)	10.1 (0.5)*	8.9 (0.7)*	8.3 (3.2)*	11.8 (1.1)	10.0 (1.7)	8.5 (1.3)
6	15.0 (1.0)	11.8 (1.2)	12.0 (1.2)	8.3 (1.2)*	3.5 (0.5)*	10.6 (0.8)	12.5 (1.9)	11.3 (0.9)

*: $p<0.05$

Phototaxis Responses to NaCl and Ag

NaCl Study

No statistically significant differences ($p<0.05$) were observed for phototaxis behavior when data from all six broods were considered. Of NaCl control broods, the 2nd

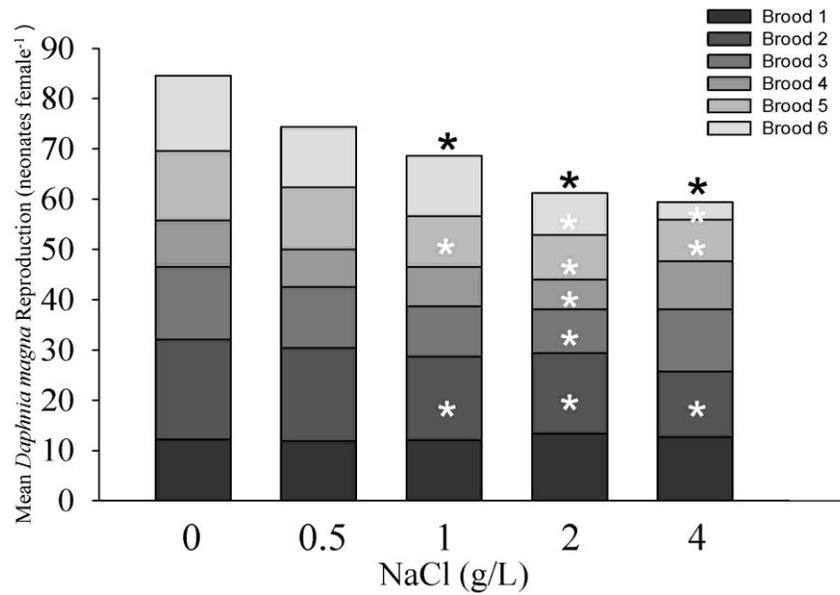


Figure 5. *Daphnia magna* mean fecundity and clutch size (neonates female⁻¹) over a 21-day salinity (NaCl) chronic toxicity experiment with treatments of 0, 0.5, 1.0, 2.0, 4.0 and 8.0 g/L. The highest concentration is not shown due to 100% mortality. *: $p < 0.05$

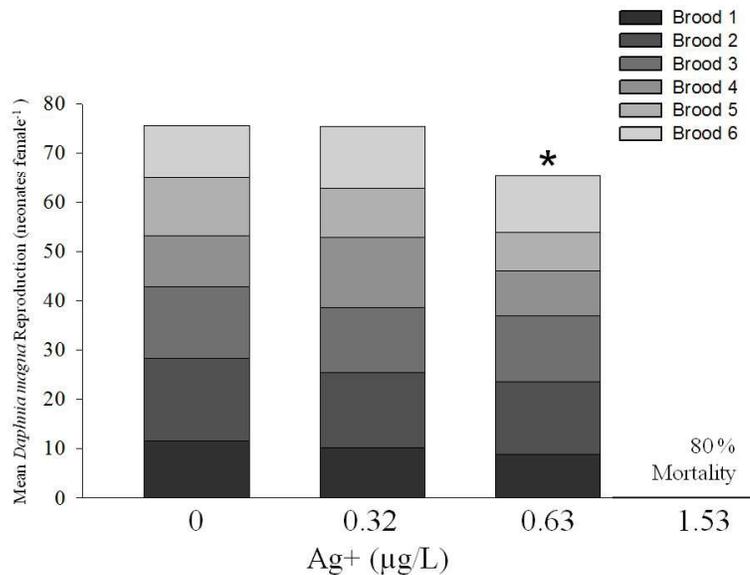


Figure 6. *Daphnia magna* mean fecundity and clutch size (neonates female⁻¹) responses over a 21-d dissolved Ag⁺ study with measured treatment levels of 0, 0.32, 0.63, 1.53 µg Ag⁺/L. Higher treatment levels not shown due to 100% mortality. *: $p < 0.05$

brood had significantly increased negatively phototactic behavior compared to other control broods as calculated by the phototactic index (ANOVA, $p < 0.05$). Because of significant differences in mortality of *D. magna* adult mothers at the 4 g/L and 8 g/L NaCl treatment levels (Fisher's exact test, $p < 0.05$), only neonates from the control, 0.5, 1, and 2 g/L treatment levels were assessed for phototaxis behavior effects for all 6 broods produced during the 21 d experiment period. No significant differences in phototactic behavior between control and treatment groups were observed of brood 1 of the NaCl experiment ($p < 0.05$); however, broods 2-6 were significantly less phototactic than controls at the 2 g NaCl/L treatment level ($p < 0.05$; Figure 7, Table 5). At the 1 g/L treatment level, broods 2, 3, 5, 6 exhibited significantly lower ($p < 0.05$) phototactic behavior than controls ($p < 0.05$), while broods 3, 5, and 5 were observed to be significantly less phototactic from the corresponding control broods at the 0.5 g/L treatment level.

Ag⁺ Study

In contrast to the NaCl experiment, phototaxis behavior of the Ag^+ study did not differ significantly among control broods ($p < 0.05$). Because the 2.5 and 5 $\mu\text{g/L}$ Ag^+ treatment levels resulted in significantly higher mortality (Fisher's exact test, $p < 0.05$) of *D. magna* mothers (Table 5), phototactic behavior of neonates from the 0.625 and 1.25 $\mu\text{g/L}$ treatment levels were assessed only. Similar to the NaCl experiment, no significant differences in the phototactic behavior of brood 1 neonates were observed among controls and treatment levels. At the fecundity LOEC treatment level (1.25 $\mu\text{g/L}$ nominal, 0.63 $\mu\text{g/L}$ measured), phototactic behavior was significantly decreased in broods 2, 4, 5 and 6. Further, at or below the reproductive NOEC treatment level (0.625

µg/L nominal, 0.32 µg/L measured) (Figure 8, Table 5), broods 2, 5 and 6 were less phototactic than controls. The phototactic assay results of both studies (NaCl, Ag+) and significant differences from control values are reported are reported in Table 5. It is interesting to note that phototactic behavior of every brood appeared more sensitive, based on LOEC/NOEC thresholds, than corresponding mean brood neonate production (Table 6).

Table 5. Phototactic index values of *Daphnia magna* progeny (F1) by brood from NaCl and dissolved Ag+ treatments over 21-day toxicity experiments

Brood	NaCl (g/L) Mean Phototaxis Index Values (I _p) (N=5, ±SD)				Ag+ (µg/L) Mean Phototaxis Index values (I _p) (N=5,±SD)		
	Control	0.5	1	2	Control	0.32	0.63
1	-0.13 (0.11)	-0.23 (0.23)	-0.15 (0.30)	-0.24 (0.14)	-0.23 (0.08)	-0.10 (0.10)	-0.39 (0.06)
2	0.43 (0.10)	0.08* (0.14)	-0.10* (0.12)	-0.20* (0.15)	0.16 (0.06)	-0.52* (0.01)	-0.51* (0.06)
3	-0.22 (0.05)	-0.61* (0.10)	-0.38 (0.43)	-0.50* (0.04)	0.10 (0.15)	-0.37 (0.16)	-0.32 (0.19)
4	-0.23 (0.05)	-0.19 (0.12)	-0.47 (0.06)	-0.66* (0.08)	-0.01 (0.09)	-0.26 (0.08)	-0.42* (0.09)
5	-0.03 (0.08)	-0.44* (0.08)	-0.44* (0.08)	-0.56* (0.07)	-0.06 (0.04)	-0.36*(0.08)	-0.44* (0.14)
6	0.05 (0.06)	-0.45* (0.06)	-0.46* (0.08)	-0.49* (0.10)	-0.08 (0.10)	-0.65*(0.04)	-0.77* (0.05)

*: $p < 0.05$; Positive I_p indicate stronger negative phototaxis (RLC) response, negative I_p values indicate no to minimal response to phototactic stimulus.

Table 6. NOEC and LOEC concentrations of dissolved Ag+ and NaCl with endpoints determined by *Daphnia magna* phototaxis index and reproduction (neonates female⁻¹) over a brood specific basis during 21-day experiments (NaCl, Ag+)

Brood Number	NaCl (g/L)						Ag+ (µg/L)					
	Reproduction			Phototaxis			Reproduction			Phototaxis		
	NOEC (0.5 g/L) ^a	LOEC (1.0 g/L) ^a	ACR (EC 50/ NOEC)	NOEC	LOEC	ACR (EC 50/ NOEC)	NOEC (0.32 µg/L) ^b	LOEC (0.63 µg/L) ^b	ACR (EC 50/ NOEC)	NOEC	LOEC	ACR (EC50/ NOEC)
1	-	8*	-	-	-	-	0.63	1.53*	1.3	-	-	-
2	1.0	2.0	4.0	-	0.5	-	0.63	1.53*	1.3	-	0.32	-
3	1.0	2.0	4.0	-	0.5	-	0.63	1.53*	1.3	-	-	-
4	1.0	2.0	4.0	1.0	2.0	4.0	0.63	1.53*	1.3	0.32	0.63	2.5
5	0.5	1.0	8.1	-	0.5	-	0.63	1.53*	1.3	-	0.32	-
6	1.0	2.0	4.0	-	0.5	-	0.63	1.53*	1.3	-	0.32	-

*LOEC value determined by survival; ^aCumulative reproduction endpoint concentration for NaCl; ^bCumulative reproduction endpoint concentration for dissolved Ag+; EC50 Value for the NaCl experiment was 4.04 g/L NaCl; EC 50 value for the dissolved Ag+ experiment was 0.81 µg Ag+/L.

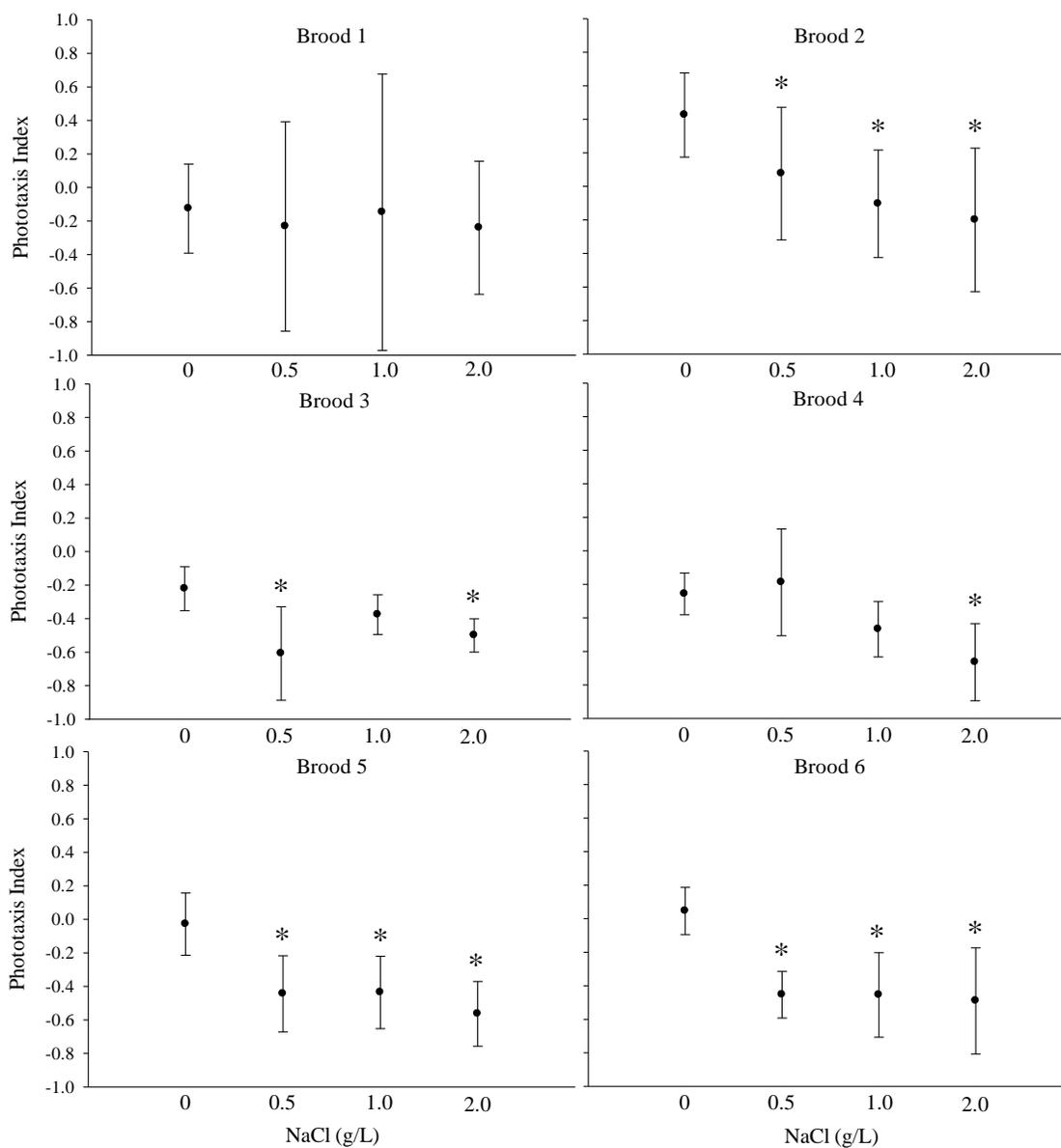


Figure 7. Mean ($n=5$; \pm SD) phototaxis behavior index values of *D. magna* progeny (F1) by brood from NaCl treatment levels of a 21 d toxicity experiment. Positive phototactic index values indicate greater mean negatively phototactic response to light stimuli (RLC). *: $p < 0.05$

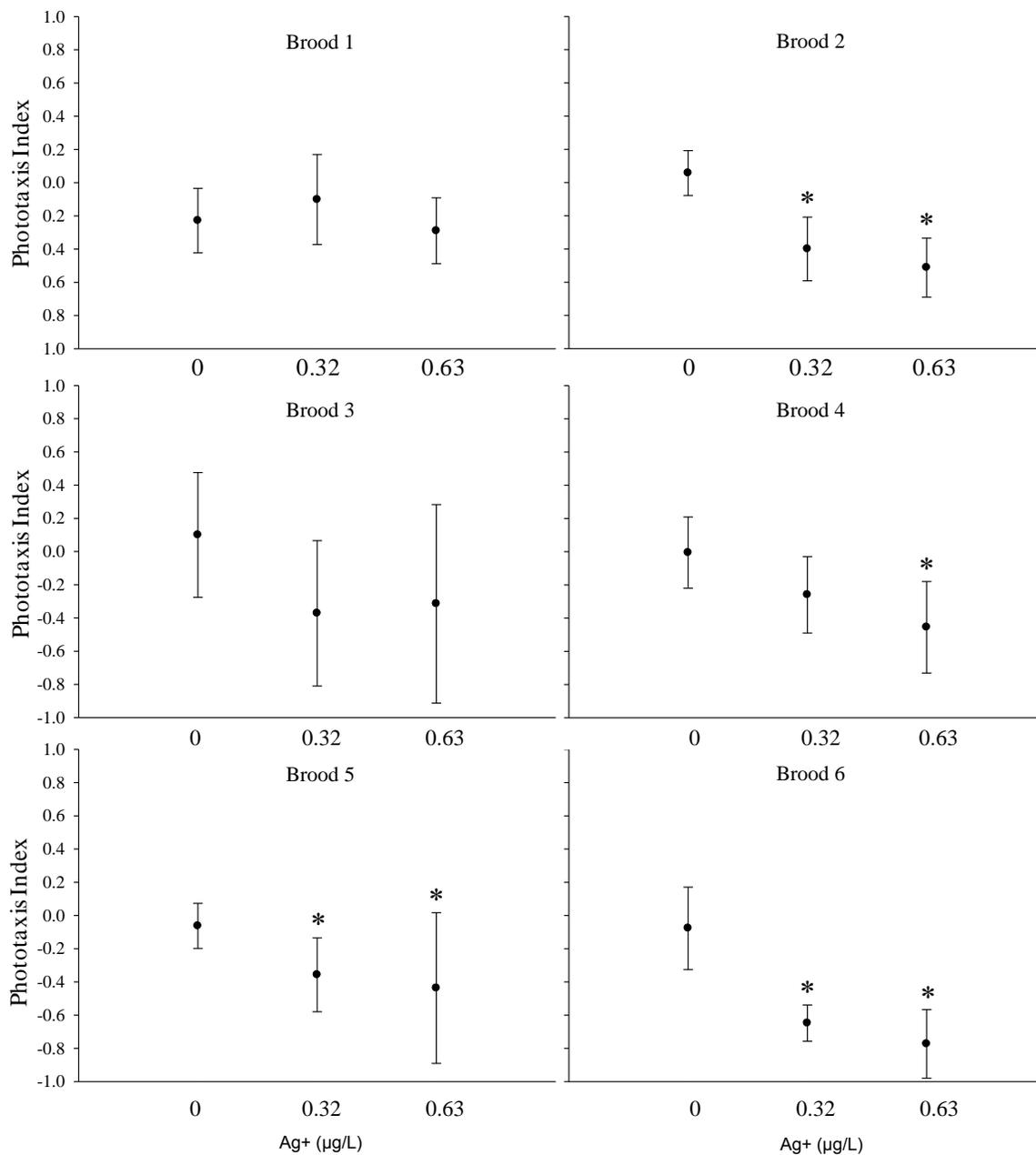


Figure 8. Mean ($n=5$; \pm SD) phototaxis behavior index values of *Daphnia magna* progeny (F1) by brood from treatments of dissolved Ag+ over a 21 d toxicity experiment. Positive phototactic index values indicate greater mean negatively phototactic response to light stimuli (RLC). *: $p < 0.05$

CHAPTER FOUR

Discussion

The primary objective of this study was to examine the comparative sensitivity of *D. magna* neonate phototaxis behavior and standardized endpoints of mortality and reproduction for two model contaminants (NaCl, Ag⁺). While phototaxis has been used as a sublethal endpoint for acute assessments of toxicant exposure for a broad spectrum of chemicals, including organics, metals, and mixtures (Martins et al. 2007, Wu et al. 2008), very few studies have assessed the use of *D. magna* neonate phototaxis as an indicator of chronic toxicity (Flickringer et al. 1982, Whitman and Miller 1982). Even less understood are the consequences of prenatal exposure on neonate phototactic behavior. Here, I tested the hypothesis that *D. magna* neonate phototaxis is more sensitive an endpoint over a chronic study period than mortality and reproduction. Typical 21-d experimental designs with *D. magna* assess contaminant effects on adult mortality and the total number of neonates released from all broods (e.g., typically 6-7 broods; 6 broods in this study). In the present study, significant differences in neonate phototactic behavior was observed among broods, indicating that maternal exposure to sublethal levels of NaCl and Ag⁺ may have impacted offspring. In fact, progeny phototactic response was significantly affected at or below 21-day LOEC thresholds for fecundity in broods 2, 3, 5 and 6 of the NaCl experiment and in broods 2, 4, 5 and 6 of the dissolved Ag⁺ study. If endpoints were to be ranked in order of increasing sensitivity

of detection of effects, the order would be lethality < clutch size < fecundity, with neonate phototaxis in most cases more sensitive than fecundity.

Investigations of toxicant effects and linkages to behavioral, energy, and neurochemistry of fish are of significant but emerging study (Amiard-Triquet 2009), yet it may be argued that understanding of behavioral linkages of aquatic invertebrates may be even less understood. Presently major aquatic invertebrate neurological-mechanistic linkages for behaviors such as avoidance and migration are only beginning to be elucidated (McCoole al. 2011, Guler and Ford 2010). However the relatively high sensitivity of phototaxis behavioral activity of *D. magna* to toxicants seems plausible. For example, Guler and Ford (2010) found that exposure of amphipods to levels of fluoxetine at what may be considered potential sewage treatment plant effluent levels (ng to $\mu\text{g l}^{-1}$) affected phototaxis behavior. While this effect may be specific to the mode of action of pharmaceuticals such as fluoxetine, other toxicants may result in similar effects (e.g. reduced phototaxis) through more general mechanisms (e.g. chronic stress) that affect energy budgets of *D. magna*.

It is proposed that neonate phototaxis, as characterized in this study, may be affected by the following mechanisms: one, chronic toxicant effects occurring over the course of embryonic and prenatal development that result in poorer performing neonates, and two, acute effects that occur after release from the brood chamber. Given the relatively increased sensitivity of later broods compared to earlier broods, acute effects on progeny may be of lesser importance. However other reasons for observed phototaxis perturbations cannot be ruled out, such as stress related avoidance behavior of toxicants,

or mechanistic specific effects related to *D. magna* photoreception and physiological function.

Relatively similar results in phototactic endpoints sensitivity at or below LOEC levels were obtained from chronic experiments of both NaCl and Ag⁺, however the causes of observed phototaxis perturbations may result from different chronic (over 21 d) mechanisms of toxicity. Neonate phototactic performance of the NaCl study may have been affected by more general mechanisms of energy budget stress from exposure to NaCl, whereas the trace level toxicity of the Ag⁺ study may have had additional compounded effects due to transgenerational effects, and potentially specific effects resulting from disposition of Ag⁺ in the eye region of *D. magna* and disruption or stress of photoreception (Bianchini and Wood 2005).

The preponderance of later broods exhibiting lower phototaxis performance at toxicant levels below LOEC thresholds relative to controls suggests that prolonged chronic exposure may affect progeny behavior. This observation is novel compared to previous observed phototactic effects, which are typically observed after <24 h of exposure to adult *D. magna*. The primary causes of the observed decrease in phototaxis performances of neonates are proposed to be due to physiological energy allocation stress (egg development, somatic maintenance, growth) of adult *D. magna* and associated prenatal effects that may impact neonate swimming and phototactic performance. Maintenance costs of phototaxis vis-à-vis the metabolic cost and maintenance of photoreceptors may be quite high, depending on species investment in photoreception and interneurons in the compound eye (Laughlin et al. 1998). As photoreception and signaling costs a large investment of ATP, reduction in overall energy level of organisms

such as *D. magna* may reduce phototactic ability (Laughlin et al. 1998). Repeated assessment of the phototactic magnitude of neonates, as performed here, may differentiate progeny of optimal or suboptimal physiological status. Many toxicants (linear alkylbenzene sulfonic acid, sodium pentachlorophenolate, and 2,4-dichlorophenoxyacetic acid, CdCl₂, K₂Cr₂O₇, tributyltin chloride) have been shown to affect the energy consumption, assimilation, and reserves of *D. magna* in 21-d chronic toxicity tests (Coen and Janssen 2003). Jemec et al. 2008 found that exposure to chromium (IV) or cadmium reduced protein content, cholinesterase, catalase, and glutathione S-transferase (GST) of *D. magna* over chronic exposure conditions, with afore mentioned biomarkers affected at levels below 21-d reproduction endpoints.

Characterizing energy status parameters such as biomarkers of *D. magna* mothers and progeny may elucidate behavioral effects such as phototaxis perturbations below levels of toxicity indicated by standardized endpoints (lethality, reproduction). Biomarkers, which may be potential indicators of *D. magna* energy status (Coen and Janssen 2003), have been linked to chronic and trace level exposure-effects relationships. For example, *D. magna* cholinesterase levels have been shown to be altered from chronic exposure to 0.082 µg/L of Cd (Jemec et al. 2008). Given the sensitivity of the energy budget and metabolic character of *D. magna*, it seems plausible that sensitive behavioral endpoints such as phototaxis are a linkage between energy status and individual behavioral performance (Amiard-Triquet 2009).

The use of biomarkers and behavioral assays to assess potential linkages among endpoints has begun to receive more attention. Sancho et al. (2009) used four biomarkers of protein, glycogen, lipids, caloric content correlated with feeding behavior studies after

exposure to 4 sublethal concentration of tebuconazole (0.41, 0.52, 0.71, 1.14 mg/L). *D. magna* energy content decreased as tebuconazole concentration increased, and catabolism increased in relation to adverse effects observed from feed behavior (Sancho et al. 2009). Natural stressors have effects on energy reserves, with alterations observed in lipid, protein and sugar content across culturing *D. magna* under different temperature regimes (16-26°C) (Filho et al. 2011). The phototactic perturbations in this study observed below reproduction LOEC or NOEC levels may be linked to general bioenergetic stress and poor neonate energy status/physiologic condition, or for other mechanistic effects.

In the present study, bioenergetic stresses of *D. magna* adults may have carry over effects on *D. magna* offspring, potentially impacting their metabolic state, and phototactic performance. Energy budget stress typically begins with poor food assimilation which reduces or inhibits *D. magna* capacity for growth, reproduction, and maintenance and metabolism (Pane et al. 2003). Optimal assimilation of food is highly important for development of progeny, as embryonic respiration has been found to be lowest in *D. magna* raised at lower food levels (Barata et al. 2005). Pane et al. (2003) observed decreased energy states of progeny born of mothers exposed to nickel, indicated by reduced growth and metabolites of unexposed progeny.

Decreased progeny energy status may first be preceded by energy budget deficits associated with maternal egg production. Decreased metabolic energy allocation towards egg production has been shown to contribute to dysfunction of *D. magna* neonate acclimation and maintenance processes (De Coen and Janssen 2003). Under cadmium acclimation of *D. magna* across generations, smaller *D. magna* progeny had increased

sensitivity to other toxicants apart from cadmium, with reduced neonate size possibly due to reduced nutrition caused by stress (Guan and Wang 2006). Low food quantity and stressors of *D. magna* have been shown to affect egg production, where prenatal and postnatal conditions affect the maturation process (Enserink et al. 1995). Frost et al. (2009) observed trans-generational effects concerning phosphorus food limitation, whereby offspring were smaller and more susceptible to infection from a virulent bacterium.

In addition to toxicant/stressor related effects on energy budgets of *D. magna* progeny, energy allocation strategies may also affect progeny development. *D. magna* maintenance (somatic) has been observed as a higher priority for energy allocation than reproduction or growth (Glazier and Calow 1992). Young adult *D. magna* put as much or more priority on reproduction than growth, whereas older adults may put more priority on growth (Glazier and Calow 1992). Apparent differences in growth allocation patterns based on age may impact energy allocation to offspring, resulting in progeny of different energy status. Juvenile *D. magna* focus energy budgets on respiration and growth, which are known endpoints to be affected by toxicant exposure (Glazier and Calow 1992). Seemingly the *D. magna* energy budget tied to offspring production is highly sensitive to stress, given the wide range of responses observed in these transgenerational studies. However rarely have potential behavioral effects of offspring across generations been characterized, if at all.

Chronic exposure to toxicants such as NaCl may decrease the energy budgets of *D. magna* and result in progeny born with decreased energy status, and consequently decreased phototactic performance of treatment progeny. Small changes in salinity at

chronic, trace levels are known to have significant effects on freshwater macroinvertebrates (Hassel et al. 2006). Hassel et al. (2006) reported decreased number of emerging *Chironomus sp.* and delayed time to emergence, and disturbed growth processes as result of changes in environmental salinity. *Daphnia spp.* relative sensitivity to changes in salinity over life history assessments appears to be strain and species dependent (Martínez-Jeronimo 2007). Gonçalves et al. (2007) conducted 21-d life history studies with *D. magna* and *D. longispina*, and reported a 21-d *D. magna* NOEC of 4.55 g/L NaCl, but for *D. longispina* reported a NOEC of 1.55 g/L. Cowgill and Milazzo (1991) reported a NOEL for NaCl of 1.2g/L, comparable to the NOEC reported in this study (1.0 g/L). The *D. magna* clone in the present appears to be relatively sensitive to changes in salinity in comparison to some previous strains studied in chronic toxicity tests (Martínez-Jeronimo 2007). Therefore chronic exposure of NaCl to adult cladocerans most likely affected phototaxis performance of neonates. If *D. magna* fail to acclimate to toxicant conditions, budgets of energy devoted and divided among promoting individual neonate growth may be expected to decrease, with potential effects compounded over time. This may explain the preponderance of poorer neonate performance in later broods (e.g., broods 3-6).

In contrast to the relative large doses (1.0 g/L) of NaCl required to elicit chronic effects on *D. magna*, dissolved silver is known to cause acute and chronic effects at trace levels (Bianchini et al. 2002). Aquatic invertebrate effects of ionic silver (Ag^+) are known to be primarily ionoregulatory, affecting Na^+/K^+ ATPase mediated transport under acute and chronic exposure conditions (Bianchi et al. 2002). Ionic silver is known to disrupt ionoregulatory Na^+/K^+ ATPase pumps of *D. magna*, with up to 90% inhibition of

enzyme activity at exposure concentrations of 0.142 $\mu\text{g/L}$ (Bianchi and Wood 2003). The toxic effect of due to dissolved Ag^+ is known to occur rapidly in *D. magna*, with lethality primarily occurring within 1-3 hours of exposure (Bianchi and Wood 2008). Concerning Ag^+ , acute effects, in contrast to chronic effects, may be considered as a component, if not a predominant cause of phototaxis perturbations observed. However, the relatively lower phototactic response of later broods in comparison to earlier broods, as well as the preponderance of increased sensitivity of later broods, suggests that chronic effects may not be ruled out as a dominant cause of phototaxis perturbation.

Bianchi and Wood (2008) have suggested that maternal transfer of Ag^+ may occur, as significant 2nd generation growth inhibition effects on *D. magna* raised in silver-free media have been observed while conducting chronic toxicity tests. Lam and Wang (2006) found that maternal transfer of Ag^+ was low, estimating Ag^+ efflux as maternal transfers at approximately 0.1-3.9% of Ag^+ efflux, whereas regeneration of Ag^+ into the dissolved phase was the primary source of efflux (83-94%). Toxicants such as Ag^+ may affect assimilation efficiency of food (Tsui and Wang 2007). For example, Tsui and Wang (2007) found that assimilation efficiency of Ag^+ from food is primarily based on food density. Bianchini and Wood (2005, 2008) suggest that latent Ag^+ effects on neonates may be quite high. Concerning *D. magna* physiology related to phototaxis, Bianchini and Wood specifically reported that *D. magna* accumulates high amounts of silver in the compound eye region (2005). Accumulation of silver in this region is possibly due to increased activity of compounds and enzymes to protect oxidative stress of these vital structures including glutathione and glutathione S-transferase (Bianchini 2005). A high proportion of silver accumulation is clearly seen and reported by

autoradiograms performed by Bianchini and Wood (2005). It may be that developmental exposure and silver body burdens affected neonate phototaxis behavior of treatments observed in the present study due to increased maintenance associated with the compound eye region of *D. magna*, which is critical to neurology and behavior associated with swimming and orientation (Smith and Macagno 1989, Ringelberg 1999, McCooles et al. 2011).

Because the phototaxis assessment elicits rapid photoreception and kinetic activity of neonates, poor quality neonates would seemingly be at a disadvantage compared to control neonates. In addition to photoreception costs, *D. magna* kinetic muscular activity due to stress may contribute to inhibition of phototaxis. Baillieul et al. (1998) have shown that *D. magna* exposed to high levels of salinity (20-80 ppt) have decreased swimming velocities when compared to control groups, with acclimation leading to increased swimming velocities occurring over several days. Short term exposure to metals has been shown to decrease *D. magna* mobility at sublethal levels (Baillieul and Blust 1999, Untersteiner et al. 2003). A decrease in *D. magna* swimming velocity over a 10 day chronic exposure period to 10^{-8} M Cd²⁺ was observed when compared to control, regardless of food intake and body size (Baillieul and Blust 1999). Copper exposure to *D. magna* after 14 hours reduced average swimming velocity at 10 µg/L Cu, but not 5 and 1 µg/L (Untersteiner et al. 2003). Concentrations of cadmium exposure from 3.5 to 5.0 µg/L in combination with increased temperature regimes, decreased *D. magna* swimming velocity after 24 hours (Wolf et al. 1998). Ultimately the energy budget stress and suborganism effects induced by chronic exposure of NaCl and

Ag⁺ in the present study may have impacted phototaxis mechanisms and kinetic muscular activity of *D. magna* neonates assessed in this study.

The lack of phototactic effects observed in the first brood of both studies (NaCl, Ag⁺) at all treatment levels assayed may be due to life cycle strategies of *D. magna* reproduction. Boersma (1997) observed that the fitness of *D. magna* of the F1 generation in terms of optimal effort per offspring is greatest from neonates of the first brood, whereas later broods show less fitness, due to tradeoffs in the physical size and number of offspring. In other cases exposure to low levels of toxicants may trigger differing life strategies of *D. magna*, including generational hormesis when exposed to low levels of cadmium (Bodar et al. 1987). When exposed to humic acid substances with bound silver, *D. magna* have been shown to increase growth and reproduction in a hormesis like effect (Glover and Wood 2004). Phototaxis effects in this study were characterized at levels of toxicants (NaCl, Ag⁺) where very little mortality occurs, and it is conceivable that slight changes in life strategies of *D. magna* in response to low level toxicant exposure may have influenced the quality of neonates in earlier broods, or in some fashion maintained phototactic capacity of progeny of early broods so that the phototactic assay could not detect significant changes in phototactic performance between cohorts.

Future investigation of behaviorally relevant biomarkers in combination with behavioral assays may improve characterization of chronic effects, as there is a paucity of information on the effects of chronic toxicity (e.g. metals) on *D. magna* (Jemec et al. 2008). This study contributes to a growing body of evidence of the high sublethal behavioral sensitivity of *D. magna* to stressors. Improving the characterization of behavioral effects such as phototaxis performance, as an integral component of diel

vertical migration, may contribute to an understanding of suborganism and organism level effects of stressors on aquatic organisms such as *D. magna*.

Future Directions

Whereas *D. magna* phototaxis may be a sensitive indicator of sublethal stress, behavior endpoints must have ecological importance to be useful within the ecological risk assessment paradigm. Inhibition of *D. magna* phototaxis behavior as an integral mechanism of DVM may have direct implications for viability of cladoceran populations. In other pelagic invertebrates, such as the marine copepod *Psuedocalanus sp.*, DVM behavior has been shown to reduce mortality due to predator avoidance and thus significantly increase fitness of individuals (Ohman 1983). While DVM of *D. magna* has been observed to have fitness costs in terms of growth and optimal grazing patterns (Loose and Dawidowicz 1994, Lampert 1989), DVM as an adapted predator avoidance mechanism is thought to ultimately result in fitness benefits where the costs of grazing pressure (e.g. ingestion by fish or invertebrates) outweighs the costs (Rinke and Petzoldt 2008). Alterations of *D. magna* DVM behavior in the environment as a consequence of contaminants may have significant ecological consequences. Alterations in zooplankton predator avoidance behavior affects population stability by eliminating fitness benefits associated with optimal depth position (Dodson and Hanazato 1995).

Avoidance behavior of zooplankton may also be vital to coping with toxicant stress. Lopes et al. (2004) have observed *Daphnia longispina* conduct avoidance behavior of copper exposure gradients of 3-87 $\mu\text{g/L}$ in large laboratory containers. Avoidance of copper was determined to be more sensitive than lethality, with differential avoidance behavior among acclimated and non-acclimated *D. longispina* sampled from

reference and contaminated sites (Lopes et al. 2004). Consensus of avoidance behavioral assessments suggests that avoidance behavior may contribute as a complementary tool in risk assessment studies (Lopes et al. 2004).

In freshwater autotrophic communities, DVM behavior of *D. magna* may impact phytoplankton trophic interactions within lake systems. Haupt et al. (2009) investigated the effects of migrating and non-migrating grazing on phytoplankton abundance and diversity in lake mesocosm experiments. In lake mesocosm experiments containing migrating *D. magna*, higher phytoplankton diversity was observed and phytoplankton abundance increased, with species specific impacts on phytoplankton development (Haupt et al. 2009). Reichwaldt et al. found that changes in nighttime grazing regimes of *D. magna* influenced shifts in algal species ratios, and may impact qualitative phytoplankton structure (2005). Field experiments support observations of changes in phytoplankton community structure, with fast growing algae benefiting from discontinuous grazing by migrating zooplankton (Reichwaldt et al. 2004). Zooplankton behavior such as DVM impacts predator-prey relationships as well, with “behavioral cascades” occurring in the environment, indicating the importance of optimal behavioral functions among trophic levels (Fiksen et al. 2005, Bollens et al. 2010). Based on observations in this study and others, further study of the community and trophic impacts of zooplankton behavior including DVM, and the potential effects of anthropogenic contaminants, are warranted (Dodson and Hanazato 1995).

D. magna DVM and phototaxis behavior may also serve as a suitable assessment tool for sublethal effects of emerging contaminants. Brausch et al. (2010) found fullerenes and functionalized fullerenes (c^{60} , $f c^{60}$) affected *D. magna* swimming behavior

and phototaxis behavior at sublethal levels ($545.4 \mu\text{g/L c}^{60}$, $545.6 \mu\text{g/L } ^f\text{c}^{60}$). Recent work by McCool et al. (2011) determined that histamine has a strong role in regulating photoreception of *D. magna*, with exposure of *D. magna* to the broad spectrum H2 receptor antagonist cimetidine resulting in inhibition of *D. magna* negative phototactic response to UV exposure (McCool et al. 2011). In many arthropods, histamine is synthesized and stored in photoreceptors, undergoes Ca-dependent release, thereby inhibiting postsynaptic interneurons by gating Cl⁻ channels (Stuart et al. 2007). Environmental contaminants that affect histaminergic function of *D. magna* may alter phototaxis and DVM behavior, resulting in zooplankton population consequences (McCool et al. 2011). Zooplankton behavior has been proposed to be utilized in a precaution system for aquatic metal pollution as part of a comprehensive approach with other methods (Zhou et al. 2008). Behavior monitoring is advantageous as it is a direct monitoring approach, where behavior alterations due to environmental contaminants may be quickly and easily identified (Zhou et al 2008). Further investigation of seemingly strong linkages between behavior (e.g. swimming, foraging, feeding, and migration) and suborganism status is warranted (Amiard-Triquet 2009). Results from the present study with Ag disruption of neonate phototaxis behavior has important implications for developing an understanding of nanosilver in the environment, particularly because ionic silver contributes to the apparent toxicity of nanosilver (Park and Choi 2010, Powers et al. 2011).

Conclusion

Behavioral responses in ecotoxicology are increasing in frequency of use and have been proven to be more sensitive than most standardized acute endpoints such as lethality. Behavioral assessments that target sensitive life stages of model organisms such as *D. magna* may prove useful in broadening the applicability of behavioral assessments. Light intensity change as a simple method for phototaxis assay of negatively phototactic clones appears relatively robust. In present study neonate phototaxis behavior of *D. magna* broods exposed during development were more sensitive to NaCl and Ag⁺ than brood specific fecundity, suggesting that behavioral assessments of negatively phototactic neonates provide an additional measurement within 21-d chronic toxicity test designs. Alternatively, an assessment factor could be developed in future studies that accounts for differences among standardized endpoints and such behavioral perturbations. Further investigations of *D. magna* neonate effects from exposures during development may allow for a greater understanding of linkages among organism and suborganism stress and behavior.

BIBLIOGRAPHY

- Allan IJ, Vrana B, Greenwood R, Mills GA, Roig B, Gonzalez C. 2006. A “toolbox” for biological and chemical monitoring requirements for the European Union’s Water Framework Directive. *Talanta* 69:302-322.
- Amiard-Triquet, Claude. 2009. Behavioral Disturbances: The missing link between sub-organismal and supra-organismal responses to stress? Prospects based on aquatic research. *Human and Ecological Risk Assessment* 15:87-110.
- Baillieul M, De Wachter B, Blust R. 1998. Effect of salinity on the swimming velocity of the water flea *Daphnia magna*. *Physiological Zoology* 71:703-7.
- Baillieul M. 1999. Analysis of the swimming velocity of cadmium-stressed *Daphnia magna*. *Aquatic Toxicology* 44:245-254.
- Barata C, Navarro JC, Varo I, Rivaa MC, Arunc S, Porte C. 2005. Changes in antioxidant enzyme activities, fatty acid composition and lipid peroxidation in *Daphnia magna* during the aging process. *Comparative Biochemistry and Physiology. Part B, Biochemistry & Molecular Biology* 140:81-90.
- Benn TM, Westerhoff P. 2008. Nanoparticle silver released into water from commercially available sock fabrics. *Environmental Science & Technology* 42:4133-9.
- Berninger JP, Du B, Connors KA, Eytcheson SA, Kolkmeier MA, Prosser KN, Valenti TW Jr., Chambliss KC, Brooks BW. 2011. Effects of the antihistamine diphenhydramine on selected aquatic organisms. *Environmental Toxicology and Chemistry* 30:2065-72.
- Bianchini A, Wood CM. 2003. Mechanism of acute silver toxicity in *Daphnia magna*. *Environmental Toxicology and Chemistry* 22:1361-7.
- Blaser SA, Scheringer M, Macleod M, Hungerbühler K. 2008. Estimation of cumulative aquatic exposure and risk due to silver: Contribution of nano-functionalized plastics and textiles. *Science of the Total Environment* 390:396-409.
- Bodar CWM, Van Leeuwen CJ, Voogt PA, Zandee DI. 1988. Effect of cadmium on the reproduction strategy of *Daphnia magna*. *Aquatic Toxicology* 12:301-309.
- Boersma M. 1997. Offspring size and parental fitness in *Daphnia magna*. *Evolutionary Ecology* 11:439-450.

- Bollens SM, Rollwagen-Bollens G, Quenette JA, Bochsansky AB. 2010. Cascading migrations and implications for vertical fluxes in pelagic ecosystems. *Journal of Plankton Research* 33:349-355.
- Boyd WA, Brewer SK, Williams PL. 2002. Altered behaviour of invertebrates living in polluted environments. In Dell’Omo G, ed, *Behavioural Ecotoxicology*. J Wiley & Sons, Chichester, UK, p. 295.
- Bossuyt B. 2003. Acclimation of *Daphnia magna* to environmentally realistic copper concentrations. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* 136:253-264.
- Brausch, KA., Anderson, TA., Smith, PN. and Maul, JD. 2011. The effect of fullerenes and functionalized fullerenes on *Daphnia magna* phototaxis and swimming behavior. *Environmental Toxicology and Chemistry* 30:878–884.
- Brooks BW, Riley TM, Taylor RD. 2006. Water quality of effluent-dominated ecosystems: ecotoxicological, hydrological, and management considerations. *Hydrobiologia* 556:365-379.
- De Coen WM, Janssen CR. 2003. The missing biomarker link: relationships between effects on the cellular energy allocation biomarker of toxicant-stressed *Daphnia magna* and corresponding population characteristics. *Environmental Toxicology and Chemistry* 22:1632-41.
- Clarke GL. 1930. Change of phototropic and geotropic signs in *Daphnia* induced by changes of light intensity. *The Journal of Experimental Biology*. 7:109-130.
- Cousyn C, De Meester L, Colbourne JK, Brendonck L, Verschuren D, Volckaert F. 2001. Rapid, local adaptation of zooplankton behavior to changes in predation pressure in the absence of neutral genetic changes. *Proceedings of the National Academy of Sciences of the United States of America* 98:6256-60.
- Cowgill, UM, Milazzo DP. 1991. Demographic effects of salinity, water hardness and carbonate alkalinity on *Daphnia magna* and *Ceriodaphnia dubia*. *Hydrobiologia*. 122:35–56.
- Dauvin JC, Desroy N, Denis L, Ruellet T. 2008. Does the *Phaeocystis* bloom affect the diel migration of the suprabenthos community? *Marine Pollution Bulletin*. 56:77-87.
- De Meester L. 1993. Genotypes, fish-mediated chemicals, and phototactic behavior in *Daphnia magna*. *Ecology* 74:1467–1474.

- Dodson S, Hanazato T. 1995. Commentary on effects of anthropogenic and natural organic chemicals on development, swimming behavior, and reproduction of *Daphnia*, a key member of aquatic ecosystems. *Environmental Health Perspectives* 103:7-11.
- Dzialowski EM, Turner PK, Brooks BW. 2006. Physiological and reproductive effects of beta adrenergic receptor antagonists in *Daphnia magna*. *Archives of Environmental Contamination and Toxicology* 50:503-10.
- Filho TU, Soares AMVM, Loureiro S. 2011. Energy budget in *Daphnia magna* exposed to natural stressors. *Environmental Science and Pollution Research International* 18:655-62.
- Flickringer AL, Bruins RJF, Winner RW, Skillings JH. 1982. Filtration and phototactic behavior as indices of chronic copper stress in *Daphnia magna* Straus. *Environmental Contamination and Toxicology* 11:457-463.
- Forward R, Rittschof D. 2000. Alteration of photoresponses involved in diel vertical migration of a crab larva by fish mucus and degradation products of mucopolysaccharides. *Journal of Experimental Marine Biology and Ecology* 245:277-292.
- Frost PC, Ebert D, Marcus MA, Zalewski A, Larson JH, Wagner ND. 2010. Transgenerational effects of poor elemental food quality on *Daphnia magna*. *Oecologia* 162:865-72.
- Gerhardt A, Janssens de Bisthoven L., Schmidt S. 2006. Automated recording of vertical negative phototactic behavior in *Daphnia magna* Straus (Crustacea). *Hydrobiologia* 559:433-441.
- Gerhardt A. 2007. Aquatic behavioral ecotoxicology - prospects and limitations. *Human and Ecological Risk Assessment* 13:481-491.
- Glazier DS, Calow P. 1992. Energy allocation rules in *Daphnia magna* : clonal and age differences in the effects of food limitation. *Oecologia* 90:540-549.
- Gliwicz ZM, Guisande C. 2011. Family planning in *Daphnia*: resistance to starvation in offspring born to mothers grown at different food levels. *Ecology* 91:463-467.
- Glover CN, Wood CM. 2004. Physiological interactions of silver and humic substances in *Daphnia magna*: effects on reproduction and silver accumulation following an acute silver challenge. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* 139:273-280.

- Glover CN, Wood CM. 2005. Physiological characterization of a pH and calcium dependent sodium uptake mechanism in the freshwater crustacean, *Daphnia magna*. *The Journal of Experimental Biology* 208:951-9.
- Gonçalves AMM, Castro BB, Pardal MA, Gonçalves F. 2007. Salinity effects on survival and life history of two freshwater cladocerans (*Daphnia magna* and *Daphnia longispina*). *International Journal of Limnology* 43:13-20.
- Gool EV, Ringelberg J. 1997. The effect of accelerations in light increase on the phototactic downward swimming of *Daphnia* and the relevance to diel vertical migration. *Journal of Plankton Research* 19:2041-2050.
- Gool EV. 1997. Light-induced swimming of *Daphnia*: can laboratory experiments predict diel vertical migration changes. *Hydrobiologia* 360:161-167.
- Gool EV, Ringelberg J. 1998. Quantitative effects of fish kairomones and successive light stimuli on downward swimming responses of *Daphnia*. *Aquatic Ecology* 291:291-296.
- Guan R, Wang WX. 2006. Comparison between two clones of *Daphnia magna*: effects of multigenerational cadmium exposure on toxicity, individual fitness, and biokinetics. *Aquatic Toxicology* 76:217-29.
- Guler Y, Ford AT. 2010. Anti-depressants make amphipods see the light. *Aquatic Toxicology* 99:397-404.
- Harris JE, Mason P. 1956. Vertical migration in eyeless *Daphnia*. *Proceedings of The Royal Society of Biological Sciences*. 145:280-290.
- Hassell KL, Kefford BJ, Nuggeoda D. 2006. Sub-lethal and chronic salinity tolerances of three freshwater insects: *Cloeon sp.* and *Centroptilum sp.* (Ephemeroptera: Baetidae) and *Chironomus sp.* (Diptera: Chironomidae). *The Journal of Experimental Biology* 209:4024-32.
- Haupt F, Stockenreiter M, Baumgartner M, Boersma M, Stibor H. 2009. *Daphnia* diel vertical migration: implications beyond zooplankton. *Journal of Plankton Research*. 31:515-524.
- Hemming JM, Turner PK, Brooks BW, Waller WT, La Point TW. 2002. Assessment of toxicity reduction in wastewater effluent flowing through a treatment wetland using *Pimephales promelas*, *Ceriodaphnia dubia*, and *Vibrio fischeri*. *Archives of Environmental Contamination and Toxicology* 42:9-16.
- Jemec A, Tisler T, Drobne D, Sepcic K, Jamnik P, Ros M. 2008. Biochemical biomarkers in chronically metal-stressed daphnids. *Comparative Biochemistry and Physiology*. 147:61-68.

- Kaushal SS, Groffman PM, Likens GE, Belt KT, Stack WP, Kelly VR, Band LE, Fisher GT. 2005. Increased salinization of fresh water in the northeastern United States. *Proceedings of the National Academy of Sciences of the United States of America* 102:13517-20.
- Kieu ND, Michels E, De Meester L. 2001. Phototactic behavior of *Daphnia* and the continuous monitoring of water quality: interference of fish kairomones and food quality. *Environmental Toxicology and Chemistry* 20:1098-103.
- Knight JT, Waller WT, 1992. Influence of the addition of Cerophyl® in the *Selenastrum capricornutum* diet of the cladoceran *Ceriodaphnia dubia*. *Environmental Toxicology and Chemistry* 11:521–534.
- Lam IKS, Wang WX. 2006. Accumulation and elimination of aqueous and dietary silver in *Daphnia magna*. *Chemosphere* 64:26-35.
- Lam IKS, Wang WX. 2006. Transgenerational retention and maternal transfer of selenium in *Daphnia magna*. *Environmental Toxicology and Chemistry* 25:2519-25.
- Lampert, W. 1989. The adaptive significance of diel vertical migration of zooplankton. *Functional Ecology* 3:21-27.
- Lampert W. 2005. Vertical distribution of zooplankton: density dependence and evidence for an ideal free distribution with costs. *BMC Biology* 3:10.
- Laughlin SB, Ruyter van Steveninck RR, Anderson JC. 1998. The metabolic cost of neural information. *Nature Neuroscience* 1:36-41.
- Loose, CJ, Dawidowicz, P. 1994. Trade-offs in diel vertical migration by zooplankton: the costs of predator avoidance. *Ecology* 75:2255-2263.
- Martínez-Jerónimo F, Martínez-Jerónimo L. 2007. Chronic effect of NaCl salinity on a freshwater strain of *Daphnia magna* Straus (Crustacea: Cladocera): a demographic study. *Ecotoxicology and Environmental Safety* 67:411-6.
- Martins J, Soares ML, Saker ML, Oliveteles L, Vasconcelos VM. 2007. Phototactic behavior in *Daphnia magna* Straus as an indicator of toxicants in the aquatic environment. *Ecotoxicology and Environmental Safety* 67:417-422.
- McCooole MD, Baer KN, Christie AE. 2011. Histaminergic signaling in the central nervous system of *Daphnia* and a role for it in the control of phototactic behavior. *The Journal of Experimental Biology* 214:1773-82.

- Michels E., Leynen M., Cousyn C., De Meester L., Ollevier F. 1999. Phototactic behavior of *Daphnia* as a tool in the continuous monitoring of water quality: experiments with a positively phototactic *Daphnia magna* clone. *Water Resources* 33:401-408.
- Michels E., Semsari S., Bin C., De Meester L. 2000. Effect of sublethal doses of cadmium on the phototactic behavior of *Daphnia magna*. *Ecotoxicology and Environmental Safety* 47:261–265.
- Mitchell SE, Read AF. 2005. Poor maternal environment enhances offspring disease resistance in an invertebrate. *Proceedings of Biological Sciences of The Royal Society* 272:2601-7.
- Naddy RB, Gorsuch JW, Rehner AB, Mc Nerneya GR, Bell RA, Kramer JR. 2007. Chronic toxicity of silver nitrate to *Ceriodaphnia dubia* and *Daphnia magna*, and potential mitigating factors. *Aquatic Toxicology* 84:1-10.
- Ohman MD, Frost, BW, Cohen EB. 1983. Reverse diel vertical migration –an escape from invertebrate predators. *Science* 220:1404–1407.
- Pane EF, McGeer JC, Wood CM. 2004. Effects of chronic waterborne nickel exposure on two successive generations of *Daphnia magna*. *Environmental Toxicology and Chemistry* 23:1051-6.
- Park EJ, Yi J, Kim Y, Choi K, Park K. 2010. Silver nanoparticles induce cytotoxicity by a Trojan-horse type mechanism. *Toxicology in vitro* 24:872-8.
- Powers CM, Slotkin TA, Seidler FJ, Badireddy AR, Padilla S. 2011. Neurotoxicology and teratology silver nanoparticles alter zebrafish development and larval behavior : distinct roles for particle size, coating and composition. *Neurotoxicology and Teratology* February 1-7
- Ren ZM, Zha JM, Ma M, Wang ZJ, Gerhardt A. 2007. The early warning of aquatic organophosphorus pesticide contamination by on-line monitoring behavioral changes of *Daphnia magna*. *Environmental Monitoring and Assessment*. 134:373-383.
- Rensing L, Ruoff P. 2002. Temperature effect on entrainment, phase shifting, and amplitude of circadian clocks and its molecular bases. *Chronobiology International* 19:807-864.
- Reichwaldt ES, Wolf ID, Stibor H. 2010. The effect of different zooplankton grazing patterns resulting from diel vertical migration on phytoplankton growth and composition: a laboratory experiment. *Ecology* 141:411-419.

- Reichwaldt ES, Stibor H. 2005. The impact of diel vertical migration of *Daphnia* on phytoplankton dynamics. *Oecologia* 146:50-6.
- Ringelberg J. 1964. The positively phototactic reaction of *Daphnia magna* Straus: A contribution to the understanding of diurnal vertical migration. *Netherlands Journal of Sea Research*. 2:320-406.
- Ringelberg J, Flik BJ. 1994. Increased phototaxis in the field leads to enhanced diel vertical migration. *Limnology and Oceanography* 39:1855-1864.
- Ringelberg J. 1995. An account of a preliminary mechanistic model of swimming behavior in *Daphnia*: its use in understanding diel vertical migration. *Hydrobiologia* 307:161-165.
- Ringelberg, J. 1999. The photobehavior of *Daphnia spp.* As a model to explain diel vertical migration in zooplankton. *Biological Reviews*. 74:397-423.
- Ringelberg J, Van Gool E. 2003. On the combined analysis of proximate and ultimate aspects in diel vertical migration (DVM) research. *Hydrobiologia*. 491:85-90.
- Sancho E, Villarroel MJ, Andreu E, Ferrando MD. 2009. Disturbances in energy metabolism of *Daphnia magna* after exposure to tebuconazole. *Chemosphere* 74:1171-1178.
- Semsari S, Megateli S. 2007. Effect of cadmium toxicity on survival and phototactic behavior of *Daphnia magna*. *Environmental Technology* 28:799-806.
- Kramer JR., Benoit G, Bowles KC, DiToro DM, Herrin RT, Luther III GW, Manolopoulos H, Robillard KA, Shafer MM, Shaw JR, 2002. Environmental chemistry of silver. In: Andren, A.W., Bober, T.W. (Eds.), *Silver in the Environment: Transport, Fate and Effects, Research Findings of the Argentum International Conference Series 1993–2000*. SETAC Press, Pensacola, pp. 1–25
- Smith KC, Macagno ER. 1990. UV photoreceptors in the compound eye of *Daphnia magna* (Crustacea, Branchiopoda). A fourth spectral class in single ommatidia. *Journal of Comparative Physiology Neuroethology Sensory Neural and Behavioral Physiology* 166:597-606.
- Smolders R, Baillieul M, Blust R. 2005. Relationship between the energy status of *Daphnia magna* and its sensitivity to environmental stress. *Aquatic Toxicology (Amsterdam, Netherlands)* 73:155-70.
- Stanley JK, Ramirez AJ, Chambliss CK, Brooks BW. 2007. Enantiospecific sublethal effects of the antidepressant fluoxetine to a model aquatic vertebrate and invertebrate. *Chemosphere* 69:9-16.

- Storz UC, Paul RJ. 1998. Phototaxis in water fleas (*Daphnia magna*) is differently influenced by visible and UV light. *Journal of Comparative Physiology A: Sensory, Neural, and Behavioral Physiology* 183:709-717.
- Stuart AE, Borycz J, Meinertzhagen IA. 2007. The dynamics of signaling at the histaminergic photoreceptor synapse of arthropods. *Progress in Neurobiology*. 82:202-27.
- Szulkin M, Dawdidowicz P, Dodson SL. 2006. Behavioural uniformity as a response to cues of predation risk. *Animal Behaviour* 71:1013-1019.
- Tsui MTK, Wang WX. 2004. Maternal transfer efficiency and transgenerational toxicity of methylmercury in *Daphnia magna*. *Environmental Toxicology and Chemistry* 23:1504-11.
- Tsui MTK, Wang WX. 2005. Influences of maternal exposure on the tolerance and physiological performance of *Daphnia magna* under mercury stress. *Environmental Toxicology and Chemistry*. 24:1228-34.
- ¹USEPA. 2008. Registration Eligibility Decision (RED) Dimethoate. US EPA, Office of Prevention, Pesticides and Toxic Substances, Washington, DC. EPA-HQ-OPP-2005-0084.
- ²USEPA. 1995. Registration Eligibility Decision (RED) Linuron. US EPA, Office of Prevention, Pesticides and Toxic Substances, Washington, DC. EPA 738-R-95-003.
- ³USEPA. 2004. Registration Eligibility Decision (RED) MCPA (2-methyl-4-chlorophenoxyacetic acid). US EPA, Office of Prevention, Pesticides and Toxic Substances, Washington, DC. List A Case 0017.
- ⁴USEPA. 2003. Ambient Aquatic Life Water Quality Criteria for Tributyltin (TBT). US EPA, Office of Water Washington, DC. EPA 822-R-03-031.
- ⁵USEPA. 2004. Registration Eligibility Decision (RED) Thiram. US EPA, Office of Prevention, Pesticides and Toxic Substances, Washington, DC. EPA 38-R-04-012
- ⁶USEPA. 1980. Ambient Aquatic Life Water Quality Criteria for Naphthalene. US EPA, Office of Water Washington, DC. EPA 440-5-80-059.
- ⁷USEPA. 1980. Update of Ambient Water Quality Criteria for Cadmium. US EPA, Office of Water Washington, DC. EPA 822-R-01-001.
- USEPA. 2002. Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms Fourth Edition

- USEPA. 1996. Ecological Effects Test Guidelines. OPPTS 850.1300 Daphnid Chronic Toxicity Test. Office of Prevention, Pesticides and Toxic Substances.
- Untersteiner H, Kahapka J, Kaiser H. 2003. Behavioural response of the cladoceran *Daphnia magna* Straus to sublethal Copper stress — validation by image analysis. *Aquatic Toxicology* 65:435-442.
- Valenti TW, Perez-Hurtado P, Chambliss CK, Brooks BW. 2009. Aquatic toxicity of sertraline to *Pimephales promelas* at environmentally relevant surface water pH. *Environmental Toxicology and Chemistry* 28:2685–2694.
- Villarroel MJ, Ferrando MD, Sancho E, Andreu E. 1999. *Daphnia magna* Feeding Behavior after Exposure to Tetradifon and Recovery from Intoxication. *Ecotoxicology and Environmental Safety* 44:40-46.
- Villegas-Navarro A. 2003. The heart of *Daphnia magna*: effects of four cardioactive drugs. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* 136:127-134.
- Ward TJ, Kramer JR. 2002. Silver speciation during chronic toxicity tests with the mysid, *Americamysis bahia*. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* 133:75-86.
- Ward TJ, Boeri RL, Hogstrand C, Kramer JR, Lussier SM, Stubblefield WA, Wyskiel DC, Gorsuch JW. 2006. Influence of salinity and organic carbon on the chronic toxicity of silver to mysids (*Americamysis bahia*) and silversides (*Menidia beryllina*). *Environmental Toxicology and Chemistry* 25:1809-16.
- Ward JT, Robinson E. 2005. Evolution of Cadmium resistance in *Daphnia magna*. *Environmental Toxicology and Chemistry* 24:2341-2349.
- Weis JS, Smith G, Zhou T, Santiago-Bass C, Weis P. 2001. Effects of contaminants on behavior: biochemical mechanisms and ecological consequences. *BioScience* 51:209.
- Whitman LJ, Miller RJ. 1982. The phototactic behavior of *Daphnia magna* as an indicator of chronic toxicity. *Proceedings of the Oklahoma Academy of Sciences* 33:22-33.
- Wolf G., Scheunders P, Selens M. 1998. Evaluation of the swimming activity of *Daphnia magna* by image analysis after administration of sublethal Cadmium concentrations. *Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology* 120:99-105.

Wu Y, Lin C, Yuan L. 2008. Phototaxis index of *Daphnia carinata* as an indicator of joint toxicity of copper, cadmium, zinc, nitrogen and phosphorus in aqueous solutions. *Ecological Indicators* 8:69-74.

Ziegler TA, Cohen JH, Forward RB. 2010. Proximate control of diel vertical migration in Phyllosoma larvae of the Caribbean spiny lobster *Panulirus argus*. *The Biological Bulletin* 219:207-19.